A COMPARISON OF THE REMOVAL OF ESTROGEN BETWEEN DIFFERENT WASTEWATER TREATMENT PROCESSES

By

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ABSTRACT

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Pharmaceuticals are found in water systems and are classified as contaminants due to their adverse effects on numerous types of aquatic organisms. Estradiol, a natural occurring estrogen and ethinylestradiol, a common synthetic estrogen found in birth control, are a class of pharmaceuticals called endocrine-disruptive contaminants (EDC). These contaminants have been shown to cause developmental delays in fish as well as feminizing male fish in concentrations as low as 1 ng/L for both estradiol and ethinylestradiol, and bioaccumulating in mollusks and oysters. There are many natural and human-assisted methods for removing pharmaceuticals in water, and they are dependent on both the properties of the contaminant and the type of treatment applied. Ten different wastewater treatment processes at two different wastewater treatment plants were studied to compare the removal of estrogen out of the wastewater. The first facility, the Arcata Wastewater Treatment Plant and Wildlife Sanctuary, utilizes oxidation ponds and constructed wetlands for the final treatment processes. The second facility, in Ferndale, CA is an active sludge wastewater treatment plant. The samples were analyzed by both HPLC and ELISA, however, the data measured on the HPLC was not used due to discrepancies in the results. The concentration of estradiol was found to be from 131 ng/L

to 212 ng/L in Arcata influent and from 46 ng/L to 113 ng/L in the effluent. The concentration of ethinylestradiol was found to be from 0.49 ng/L to 0.63 ng/L in the Arcata influent and from 0.36 ng/L to 0.46 ng/L in the effluent. In Ferndale, the concentration of estradiol was found to be from 123 ng/L to 143 ng/L in the influent and 18 ng/L to 131 ng/L in the effluent while the concentration of ethinylestradiol was found to be 0.47 ng/L to 0.56 ng/L in the influent and 0.33 ng/L to 0.41 ng/L in the effluent. The concentration of estrogen increased in numerous treatments, while the oxidation pond and aeration basin were the only treatments that showed consistent decrease of concentration at either site. The oxidation pond had the highest removal efficiency with 80% of the incoming estradiol removed and 25% of the incoming ethinylestradiol removed. The concentration of estradiol at both locations was greater than 1 ng/L which is greater than levels that are safe for fish. Additional studies are recommended to determine how far from the discharge point the concentration of estrogen drops below 1 ng/L and whether the oysters in the Humboldt Bay are bioaccumulating estrogen to an unsafe concentration.

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INTRODUCTION

Pharmaceuticals have been in use by humans for hundreds of years, however, with the exception of a few papers posted in the 1970s and 1980s, they had not been recognized as a serious environmental contaminant until about 20 years ago (Hughes, Kay, and Brown 2013). The ability to detect and analyze these contaminants was progressed by the advancement of analytical instrumentation, such as gas and liquid chromatography tandem mass spectrometry (GC-MS, LC-MS) and various assays such as yeast assays and enzyme-linked immunosorbent assays (ELISA) (Gabet et al. 2007).

The types of pharmaceuticals found in the environment, especially in water systems are numerous and varied (Sui et al. 2015). Along with pharmaceuticals used specifically for human consumption, various pharmaceuticals used for veterinary practices have been found in water systems that were due to animal waste overflow, storage structure leakage, or runoff from land applications (Kolpin et al. 2002). Two of the common antibiotics used in animal feed that have been found in the water systems are sulfonamides and tetracyclines (Arikan, Rice, and Codling 2008), although these classes of antibiotics are used for human use as well. Of the pharmaceuticals for human use, the most common classes that have been found in water systems are hormones (30%), pain killers and anti-inflammatories (20%) and antibiotics (9%) (Miège et al. 2009). Different pharmaceuticals have been found in surface waters (Li 2014), ground water (Sui et al.

2015), wastewater (Gracia-Lor et al. 2012) and in small concentrations in drinking water (Huerta-Fontela, Galceran, and Ventura 2011; Yang et al. 2017).

While the concentrations are small, these different pharmaceuticals have been shown to have several negative effects on the environment. One issue that affects not only marine life, but humans as well is that the increase of antibiotics in water has led to numerous strains of bacteria becoming increasingly resistant to many of our antibiotics (Karkman et al. 2018; Baquero, Martínez, and Cantón 2008; Huerta et al. 2013). This has led to the creation of cMRSA, which is a methicillin resistant Staph Aureus that is found in community water, something that had not been seen even 40 years ago (Al-Rawahi et al. 2010). There are also pharmaceuticals that have not been shown to affect humans once in the water systems, but they do have negative affects at acute and chronic exposure to various aquatic life. In fact, over 64% of the pharmaceuticals and personal care products tested in one study showed high toxicity and were harmful to aquatic organisms (Ortiz de García et al. 2014). For instance, different antidepressants, psychiatric medications and antihistamines have been shown to induce behavioral changes in different species of fish in concentrations ranging from low ng/L to low µg/L (Brodin et al. 2014). In addition to fish, many crustaceans were found to have adverse effects from pharmaceuticals in both acute and chronic exposure (Varano, Fabbri, and Pasteris 2017). Fully grown species were not the only organisms affected, many pharmaceuticals were shown to be toxic or harmful to specific organs like fish hepatocytes (Laville et al. 2004) in addition to undeveloped organisms such as frog embryos (Richards and Cole 2006).

One class of pharmaceuticals that have been of increasing concern over the past decade are endocrine-disrupting compounds (EDCs) such as hormones, like estrogen. Estrogens are a type of steroid, and they are the primary sex hormone in women. There are many different types of naturally occurring and synthetic estrogens. Estrone (E1), Estradiol (E2), and Estriol (E3) are some of the most common naturally occurring estrogens, while Ethinylestradiol (EE2) is a synthetic estrogen commonly found in birth control (Evans and Sutton 2015). In the USA, 62% of women use contraceptives with hormonal contraceptives accounting for 35% (Jones 2012). In fact, EE2 is found in almost all combined forms of birth control pills and is the exclusive estrogen used for this purpose making it the most widely used estrogen. In addition to birth control, many of the estrogens, including E2 are found in medications used for male-to-female transgender individuals, which accounts for 0.3-0.5% of the population in the USA (Unger 2016). Both Estrone and Estradiol are synthesized from androgenic precursors. Estrone is synthesized from Androstenedione and Estradiol from Testosterone via the pathway shown in Figure 1. Both Estrone and Estradiol are synthesized by cytochrome P450 aromatase (P450 AROM) while Estrone and Estradiol can be synthesized into one another via the enzyme 17beta-hydroxy-steroid dehydrogenase (17β-HSD). Estriol can be synthesized from Estrone via two different intermediates; 2-Hydroxyestrone and 16α-Hydroxyestrone (Gao et al. 2014).

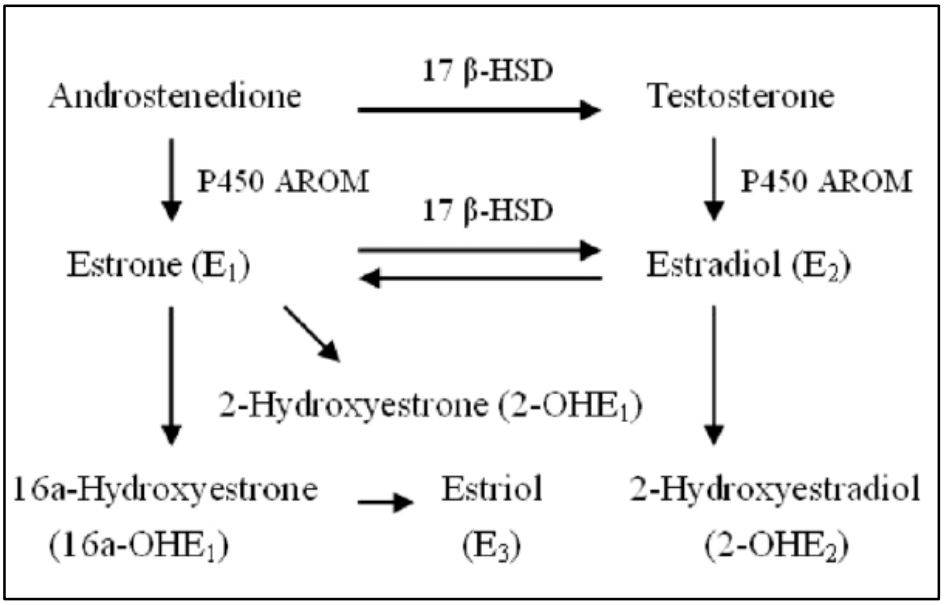

Figure 1: Biological enzymatic pathway of synthesis of estrogens. Courtesy of Gao et al. 2014

Estrogen hormones are excreted in fecal waste in the parent form (Aris, Shamsuddin, and Praveena 2014) and in urine as the glucuronide conjugate (Stanczyk, Archer, and Bhavnani 2013a). Estrogens excreted as waste generally make their way to wastewater treatment plants, however, estrogens have been found in surface water (Liu et al. 2015), ground water (Kolodziej, Harter, and Sedlak 2004), and natural water, such as lakes, and rivers (Desbrow et al. 1998). Some of this is likely due to runoff from agriculture or aquaculture operations (Kolodziej, Harter, and Sedlak 2004), and depending on the area, that non-human contribution is trivial, however, it is also likely from effluent from various wastewater treatment plants. In many rural areas leakage from septic systems can also be a source.

There are, however, numerous processes, including natural processes, that remove pharmaceuticals out of water. Biodegradation is a process that occurs when bacteria

break down a molecule to be used as an energy source and it occurs naturally in soil and water (Combalbert and Hernandez-Raquet 2010). Many pharmaceuticals have been shown to biodegrade, including E2, however, biodegradation of E2 in water happens too slowly to prevent transport further into the system (Yu, Deeb, and Chu 2013). There is also the process of photodegradation in which a molecule is altered via light, including UV light and it typically involves oxidation and hydrolysis to break down a molecule (Sornalingam, McDonagh, and Zhou 2016). Photodegradation happens naturally in aqueous and non-aqueous matrices and can be increased using various catalysts (Tanizaki, Kadokami, and Shinohara 2002a). While EE2 is less resistant to biodegradation than E2, both are susceptible to photodegradation, however, under ideal conditions, the half-life for both molecules is around 10 days (Jürgens et al. 2002; Leech, Snyder, and Wetzel 2009). Oxidation is another natural process in which oxygen itself breaks down a molecule, and it is effective at removing many organic compounds, like pharmaceuticals, specifically beta-blockers, from the system (Huerta-Fontela, Galceran, and Ventura 2011). Another natural process that removes pharmaceuticals from the water system is sorption onto sediment and soils, however, this does not necessarily mean the contaminant is removed from the system, especially in natural systems such as surface and groundwaters.

Human-assisted removal processes of pharmaceuticals can occur during water treatment. For instance, chlorination is shown to remove many pharmaceuticals, some at over 99% efficiency (Huerta-Fontela, Galceran, and Ventura 2011). However, estrogen is shown to have higher resistance to chlorination (Auriol et al. 2006). Many hydrophobic

molecules, such as warfarin, a blood thinner, tend to be removed during filtration processes, such as by trickling filters that are found in wastewater treatment plants and drinking water treatment plants, but other pharmaceuticals show less than 70% removal (Huerta-Fontela, Galceran, and Ventura 2011; Kasprzyk-Hordern, Dinsdale, and Guwy 2009). Various activated sludges, such as nitrogen activated sludge have shown great removal efficiencies for pharmaceuticals from water, however, it is likely that the removal is due to sorption onto the sludge rather than actual degradation of the compound, and while the removal is significant, often over 80% in most cases, molecules with low sorption coefficients will not be removed this way, and so biological transformation is preferred (Joss et al. 2005; Archer et al. 2017).

There are many different types of wastewater treatment plants including sewage treatment plants, for residential and business areas (Tchobanoglous, Burton, and Stensel 2003a), industrial wastewater treatment plants (Tchobanoglous, Burton, and Stensel 2003a), agricultural plants for continuous confined animal operations like milk and egg production ("Protecting Water Quality from Agricultural Runoff" 2005), and leachate treatment plants. Within sewage treatment plants (sometimes called wastewater treatment plants, or WWTP), there are different varieties such as activated sludge plants, trickling filter, submerged aerated filter and rotating disc system. The main difference in the types of WWTP are the way that they treat the raw water.

This study was conducted in Humboldt County, CA where the main types of water treatment plants are trickling filter (Eureka), active sludge (Fortuna, Ferndale, Rio Dell), stabilization pond (Blue Lake) and ponds and constructed wetland (Arcata).

Because each of the plants have different methods for removal of contaminants from water, they have different efficacy in removal of contaminants, including estrogen (Qiang et al. 2013; Song et al. 2009). Additionally, there are numerous other factors to take into consideration with the removal of pharmaceuticals from the system. Temperature of the water, kinetic behavior of the contaminant, sludge retention time and hydraulic retention times all affect the removal of contaminants (Gros et al. 2010). It is imperative that the majority of contaminants are removed from wastewater and that wastewater is properly disinfected because much of the effluent returns back into natural water bodies. In Humboldt county, the effluent often goes into Humboldt Bay (Figure 2). The Humboldt Bay watershed hosts numerous ecosystems that house over 100 plants species, 300 invertebrate species, 100 fish species, 200 bird species and additional ecosystems that are classified as endangered or threatened (Gerwin 2012).

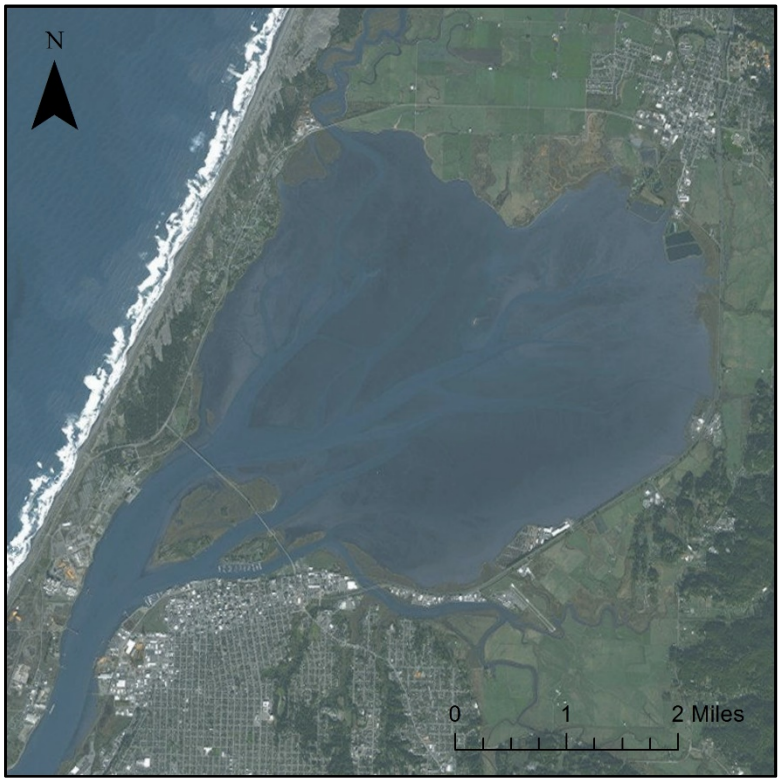

Figure 2: Image of the northern side of Humboldt Bay where the effluent from the Arcata Marsh is released. Image courtesy of Google

Numerous studies have shown that endocrine-disrupters, like estrogens, have contributed to sexual disruption and intersex, defined as the simultaneous presence of both male and female gonadal characteristics, in a variety of fish (Jobling et al. 1998; Sumpter and Jobling 1995; Barnhoorn et al. 2004) and alligators (Guillette Jr et al. 1994) at ng/L (or parts per trillion, ppt) levels (Robinson et al. 2009). This sexual disruption of fish is so widespread that vitellogenesis, or the process of yolky egg production, in male fish can be used as a biomarker for estrogenic contamination in water (Sumpter and Jobling 1995). In fish such as roach (Jobling et al. 1998) and catfish (Barnhoorn et al. 2004), this means that the fish cannot reproduce because the males have been feminized.

In Humboldt Bay, two of the more important aquatic species in this area are salmon and oysters. Estradiol has been shown to delay smolt development and downstream migration in Atlantic Salmon (Madsen et al. 2004) and it also has been shown to bioaccumulate in the oyster *Crassostrea gigas* (Le Curieux-Belfond et al. 2005).

The removal of estrogens has been studied in various types of wetlands such as engineered wetlands, and vertical wetlands (Song et al. 2009; Huang and Sedlak 2001a), however, none of them have been in a wastewater wetland constructed like the Arcata Marsh.

The purpose of this study was to look at ten different wastewater treatment processes at two different wastewater treatment plants, one more natural and the other more conventional, in the same regional area and determine which of these processes overall removes estrogen most efficiently and which of the treatment steps within the overall process is responsible for the bulk of estrogen reduction. The collection of the samples was done during the dry season to avoid seasonality variations, such as those found in other studies (Qiang et al. 2013). The Arcata marsh uses natural plants and aquatic organisms for reduction of many contaminants in wastewater. The waters in the Arcata marsh flow through different ponds and wetlands, with retention times varying with flow rate, but often around 14 days, and finally through a chlorine contact basin for disinfection. The conventional wastewater treatment plant is in Ferndale, CA, and consists of an aeration basin with activated sludge followed by a filter and then UV treatment for disinfection. The retention time of the Ferndale treatment is much shorter, often only hours long. Previous studies have shown activated sludge removing 50% of

the E2 and 70% of EE2 in the system (Miya et al. 2007; Ternes et al. 1999) and constructed wetlands only removing around 40% of estrogens from the system (Gray and Sedlak 2005; Larsson et al. 1999a). Studies have also shown less EE2 in the influent which is to be expected because there are less people taking birth control and hormone therapy than there are people producing estrogen (Baronti et al. 2000a; Belfroid et al. 1999). With these factors taken into consideration, and with the knowledge of estrogens exhibiting hydrophobicity, I hypothesized that for the Ferndale site, the trickling filter removes most of the estrogens, as the estrogens will be more attracted to the filter than they will be to the water, and the remainder of the estrogens is removed during UV treatment. However, the estrogen will have a longer time to biodegrade and photodegrade in the Arcata marsh, and it is likely that the aquatic species in the marsh mix the sediment like the aeration basin mixes sludge, so it is possible that the removal efficiencies are nearly identical, with the estrogen exhibiting a more gradual decline in the marsh than in the Ferndale WWTP.

MATERIALS AND METHODS

Site Selection and Sampling

Two sites were selected for the sampling process; the Arcata Marsh and the Ferndale Wastewater Treatment Plant. The first site, the Arcata Marsh, has primary, secondary, and tertiary treatment areas before a chlorine disinfection ("A Natural System for Wastewater Reclamation and Resource Enhancement" 1993). The primary treatment includes the headworks, clarifier and digesters. The headworks component removes the majority of the large debris such as clothes, toilet paper, and sand. From there, the water moves to the clarifiers where the water effluent suspended material settles out and the grease floats to the top and is skimmed off. The settled-out solids move to the digesters, while the liquid moves to the secondary treatment area; the oxidation ponds. The oxidation ponds work to remove any remaining suspended particles as well as about half of the Biochemical Oxygen Demand (BOD). The oxidation ponds also remove majority of the nitrogen between June and November. This is achieved with a long retention time as well as microorganisms living in the water. The water then flows into the treatment marshes which are planted with various submerged, emergent and floating plants, that are native to the area. This helps to remove additional BOD in the water. From here, much of the water is chlorinated and sent into the tertiary treatment area, which is composed of enhancement marshes where much of the residue nutrients, such as phosphorus and nitrogen, are removed from the water via plants and microorganisms. The water from the

enhancement marshes are re-chlorinated to disinfect the water a second time, and some is sent back into the marshes, while the rest is de-chlorinated and sent into Humboldt Bay ("A Natural System for Wastewater Reclamation and Resource Enhancement" 1993).

From the Arcata WWTP, four liters of water each was taken from the influent, primary clarifier effluent, oxidation pond effluent, treatment pond effluent and the final effluent which is discharged into the Humboldt bay. Samples were taken three times, in July, September, and October. These dates were chosen because they were all in the dry season and it was possible that there would be a difference between the influent concentration in Arcata between July and the other two months because most of the college students would be gone in July, but back for Fall semester starting in September.

The second site, the Ferndale Wastewater Treatment Plant, also has primary, secondary and tertiary treatment areas, but for disinfection it uses Ultraviolet light (UV). The primary treatment consists of the headworks with a comminutor and bar screen to remove large particles and break up particles into smaller ones. Those particles move on to the aero-mod which acts as the primary clarifier and removes settleable solids, and then to the aeration basin. In the aeration basin, air is added to assist in the growth of bacteria helping decrease BOD and nutrients. The water then flows to a disc filter and then finally past the UV light for disinfection. Four liters of water each were taken from the influent, the aeration basin effluent, the holding basin for the filter influent, the filter effluent and the UV light effluent.

Materials and Instrumentation

Scientific solvents used were HPLC grade. Acetonitrile and Methanol were purchased from Fisher. C-18 SPE cartridges were purchased from Supelco. The estradiol ELISA kit, estradiol stock and ethinylestradiol stock were purchased from Cayman. The ethinylestradiol ELISA kit was purchased from Aviva systems. The HPLC used was a Waters binary pump with a dual absorbance UV detector and equipped with an Xterra RP 5 µm 4.6x150 C-18 column.

Sample Preparation and Analysis

The samples were prepared according to methods outlined in previous work (Huang and Sedlak 2001b). The wastewater samples were immediately stored at 4°C and the solids were allowed to settle before filtering on a Whatman GF/B filter. The filtered samples were stored at 4 °C until extraction. The hormones were extracted on a C-18 SPE cartridge and were eluted using 30 mL of methanol. The samples were dried under a gentle stream of nitrogen, and re-suspended in 1 mL of methanol for HPLC analysis. The samples were then re-dried under a gentle stream of nitrogen and re-suspended for ELISA analysis. For HPLC analysis, the dried samples were reconstituted in methanol and filtered through a 0.22 µm glass-fiber filter. Each sample was analyzed in triplicate at both 220 and 280 nm in a 60:40 water: acetonitrile mobile phase. Both the peak area and peak height were recorded for analysis. For ELISA analysis, 100 µL of each sample was taken and dried under a gentle stream of nitrogen and reconstituted in the appropriate

ELISA buffer. The plates were then prepared according to manufacturer instructions and read on the plate reader at the suggested wavelengths. For both analysis processes, calibration curves were created using pure estradiol and ethinylestradiol in the appropriate solvent. The calibration curve for ethinylestradiol was created using a double log scatter plot, while the calibration curve and concentrations for estradiol were calculated using a four-parameter logistic curve (www.myassays.com). Removal efficiency was calculated using the following equation: $\frac{influent\left(\frac{ng}{L}\right)-effluent\left(\frac{ng}{L}\right)}{influent\left(\frac{ng}{L}\right)} * 100\%$.

RESULTS

The average concentration of estradiol and ethinylestradiol (Table 1 through Table 8) was calculated using specific calibration curves for ELISA analysis (Appendix). The calibration concentrations for ELISA were specified by the manufacturer. For estradiol, the range was from 0.61 ng/L to 10,000 ng/L and for ethinylestradiol, the range was from 24.69 ng/L to 2,000 ng/L.

When analyzed via ELISA, the concentrations of estradiol ranged from 28 ng/L to 212 ng/L at the Arcata Marsh and from 18 ng/L to 175 ng/L in Ferndale. The concentrations of ethinylestradiol ranged from 0.34 ng/L to 0.63 ng/L at the Arcata Marsh and ranged from 0.33 to 0.56 ng/L in Ferndale. This analysis showed a significant declining trend for the estradiol, but only a slight declining trend for ethinylestradiol (Figures 7 through 10).

Although measured, the HPLC data was not included due to large discrepancies with calculated results. The concentration of both estradiol and ethinylestradiol were over 4 orders of magnitude greater than those calculated using ELISA. Additionally, many of the calculated concentrations fell outside of the limit of linearity for the calibration curve. For these reasons, the results could not pass QA/QC and were not included.

Table 1: Average concentration of estradiol in the Arcata Marsh for each sampling time, analyzed via ELISA

E2 (ng/L) ELISA	Arcata 1 (July)	Arcata 2 (September)	Arcata 3 (October)
Influent	143	131	212
Primary Clarifier	169	126	118
Oxidation Pond	37.5	28	36.5
Treatment Wetland	*	37.5	45
Effluent	112.5	45.5	79.5

*These values were not collected

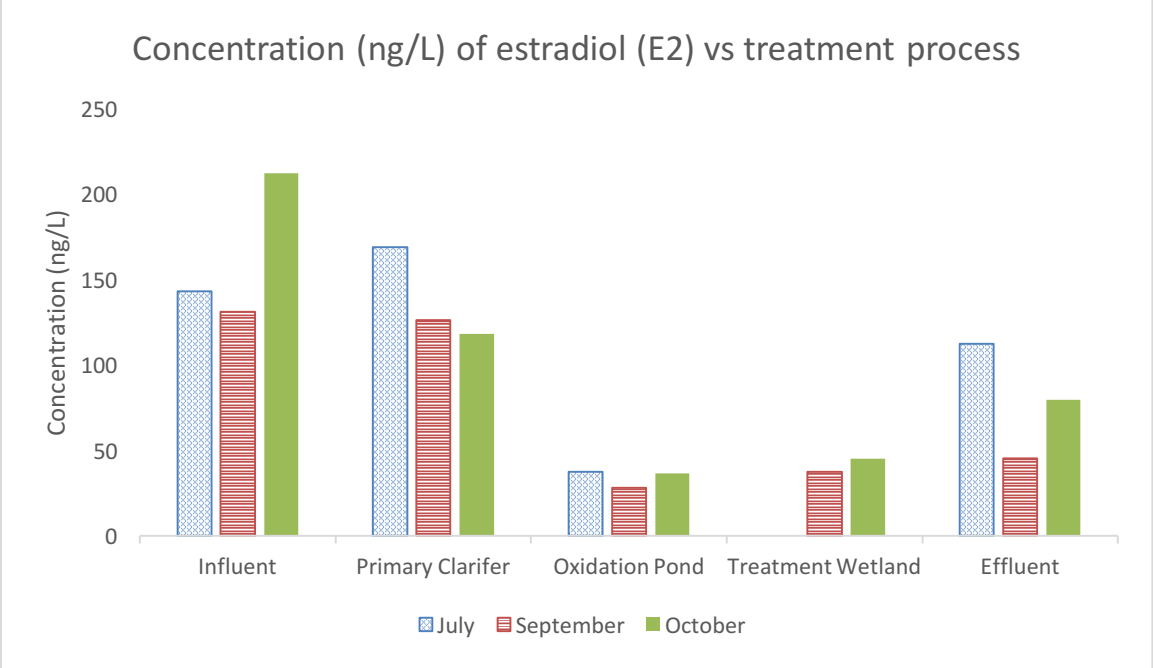

Figure 3: Average concentration (ng/L) of estradiol (E2) in the different treatment processes at the Arcata Marsh analyzed via ELISA

Table 2: Average concentration of ethinylestradiol (EE2) in the Arcata Marsh for each sampling time analyzed via ELISA

EE2 (ng/L) ELISA	Arcata 1 (July)	Arcata 2 (September)	Arcata 3 (October)
Influent	0.491	0.538	0.626
Primary Clarifier	0.582	0.526	0.603
Oxidation Pond	0.432	0.396	0.442
Treatment Wetland	*	0.340	0.381
Effluent	0.464	0.364	0.387

*These values were not collected

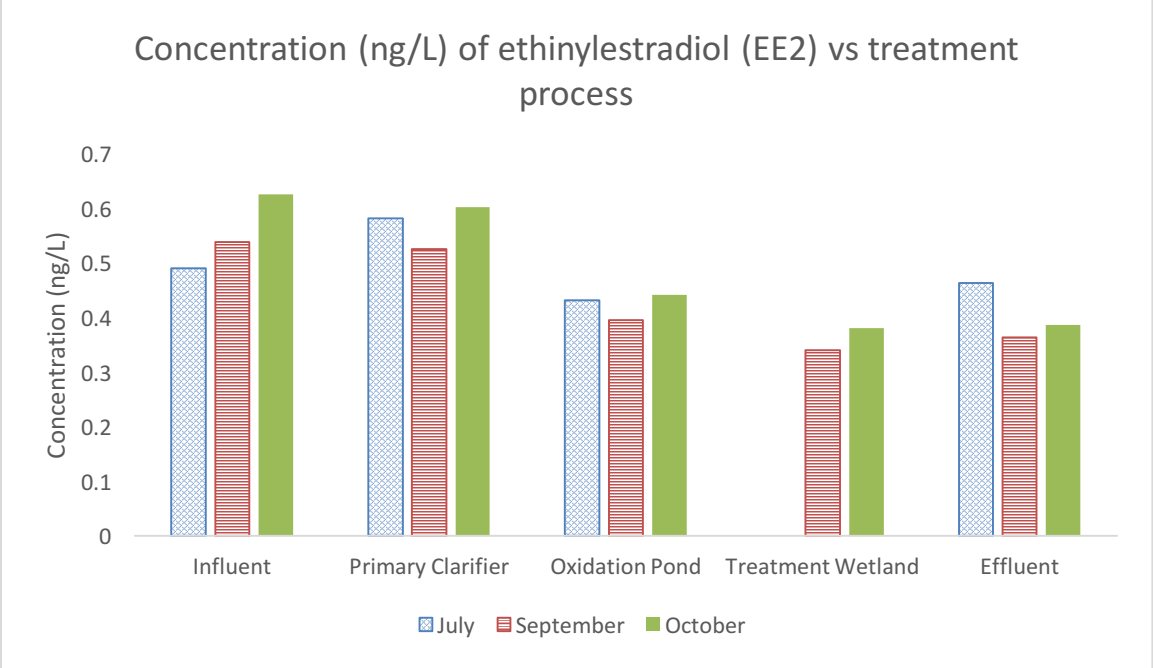

Figure 4: Average concentration (ng/L) of ethinylestradiol (EE2) in the different treatment processes at the Arcata Marsh analyzed via ELISA

Table 3: Average concentration of estradiol (E2) in the Ferndale WWTP for each sampling time analyzed via ELISA

E2 (ng/L) ELISA	Ferndale 1 (July)	Ferndale 2 (September)	Ferndale 3 (October)
Influent	124	123.5	143
Aeration Basin	29.5	24	169
Pre-Filter	29	21	37.5
Post-Filter	24	22.5	112.5
UV Effluent	17.5	17.5	131

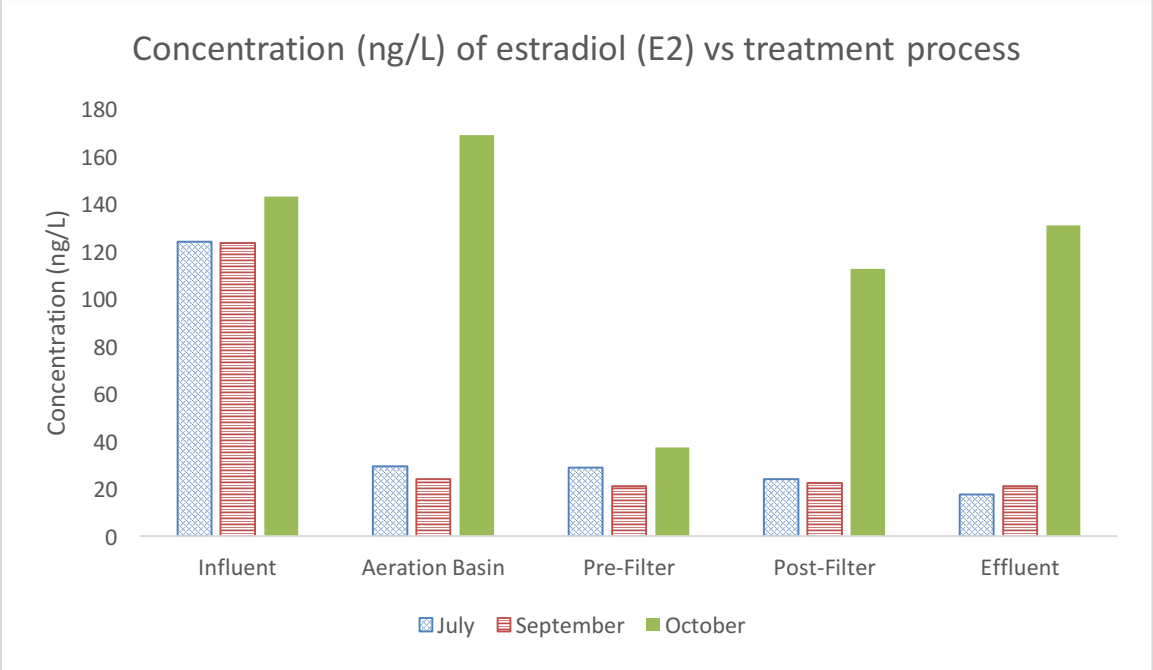

Figure 5: Average concentration (ng/L) of estradiol (E2) in the different treatment processes at Ferndale WWTP analyzed via ELISA

Table 4: Average concentration of ethinylestradiol in the Ferndale WWTP for each sampling time analyzed via ELISA

EE2 (ng/L) ELISA	Ferndale 1 (July)	Ferndale 2 (September)	Ferndale 3 (October)
Influent	0.560	0.515	0.469
Aeration Basin	0.504	0.512	0.364
Pre-Filter	0.429	0.475	0.340
Post-Filter	0.452	0.494	0.349
UV Effluent	0.409	0.383	0.332

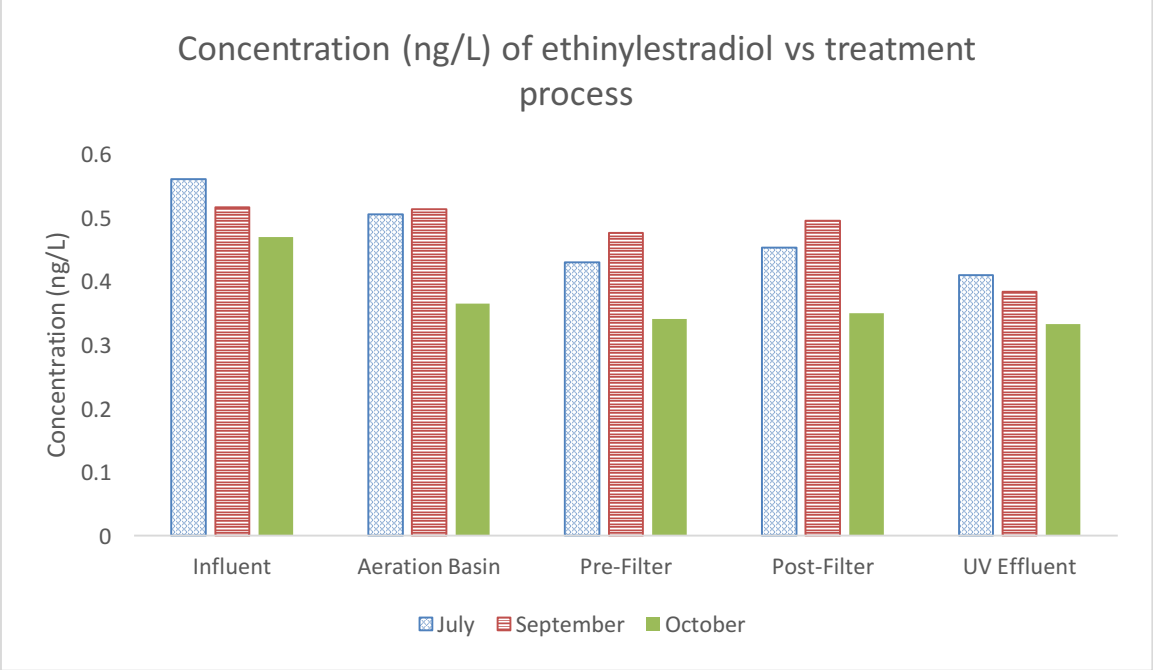

Figure 6: Graph of the concentrations of ethinylestradiol (ng/L) in Ferndale. These samples were analyzed via ELISA.

DISCUSSION

On average, the wastewater treatment processes at the Arcata Marsh removed around 100 ng/L of estradiol and 0.2 ng/L of ethinyl estradiol from the wastewater. The concentration of estradiol seemed to decrease after the primary clarifier and the oxidation ponds, and then increase in the treatment wetland and the effluent. It is likely, then, that the estradiol was being removed via degradation from UV (Larsson et al. 1999b) or possibly adsorbing onto the soil (Baronti et al. 2000b; Liu et al. 2011; Ternes et al. 2002) and then potentially desorbing from the soil back into the water column in the treatment wetland. Similarly, the ethinylestradiol was slowly removed from the system, however, ethinylestradiol is not efficiently degraded via UV (Tanizaki, Kadokami, and Shinohara 2002b) so it is more likely that this form of estrogen was adsorbing onto soil (Hamid and Eskicioglu 2012; Shi et al. 2004). It is possible that due to the long retention times it would be difficult to deduce the actual efficiency of the estrogen removal, but the standard deviation of the estradiol concentration in the influent for the Arcata Marsh was lower than that of the Ferndale WWTP (497 vs 716 respectively) so it can be concluded that the concentrations of estrogen in the influent do not vary so much that it significantly effects efficiency calculation.

At the Ferndale WWTP, the average amount of estradiol removed was also around 100 ng/L. However, unlike at the Arcata Marsh, the concentration of estradiol was not removed gradually. Except for the October data, the bulk of the estradiol was

removed after treatment in the aeration basin. The aeration basin at the Ferndale WWTP uses an activated sludge to help remove nutrients and decrease organic matter. Nitrogen activated sludge (NAS) has been shown in prior studies to be effective at removing estradiol from the water (Shi et al. 2004), however, it is unclear whether the NAS is helping to degrade the estrogen, or whether the act of aeration is increasing contact between estradiol and sludge particles which would help it to adsorb more. The secondary clarifier and disc-filter treatments removed a trivial amount of the estrogen, however, the UV treatment appeared to be quite effective. In July and September, over 40% of the remaining estradiol was removed after UV treatment. This was expected due to the many studies showing that UV light degrades estradiol (Larsson et al. 1999b). Like the Arcata Marsh, the Ferndale WWTP did not remove ethinylestradiol to the degree that it did with estradiol. On average, 70 ng/L was removed, while nearly 200 ng/L remained in the effluent.

In relation to other studies, the concentration of E2 found in the influent and effluent was higher. The EE2 concentration, however, tended to be in the same range found in previous studies. This current data follows the trend of the E2 being a higher concentration in the influent than EE2. The values from previous studies ranged from 4 ng/L of E2 in the influent to 250 ng/L of E2 in the influent. These large ranges of concentrations could be due to population density variations, seasonal variations, and temporal variations as well as variations in methods used for sample prep and sample analysis. These different studies span a 15-year range, and the most recent study was done 5 years before this project. Analytical techniques have advanced since the study

done in 1998 and better methods for analysis have been developed. Additionally, the population of the world has increased by over 2.2 billion people since 1998 which would promote an increase in the concentration of both E2 and EE2 in wastewater systems.

Table 5: Tabulated data of the influent and effluent concentrations (ng/L) of Estradiol and Ethinylestradiol measured in previous studies

Location	E2 In	E2 Out	EE2 In	EE2 Out	Source
Canada	24-66	0.1-26	0.5-5.7	0.6-9.8	Atkinson 2012
Portugal	20-250	10-190	20-190	20-140	Fonseca 2013
China	76-94	17.8-28.2	150.5-159.5	45-57	Cui 2006
Germany	12.2-19.5	9.2-12.6	6.2-10.1	3.5-7.0	Andersen 2003
England	N.M.	3.1-54	N.M.	0.1-10.7	Desbrow 1998
Italy	4.0-25	0.35-3.3	0.43-13	0.33-1.7	Baronti 2000
U.S.	N.M	0.23-4.95	N.M.	0.09-3.02	Huang 2001
Arcata*	131-212	80-112	0.54-0.63	0.36-0.46	2018
Ferndale*	123-143	18-131	0.47-0.56	0.32-0.41	2018

* This study, ELISA method.

The removal efficiency shows that the oxidation pond had the highest removal efficiency for both estradiol (80% of incoming concentration) and ethinylestradiol (25% of incoming concentration). The efficiencies of each of the treatments were calculated and displayed as bar charts for ELISA with concentration decrease in the positive direction and concentration increase in the negative direction. (Figures 7 and 8).

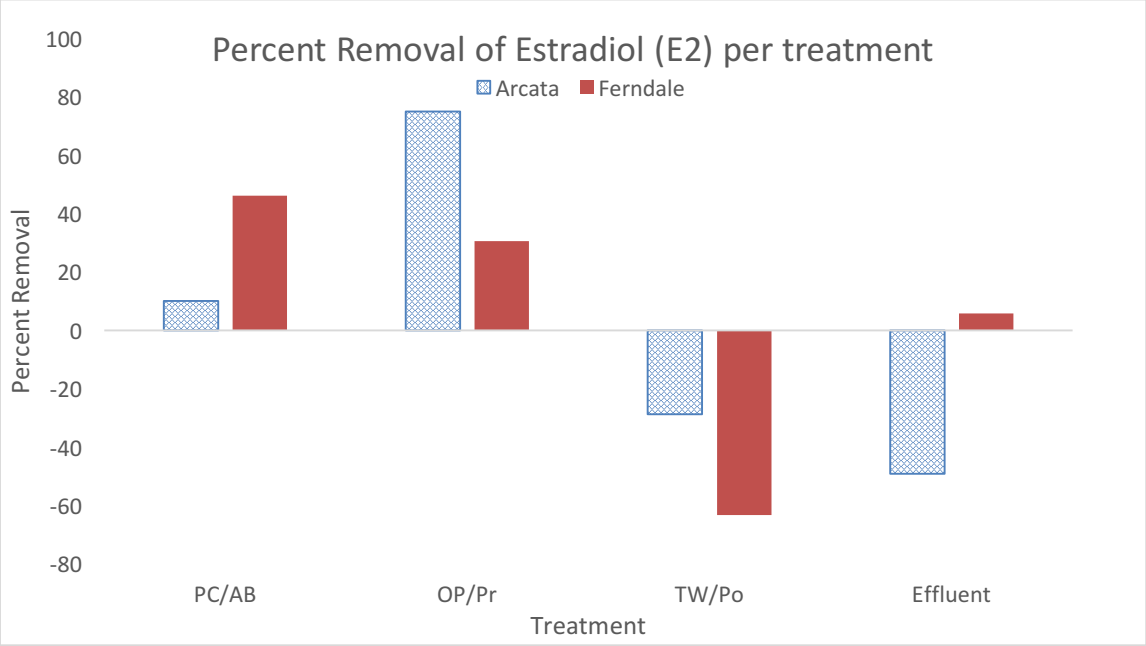

Figure 7: Average percent removal of estradiol in the different treatment processes at the Arcata Marsh and the Ferndale WWTP. The Arcata processes are PC (Primary Clarifier), OP (Oxidation Pond), TW (Treatment Wetland), and the Effluent. The Ferndale processes are AB (Aeration Basin), Pr (Pre-filtration), Po (Post-filtration), and Effluent.

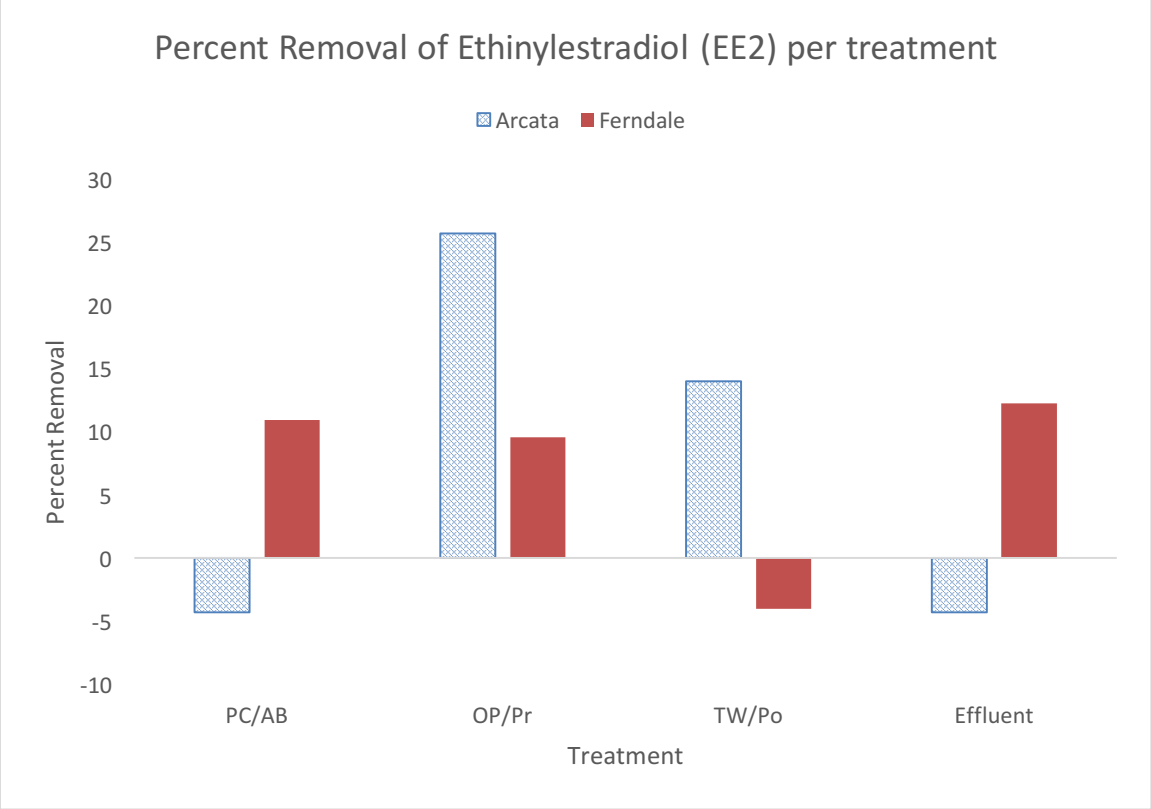

Figure 8: Average percent removal of ethinylestradiol in the different treatment processes at the Arcata Marsh and the Ferndale WWTP. The Arcata processes are PC (Primary Clarifier), OP (Oxidation Pond), TW (Treatment Wetland), and the Effluent. The Ferndale processes are AB (Aeration Basin), Pr (Pre-filtration), Po (Post-filtration), and Effluent.

Overall, the oxidation pond was the most efficient at removing both E2 and EE2. This could potentially be due to UV degradation from long retention times outside as well as due to adsorption onto the settleable particles. However, for both sites, the post-filtration treatment increased the concentration of both E2 (60%) and EE2 (5%). It is possible that the estrogen is desorbing from organic matter sometime during the treatment. Additionally, it could be that the estrogen is sticking to the filter media and then eluting off. The oxidation pond and aeration basin were the only two treatments that did not see an increase in concentration in either E2 or EE2 while the other sites either increased or decreased the concentration of estrogen.

The concentrations calculated using the HPLC were drastically different than those calculated from the ELISA, sometimes an order of magnitude greater. This could potentially be due to the estradiol and ethinylestradiol co-eluting with the glucuronide linked estradiol and ethinylestradiol. The co-elution would, in theory, make the concentration of estrogen look higher than it was. In previous studies, the linked estrogens were unlinked via an enzyme to maximize the amount of estrogen measured (Huang and Sedlak 2001b; Voller, Bartlett, and Bidwell 1978; Farré et al. 2006), however, that was not done in this study due to the desire to measure only the free estrogen in the wastewater. Conjugated estrogen can be transformed into the parent molecule by bacteria (Stanczyk, Archer, and Bhavnani 2013b) which would suggest an increase of estrogens as the treatment progressed. However, I found the rate of transformation into the parent molecule was less than the rate of removal of estrogen from the water. The ELISA analysis only detects the parent compound which might

explain why the concentrations in the influent and primary clarifier were much less when analyzed via ELISA than with HPLC. Another possible explanation for the discrepancy in values would be degradation of the estrogen because the ELISA was performed months later than the HPLC was performed. However, if this were the case, the concentrations would be expected to degrade consistently in each of the sample locations instead of primarily in the influent and primary clarifier/ aeration basin.

One of the issues with the HPLC analysis was the concentration measured in the wastewater exceeded the limit of linearity of the calibration curve. Optimally multiple dilutions would have been made for each of the samples, but due to time and material constraints, that was unfeasible. Many of the concentrations were calculated outside of the top of the limit of linearity (3000 ng/L) and the bottom of the limit of linearity (500 ng/L) so it is very likely that those concentrations were not accurate.

CONCLUSION

The concentration of both estradiol and ethinylestradiol were measured using ELISA. The concentration of both compounds was found to decrease between the influent and the effluent, however, the concentration of estradiol was found to increase in several of the treatment processes at both the Arcata Marsh and the Ferndale WWTP. The concentration of both E2 and EE2 was most reduced in the oxidation pond, although the concentrations were reduced at the aeration basin as well. The concentration of ethinylestradiol was consistently found to be under 1 ng/L while the concentration of estradiol was above 1 ng/L in the effluent at both sites, with 1 ng/L being the acceptable threshold allowed to prevent feminization of fish. This is consistent with concentrations found in previous studies.

It would be easy to say that all wastewater treatment plants should add a quaternary treatment to deal with the pharmaceutical and estrogen issue based on the results of this experiment, however, that can be incredibly costly (Guo, Englehardt, and Wu 2014) and additional studies would need to be done first. Primarily, the study would determine at what distance after the effluent mixes with the water system does the concentration of estrogen fall below 1 ng/L, the lowest concentration that causes feminization in fish, such as salmonids. Additional research is needed to determine if, the estrogen adsorbed onto the soil is taken out of the system permanently, or if it desorbs back into the water. Lastly, and mostly for regional concerns, it is necessary to determine whether the organic matter in the Humboldt Bay is bioaccumulating estrogen in their

systems. These studies could provide information necessary to make decisions regarding the necessity of further treatment of the effluent. It is possible that once the effluent mixes with the Bay water, it is rapidly diluted below the thresholds of concern. If that is the case, even during low-flow seasons, then it would be unnecessary to add additional treatments for the sake of estrogen alone. However, if it was found that even at these low concentrations the oysters are bioaccumulating estrogen past a safe point for consumption, then it would be a wise investment to research methods of estrogen removal out of the wastewater. For places, like Arcata, that are concerned with restoring salmonids to many of the water systems, it might be necessary to take additional precautions involving wastewater treatment. Although the concentrations of estrogen might be at a safe level now, the population of the area could increase to a point where so much additional estrogen is being added to the water that it is no longer conducive to the restoration of salmonids and other species in the water systems.

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APPENDIX

Table legends:

S1-8 : Standard 1-8
A: Arcata
F: Ferndale
In: Influent
PC: Primary Clarifier
OP: Oxidation Pond
TW: Treatment Wetland
Ef: Effluent
AB: Aeration Basin
PF: Pre filtration holding basin
Po: Post-filtration
(1): July Sample
(2): September Sample
(3): October Sample

Table 6: Set up of plate 1 of Estradiol for ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	S1	S1	A In 1	A In 1	A In 1	A Ef 2	A Ef 2	A Ef 2	F PF 1	F PF 1	F PF 1
B	Blank	S2	S2	A PC 1	A PC 1	A PC 1	A In 3	A In 3	A In 3	F Po 1	F Po 1	F Po 1
C	NSB	S3	S3	A OP 1	A OP 1	A OP 1	A PC 3	A PC 3	A PC 3	F Ef 1	F Ef 1	F Ef 1
D	NSB	S4	S4	A Ef 1	A Ef 1	A Ef 1	A OP 3	A OP 3	A OP 3	F In 2	F In 2	F In 2
E	B_o	S5	S5	A In 2	A In 2	A In 2	A TW 3	A TW 3	A TW 3	F AB 2	F AB 2	F AB 2
F	B_o	S6	S6	A PC 2	A PC 2	A PC 2	A Ef 3	A Ef 3	A Ef 3	F PF 2	F PF 2	F PF 2
G	B_o	S7	S7	A OP 2	A OP 2	A OP 2	F In 1	F In 1	F In 1	F Po 2	F Po 2	F Po 2
H	TA	S8	S8	A TW 2	A TW 2	A TW 2	F AB 1	F AB 1	F AB 1	F Ef 2	F Ef 2	F Ef 2

Table 7: Set up of plate 2 for Estradiol on ELISA

	1	2	3	4	5	6
A	Blank	S1	S1	F In 3	F In 3	F In 3
B	Blank	S2	S2	F AB 3	F AB 3	F AB 3
C	NSB	S3	S3	F PF 3	F PF 3	F PF 3
D	NSB	S4	S4	F Po 3	F Po 3	F Po 3
E	Bo	S5	S5	F Ef 3	F Ef 3	F Ef 3
F	Bo	S6	S6			
G	Bo	S7	S7			
H	TA	S8	S8			

Table 8: Raw Data of full plate of Estradiol

Temp	1	2	3	4	5	6	7	8	9	10	11	12
a	0.2221	0.3149	0.3291	0.4698	0.4495	0.5312	0.8573	0.8278	0.8857	1.0338	0.9819	0.9905
b	0.2310	0.4119	0.4223	0.4445	0.4307	0.4463	0.3386	0.4272	0.4578	1.0803	1.0697	1.0299
c	0.2245	0.5131	0.6014	0.8884	0.8761	1.0070	0.5094	0.5299	0.562	1.1059	1.1608	1.1320
d	0.2225	0.6323	0.5816	0.5468	0.5331	0.5598	1.0137	0.8917	0.8950	0.4485	0.4826	0.5620
e	1.3177	1.1017	1.1007	0.5030	0.4998	0.5069	0.8402	0.8210	0.9235	1.0384	1.0533	1.0917
f	1.3460	1.1581	0.9916	0.5181	0.5138	0.5092	0.6530	0.6536	0.6635	1.0789	1.1144	1.0877
g	1.3629	1.1916	1.1896	0.9820	0.9905	1.0714	0.5312	0.5127	0.5102	1.0438	1.072	1.105
h	0.2583	1.2692	1.9584	0.9423	0.9149	0.9062	1.0193	1.0014	0.9811	1.1180	1.0874	1.066

Table 9: Raw data for estradiol half plate on ELISA

Temperature(¡C)	1	2	3	4	5	6
25	0.2231	0.3231	0.3413	0.5690	0.5659	0.5630
	0.2245	0.3680	0.4189	1.1629	1.1269	1.0601
	0.2293	0.4667	0.4512	1.1775	1.1114	1.2616
	0.2302	0.5918	0.5936	1.1458	1.2232	1.2192
	1.3405	0.9074	0.7424	1.1742	1.1693	1.2609
	1.4641	1.2559	1.0080	1.5287	1.6066	1.6140
	1.4607	1.2055	1.2364	1.5685	1.5443	1.5977
	0.2576	1.4846	1.4038	1.9366	2.1004	1.7664

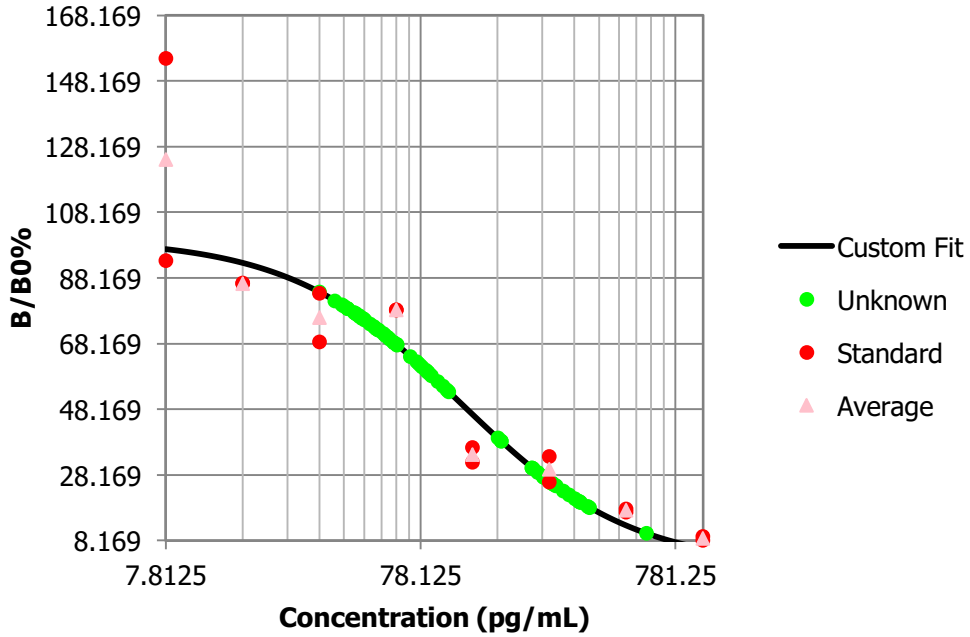

Figure 9: Four-parameter logistic regression curve for Estradiol plate 1

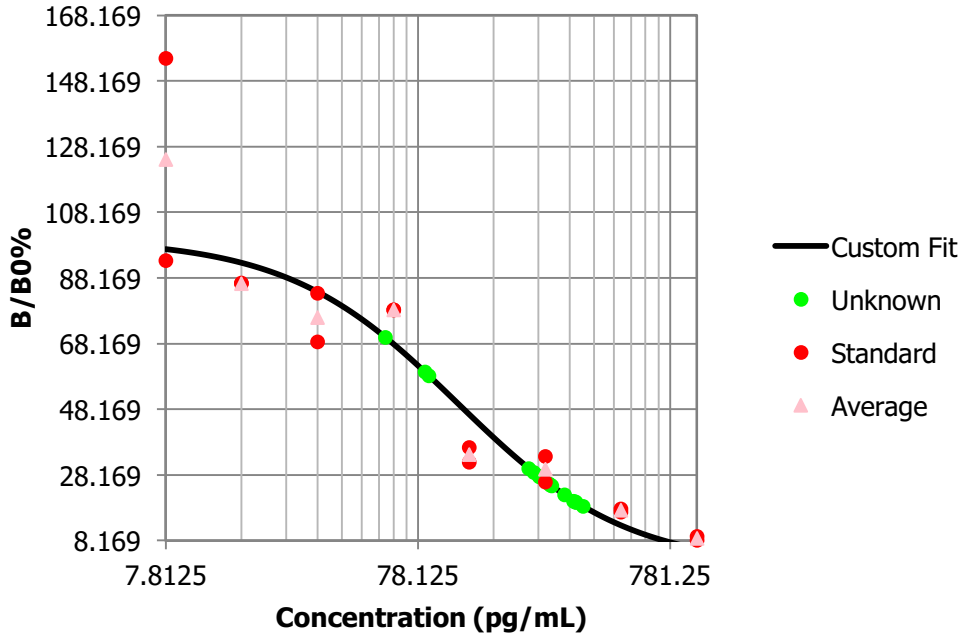

Figure 10: Four-parameter logistic curve for plate 2 for Estradiol

Table 10: Plate 1 set up for Ethinylestradiol on ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	ABS	ABS	A In 1	A In 1	A In 1	A Ef 2	A Ef 2	A Ef 2	F PF 1	F PF 1	F PF 1	F In 3
B	Blank	Blank	A PC 1	A PC 1	A PC 1	A In 3	A In 3	A In 3	F Po 1	F Po 1	F Po 1	F In 3
C	S1	S1	A OP 1	A OP 1	A OP 1	A PC 3	A PC 3	A PC 3	F Ef 1	F Ef 1	F Ef 1	F In 3
D	S2	S2	A Ef 1	A Ef 1	A Ef 1	A OP 3	A OP 3	A OP 3	F In 2	F In 2	F In 2	F AB 3
E	S3	S3	A In 2	A In 2	A In 2	A TW 3	A TW 3	A TW 3	F AB 2	F AB 2	F AB 2	F AB 3
F	S4	S4	A PC 2	A PC 2	A PC 2	A Ef 3	A Ef 3	A Ef 3	F PF 2	F PF 2	F PF 2	F AB 3
G	S5	S5	A OP 2	A OP 2	A OP 2	F In 1	F In 1	F In 1	F Po 2	F Po 2	F Po 2	
H	S6	S6	A TW 2	A TW 2	A TW 2	F AB 1	F AB 1	F AB 1	F Ef 2	F Ef 2	F Ef 2	

Table 11: Plate 2 setup for Ethinylestradiol on ELISA

	1	2	3	4
A	ABS	ABS	F PF 3	F Ef 3
B	Blank	Blank	F PF 3	F Ef 3
C	S1	S1	F PF 3	F Ef 3
D	S2	S2	F Po 3	
E	S3	S3	F Po 3	
F	S4	S4	F Po 3	
G	S5	S5		
H	S6	S6		

Table 12: Raw data for full plate of Ethinylestradiol on ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.4120	0.4166	6.6434	5.2590	4.1759	6.0571	6.2687	6.3426	5.2776	5.4932	6.0537	4.4992
B	8.1705	7.8535	4.8971	5.5512	3.7715	4.4522	4.2896	4.3872	5.4161	5.1154	5.7066	5.9898
C	0.3263	0.2654	5.7125	6.0172	5.0649	4.9341	4.3983	4.1851	5.5940	5.8013	5.9012	5.6539
D	0.4605	0.4205	5.8320	5.4409	4.7977	5.3518	5.6909	5.4141	4.7160	5.2292	4.9825	6.3663
E	1.1041	1.1302	4.9301	5.1598	4.4555	6.6022	5.8836	5.7103	4.8990	5.0622	4.9888	5.9242
F	2.5423	2.5515	4.8342	5.7094	4.3654	6.4948	5.9286	5.5883	5.4489	5.3585	4.9208	6.3870
G	4.8538	4.7854	5.9087	6.1297	5.6307	4.4328	4.9537	4.7517	5.0091	5.0591	5.2340	7.7206
H	7.6404	7.2490	6.6069	6.7766	6.1518	4.5347	5.7367	4.9979	5.9903	6.0089	6.0379	9.3526

Table 13: Raw data for half plate of Ethinylestradiol on ELISA

Temperature(C)	1	2	3	4
25	0.401789	0.411632	6.918854	6.767639
	8.488029	8.419365	6.510682	6.450884
	0.349799	0.311285	6.134516	6.584948
	0.569268	0.477615	6.448393	8.002956
	1.008499	1.139451	6.405526	7.275542
	2.86094	2.345484	6.317256	8.271984
	4.925317	4.573824	7.804363	8.572883
	5.282557	6.191824	7.740298	7.882503