Estrogenic Activity of Wastewater at Different Stages in Three Activated Sludge Municipal Wastewater Treatment Plants in Central Tennessee

by

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ABSTRACT

It is known that endocrine disrupting compounds can be found in the effluent of wastewater treatment plants. Included in this category are estrogens and estrogenic chemicals. When effluent from wastewater treatment plants enters aquatic systems, estrogens in the effluent can have a negative effect on the development and reproductive system of organisms found there. Many studies have demonstrated these effects in fish and mollusks. These estrogenic compounds are found in the effluent because wastewater treatment plants are not designed to break down pharmaceutical or hormonal compounds. The aim of the present study was to follow estrogenic activity through three similar wastewater treatment plants in Central Tennessee. Water samples were taken from each plant's influent and effluent, as well as from the oxidation ditch where the activated sludge process occurs and from water that had yet to be disinfected with ultraviolet radiation. These water samples were examined for estrogenic activity using a bioluminescent yeast estrogen screen (BLYES) assay and liquid chromatography-mass spectrometry (LC-MS). Estrogenic activity was then compared along points in the treatment process for each of the wastewater treatment plants to determine if there is a particular step in the process that significantly decreases estrogenic activity. There was no significant difference ($F_{(3,11)} = 2.87$, $p = 0.104$) of estrogenic activity among points in the treatment process, nor was there a significant difference ($F_{(3,11)} = 2.12$, $p = 0.176$) in individual estrogenic compound concentrations among treatment points. However, there does seem to be a general trend of decreasing estrogenic activity and concentration from influent to effluent.

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INTRODUCTION

The neuroendocrine system is responsible for the regulation of hormones such as estrogens. Estrogens are a group of hormones produced naturally by both males and females; however, they are typically produced in higher concentrations by females. Some of the most common estrogens are estrone (E1), 17β-estradiol (E2), estriol (E3), and synthetic 17α-ethinylestradiol (EE2) (Baronti et al. 2000). The levels of these hormones in humans vary from person to person, but there are some general trends. Levels of E1 and E2 are much higher than E3 in non-pregnant women, whereas levels of E3 are much higher than E1 and E2 in pregnant women (Ying, Kookana, and Ru 2002). E1 is the most predominant of the estrogens produced after menopause, and E2 is mainly produced before menopause (Ying, Kookana, and Ru 2002). These estrogens are responsible for the development of the female sex organs, and also play a major role in pregnancy and ovulation cycles (Baronti et al. 2000; Metcalfe et al. 2001). EE2 is a synthetic estrogen found in oral contraceptives. Oral contraceptives are usually composed of both EE2 and progestin, which is the synthetic form of the body's natural hormone progesterone.

In premenopausal women, between 10 and 100 micrograms of E1, E2, and E3 are excreted on a daily basis (Baronti et al. 2000). Women taking birth control or who are on hormone replacement therapy also excrete concentrations of EE2. However, pregnant woman can excrete up to 30 milligrams of estrogen on a daily basis (mainly E3) (Baronti et al. 2000). These estrogens are excreted in the urine as conjugates of glucuronic and sulfuric acids (Andersen et al. 2003). These conjugates are not able to function biologically, but when released into the environment, these conjugates can be

metabolized by bacteria, and the hormones can then be freed to become biologically active (Andersen et al. 2003; Baronti et al. 2000; Xu et al. 2012).

Wastewater treatment plants (WWTPs) are suspected to be a primary source of estrogens that are released into aquatic environments (Kirk et al. 2002; McAvoy 2008; Xu et al. 2012; Baronti et al. 2000). These estrogens mainly come from the urine of premenopausal women and the urine of women who are taking birth control pills or who are on hormone-replacement therapy (Baronti et al. 2000; Metcalfe et al. 2001; Shi et al. 2004). Because WWTPs use microorganisms as a stage of treatment, the conjugates excreted in urine may become metabolized (deconjugated) to become biologically active during this process (Shi et al. 2004).

Because studies have shown that exposure of aquatic organisms to these hormones can lead to abnormalities in reproductive function (Jobling et al. 2002; Jobling et al. 1998; Kidd et al. 2007; Kirk et al. 2002; Servos et al. 2005), it is important to determine whether there are significant amounts of endocrine-disrupting compounds in treatment plant effluent, as well as how estrogenic activity changes as they go through the treatment process. The level and quality of water purification varies among individual wastewater treatment plants; however, the fundamentals of water treatment are typically the same (Figure 1).

A typical WWTP first filters the water through coarse screens which have openings of 6-10 millimeters. This step rids the water of rocks, sticks, sand, gravel, or any other large objects that are in the water. As part of this primary phase, the water passes through a comminutor which is a pump that has a rotating screen that cuts large fragments of organic matter into smaller ones. In a grit chamber, small objects that are

more dense than the organic matter sink to the bottom. The last step of the primary treatment phase is sedimentation, which allows the different components of the water to settle out based on density and buoyancy (Andersen et al. 2003).

The secondary phase of wastewater treatment can vary from plant to plant. One of the most common forms of secondary treatment is the activated sludge process. In this process, oxygen is supplied to microorganisms that feed on organic matter. The microorganisms will feed on any leftover organic compounds that make it through the first phase of treatment. Nutrient removal such as denitrification occurs during this stage as well. Water mixed with microorganisms is then allowed to sit in an anoxic zone with very little dissolved oxygen. Microorganisms are then forced to obtain oxygen from the nitrates and nitrites found in the water which in turn releases the freed nitrogen molecules into the atmosphere. The water is then allowed to settle again in a clarifier so the supernatant can go through more treatment phases, if applicable (Andersen et al. 2003). For this study, all plants selected for analysis use the activated sludge treatment process.

Another common type of treatment process other than the activated sludge process is the trickling, or percolating, filter process. The secondary phase for this type of wastewater treatment process is to allow the water to percolate through a loose bed of stones covered in microorganisms that feed on organic matter. The main difference in these two processes is that for activated sludge, the oxygen is supplied to the bacteria by a mechanical source, and for the trickling process the oxygen is supplied by the environment (Andersen et al. 2003).

Some WWTPs put water through one final, or tertiary, treatment phase. In this optional phase, specific pollutants such as phosphorous and harmful microbes such as

viruses are removed from the water by using very fine filters (nanometers instead of millimeters). Also, disinfection of the water by ultraviolet (UV) radiation, chlorination, or ozone can occur in this phase (Annachhatre 2006; Andersen et al. 2003).

Even with these steps to clean wastewater, the effluent of WWTPs can still contain endocrine-disrupting compounds such as estrogen hormones, or other compounds with estrogen-like behaviors, because the treatment process is not specifically designed to remove hormones or other pharmaceuticals (Andersen et al. 2003; Zorita, Mårtensson, and Mathiasson 2009). According to previous studies, estrogen hormones found in the effluent are predominantly natural estrogens (E1, E2, and E3), but there can still be noticeable concentrations of synthetic EE2 (Ternes et al. 1999).

Estrogens are very important in all organisms for normal reproductive and developmental processes such as sexual maturation and differentiation; however, exposure to estrogens at inappropriate times or in increased concentrations can be problematic (Servos et al. 2005). For example, when exposed to estrogens during sexual differentiation, sex-reversal and even intersexuality (having both male and female gonadal characteristics) can occur in male fish, as well as less successful spermatogenesis (Jobling et al. 1998; Kidd et al. 2007; Servos et al. 2005). Many WWTPs are now trying to take steps to decrease the amount of estrogens released in the effluent (Hemminger 2005; Isabelle et al. 2011; Servos et al. 2005); however, it only takes a very small amount (<10 ng/L) to potentially cause an effect (Isabelle et al. 2011; Servos et al. 2005; Shi et al. 2004).

A method that has been used widely in various studies to measure the presence of compounds with estrogenic activity in aquatic systems is the bioluminescent yeast

estrogen screen (BLYES) (Sanseverino et al. 2005). In this assay, a strain of yeast (*Saccharomyces cerevisiae*) with the human estrogen receptor (hER-α) gene on its chromosome is transformed with the pUTK404 and pUTK407 plasmids (Sanseverino et al. 2005). The pUTK404 plasmid contains the *lux*-C, -D, and -E aldehyde synthesis genes that originate from the bacterial organism *Photorhabdus luminescens* as well as the *frp* gene from *Vibrio harveyi* that provides the cofactor $FMNH_2$. These products are essential for the bioluminescent response to occur (Sanseverino et al. 2005). A yeast internal ribosome entry site (IRES) is also included in pUTK404 to initiate the translation of the genes found on this plasmid (Sanseverino et al. 2005). The pUTK407 plasmid contains divergent GPD and ADH1 promoter sequences and multiple estrogen-response elements (ERE) (Sanseverino et al. 2005). When the estrogen receptor proteins are bound by an estrogen-like compound, they then bind to the estrogen response element. This in turn activates the transcription of *lux*-A and *lux*-B found on the pUTK407 plasmid which causes a measurable bioluminescent response (Figure 2) (Sanseverino et al. 2005).

The goal of the present study is to determine if the amount of estrogenic activity and/or the concentration of estrogenic compounds among three similar municipal wastewater treatment plants in Central Tennessee decreases significantly at one point along the treatment process to determine if there is a particular step that may help to reduce estrogenic activity or compound concentration.

MATERIALS AND METHODS

Study Design

Three municipal WWTPs were selected in Central Tennessee that serve fairly large communities and treat their water in a similar way. These plants were located in Shelbyville, Murfreesboro, and Smyrna, TN. All of these plants treat their wastewater with activated sludge during the secondary phase of treatment and UV radiation during the tertiary phase of treatment. The Shelbyville, TN WWTP is a slightly smaller plant that treats water at a rate of about 3 million gallons per day (MGD) (Bert Troxler, personal communication, April 2015). The Smyrna, TN WWTP treats about 6 MGD (Shannon Pratt, personal communication, May 2015), and the Murfreesboro, TN WWTP is larger and can treat around 16 MGD (Josh Smith, personal communication, April 2015). Samples were taken from four different points at all facilities: the raw influent, the oxidation ditch where the activated sludge process occurs, before the UV treatment process, and from the effluent. Figure 1 shows these sample points on a schematic overview of the treatment process.

Water Extraction and Sample Preparation

Two one liter samples were collected on four separate days from each of the four test points at each WWTP. Samples were then stored on ice in glass amber jars until extraction using solid phase extraction, within 8 hours of collection. One of the two samples from each of these test points from each sample date was spiked with 25µL of 200 ppm 4-nonylphenol stock solution (final concentration of 5 ppb) to act as a marker during high performance liquid chromatography (HPLC) analysis to test for extraction efficiency. Estrogenic compounds were extracted from water samples using 47 mm

Empore™ C18 solid phase extraction disks. If extraction disks became clogged during extraction of exceptionally turbid samples, multiple extraction disks were used. Estrogenic compounds were eluted from disks with 10 mL of methanol, and the samples were collected in small (10 mL) glass vials. If multiple disks were used, the methanol eluents were combined. The methanol was then evaporated under nitrogen gas, and the dried samples were then resuspended in either 0.5 mL of methanol or 0.5 mL of dimethyl sulfoxide (DMSO). The samples that had been spiked with 4-nonylphenol were resuspended in methanol for HPLC analysis. The remaining samples were resuspended in DMSO to be used for the BLYES assay and LC-MS analysis.

Initially, samples were brought up in methanol for both the HPLC analysis and the BLYES assay, however, upon reading the plates for the BLYES assay on the ChemiDoc™ MP Imaging System from Bio-Rad Laboratories it appeared that all wells had equal amounts of bioluminescence. Even the wells used for the standard curve, which should have shown a decreasing amount of luminescence as the 17β-Estradiol concentration decreased, also showed equal amounts of bioluminescence indicating that the methanol itself was having an estrogenic effect as shown by the BLYES assay (Figure 2). This led to the use of DMSO for the water samples being used for the BLYES assay, as this chemical did not have any measurable estrogenic effect. An example of a 96-well plate where DMSO was used instead of methanol is shown in Figure 2.

Bioluminescent Yeast Estrogen Screen (BLYES) Assay

Yeast (*Saccharomyces cerevisiae*) strains transformed with plasmids coding for the human estrogen receptor gene (hER-α) and (ERE)-*lux* (donated by the University of Tennessee, Knoxville, TN) were grown in 30 mL of yeast minimal media (YMM) (leu^-,

ura$^-$) in a sterilized 125 mL Erlenmeyer flask overnight at 30˚C with gentle shaking until an optical density of 1.0 at 600 nm was reached (Figure 3). A set of standards consisting of 17β-estradiol (E2) diluted in DMSO was created ($2.5x10^{-11}$-$1x10^{-5}$ M), and extracted water samples were diluted to give a range of nine different concentration factors from 50 to 2000 fold dilutions. Using 96 well plates, 4 µL aliquots of each of the standards and water sample dilutions were added to wells, along with 196 µL aliquots of transformed yeast cells. The plates were incubated at room temperature for four hours, and then bioluminescence was measured using the ChemiDoc™ MP Imaging System from Bio-Rad Laboratories. Using this system, pictures were generated with the associated Image Lab 4.0 computer program. Luminescence was measured using the ImageJ 1.48v computer program. Background luminescence was measured from wells including only DMSO, and this was subtracted from all other luminescence readings.

A standard curve was plotted using a logarithmic scale for concentration. Sample dilutions were compared to the linear portion of the standard curve, and a dilution with a luminescence value measuring near the center of the standard curve was used to determine initial water sample estrogenic activity expressed in ng/L of E2 equivalents.

Extraction Efficiency and Estrogen Concentration Analysis

Liquid-chromatography-mass spectrometry (LC-MS) analysis was carried out on each of the 4-nonylphenol-spiked samples to determine the efficiency of the extraction technique. For LC-MS analysis, a Dionex U3000 HPLC system was used with a LPG-3400SD pump and a WPS-3000SL autosampler. The column compartment used was a TCC-3000SD, and the column was a Thermo Accucore C18 column sized at 150mm x 2.1mm x 2.6µm. A MS Thermo MSQ Plus mass spectrometer system was used under

control of Chromeleon version 6.8 software. The mobile phases used were water and methanol, which both had a $MeNH_3OH$ concentration of 0.5 mM.

On two of the four sets of extracted water samples, LC-MS analysis was implemented in addition to the BLYES assay to determine estrone (E1), 17β-estradiol (E2), estriol (E3), and 17α-ethinylestradiol (EE2) concentrations along the treatment process.

Standards of each estrogenic compound were made by dissolving each compound in DMSO to produce a linear curve ranging from 0.1 to 10 ppm. Table 1 shows the running conditions for each sample, and Table 2 shows the channels used for each compound during mass spectrometry.

Statistical Analysis

The three WWTPs were considered replicates based on the fact that each plant treats their water in the same way, and the individual measurements taken at each point in the treatment process were averaged together before statistical analysis.

To determine if the stages of treatment tested for each WWTP had an effect on the estrogenic activity or E1, E2, E3, or EE2 concentration found, a one-way analysis of variance (ANOVA) was used to analyze the data using the JMP software created by SAS. The data sets from both the BLYES assay and LC-MS analysis failed to meet the assumption of equal variance, so the data sets were both log-transformed to meet this assumption. The significance value was $\alpha = 0.05$.

RESULTS

Extraction Efficiency Analysis

Extraction efficiencies for several of the samples were below 50%, yet all samples were still included in the final analysis. After the technique was performed a few times, however, extraction efficiencies ranged from 60-90%. Because extraction efficiencies are based on 4-nonylphenol, these are estimates of extraction efficiency for all estrogenic compounds. Since it is not known for sure which chemicals and/or compounds contribute to the overall estrogenic activity of the water samples, all estimates of estrogenic activity obtained during the BLYES assay could be conservative estimates.

BLYES Assay

The mean concentration of estrogenic compounds determined from the BLYES assay in terms of 17β-estradiol (E2) equivalents (ng/L) ranged from ~5-2,000 ng/L across all sites averaged together (Table 3). Estrogenic activity was not different based on stage of the WWTP process ($F_{(3,11)} = 2.87$, $p = 0.104$). There did, however, seem to be a general trend showing a decrease in estrogenic activity along the points in the treatment process (Figure 4).

Estrogen Concentration Analysis

The concentration of each estrogenic compound found in the WWTPs ranged from 128-24,658 ppt (ng/L) (Table 4). Due to a limited number of detections (some possibly caused by matrix interference) for E1, E2, and EE2, statistical analysis could only be performed on data collected for estriol (E3). There was no statistical difference found among stages of the treatment process for this compound ($F_{(3,11)} = 2.12$, $p = 0.176$) (Figure 5).

DISCUSSION

Estrogenic chemical concentrations of as little as 1-10 ng/L have been shown to have impacts such as intersexuality, decreased spermatogenesis success, or complete sex-reversal on aquatic organisms in previous studies (Isabelle et al. 2011; Jobling et al. 1998; Kidd et al. 2007; Servos et al. 2005; Shi et al. 2004). The concentration of estrogenic compounds (or activity converted to E2 equivalents) found for most samples in the WWTPs in the present study were greater than this threshold, indicating the potential for effects on aquatic organisms located downstream of WWTPs. This is especially important since these compounds can be very potent and can have synergistic effects when combined with one another (Vajda et al. 2008; Thorpe et al. 2006; Servos et al. 2005).

It is important to note that all of the concentrations of estrogenic compounds found in this study are undiluted. Once the effluents from the WWTPs are released into streams, the concentrations would decrease significantly depending on stream size and flow (Johnson 2010; Keller et al. 2014).

It is interesting that E3 was the only estrogenic compound that was consistently above the detection limit since other estrogens are more readily excreted by the average female population. It is possible that a large number of pregnant women could be in the areas serviced by the treatment plants tested, but this does not seem likely. It is important to note that E3 *is* excreted at several orders of magnitude higher in pregnant women than E1 or E2 in non-pregnant women (Baronti et al. 2000), so E3 could arrive at the plant at relatively high concentrations even if there is not a high concentration of pregnant women in the area.

It is not surprising that no particular step in the wastewater treatment process was found to significantly decrease the estrogenic activity since WWTPs are not currently designed to remove pharmaceutical or hormone compounds (Andersen et al. 2003; Zorita, Mårtensson, and Mathiasson 2009). However, in the present study, estrogenic activity and E3 concentration generally seemed to decrease as treatment progressed, with higher estrogenic activity found in the influent compared to the effluent (Figures 4 and 5). Since there is no certain stage that seemed to cause this decrease, further analysis should be done with a larger sample size to decrease the variance among samples, which could potentially be hiding statistical differences between treatment points.

A potential cause of the seemingly lower estrogen activity and E3 concentration found in the effluent could be that the estrogenic compounds were not only deconjugated by the microbial organisms during treatment, but also degraded by these same organisms. For example, studies have shown that E2 can become oxidized by bacteria to become E1, which is more easily degraded by microbes during the treatment process (Shi et al. 2004).

Another aspect to consider is that for the present study, only "grab" samples were obtained for each point in the treatment process. This means that the water samples for different stages within each treatment plant could potentially be different "batches" of water. The holding time for the water in each of the locations tested varies depending on day, antecedent rain events, and other various factors. Since all samples for each WWTP were gathered simultaneously, this could mean that the trends shown here could be significantly different than if the same "batch" of water was followed throughout the treatment process.

Wastewater treatment plants are in need of ways to decrease the amount of

compounds like estrogenic hormones reaching the effluent. Future research should focus on expanding this study to include other types of treatment plants and treatment plants that use varying types of tertiary treatment.

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APPENDICES

APPENDIX A: TABLES

Table 1. Running conditions used for each sample during liquid chromatography-mass spectrometry (LC-MS) analysis to determine estrogenic compound concentration at each test point.

LC-MS Conditions

Sample Size:	10 µL
Initial Flow:	0.27 mL/min
Initial $MeNH_3OH$ concentration:	55%
Column Temperature:	35ºC
Hold Time:	3 min
Ramp 1 of Methanol:	80%
Ramp 1 Time Period:	4 min
End Acquisition at:	8 min
Ramp 2 of Methanol:	55%
Ramp 2 Time Period:	0.1 min
End of Program:	12 min

Table 2. Channels used for each sample during liquid chromatography-mass spectrometry (LC-MS) analysis to determine concentration of estrogenic compounds at each test point.

Compound	MS Channel
Estrone (E1)	269.20 ± 1.00 amu
17β-estradiol (E2)	271.30 ± 1.00 amu
Estriol (E3)	287.30 ± 1.00 amu
17α-ethinylestradiol (EE2)	295.50 ± 1.00 amu

Table 3. Mean concentration in ppt (ng/L) of 17β-Estradiol (E2) equivalents in each sample as determined by the BLYES assay.

Location	Sample	Mean Concentration (ppt)
Shelbyville	Influent	2.21×10^{2}
	Oxidation ditch	6.58×10^{2}
	Pre-UV	2.83×10^{2}
	Effluent	4.72×10^{1}
Smyrna	Influent	1.99×10^{3}
	Oxidation ditch	2.08×10^{1}
	Pre-UV	3.83×10^{1}
	Effluent	9.75×10^{0}
Murfreesboro	Influent	1.33×10^{2}
	Oxidation ditch	3.75×10^{1}
	Pre-UV	5.25×10^{1}
	Effluent	5.26×10^{0}

Table 4. Concentration in ppt (ng/L) of E1, E2, E3, and EE2 at each location and treatment point. Analytical chemistry results from duplicate samples are shown. A dash indicates that the concentration was below the detection limit.

Location	Treatment Point	Estrone (E1) ppt	17β-Estradiol (E2) ppt	Estriol (E3) ppt	17α-Ethinylestradiol (EE2) ppt
Shelbyville	Influent	2.26×10^2	-	1.895×10^3	1.28×10^2
	Influent	-	-	1.8311×10^4	-
	Oxidation Ditch	-	-	6.678×10^3	-
	Oxidation Ditch	-	-	3.502×10^3	-
	Pre-UV	-	-	4.10×10^2	-
	Pre-UV	-	-	4.40×10^2	-
	Effluent	-	-	3.645×10^3	-
	Effluent	-	-	3.435×10^3	-
Smyrna	Influent	-	-	2.4658×10^4	-
	Influent	-	-	4.875×10^3	-
	Oxidation Ditch	-	-	1.500×10^3	-
	Oxidation Ditch	-	-	2.253×10^3	-
	Pre-UV	-	-	9.28×10^2	-
	Pre-UV	-	3.75×10^2	2.252×10^3	-
	Effluent	-	-	2.752×10^3	-
	Effluent	-	-	5.598×10^3	-
Murfreesboro	Influent	-	-	8.07×10^2	-
	Influent	16.541×10^3	-	3.819×10^3	-
	Oxidation Ditch	-	-	2.967×10^3	-
	Oxidation Ditch	-	-	2.873×10^3	-
	Pre-UV	-	-	1.072×10^3	-
	Pre-UV	-	-	6.877×10^3	-
	Effluent	-	-	6.47×10^2	-
	Effluent	-	-	5.318×10^3	-

APPENDIX B: FIGURES

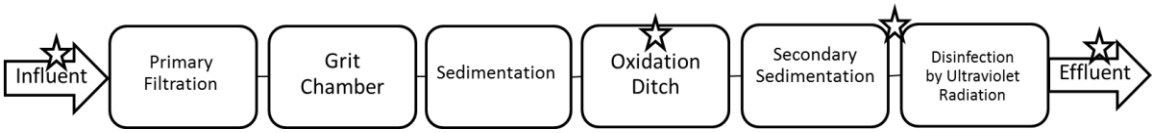

Figure 1. A schematic overview of an activated sludge wastewater treatment plant. Stars represent points along the process where water samples were collected for analysis of estrogenic activity and estrogenic compound concentration.

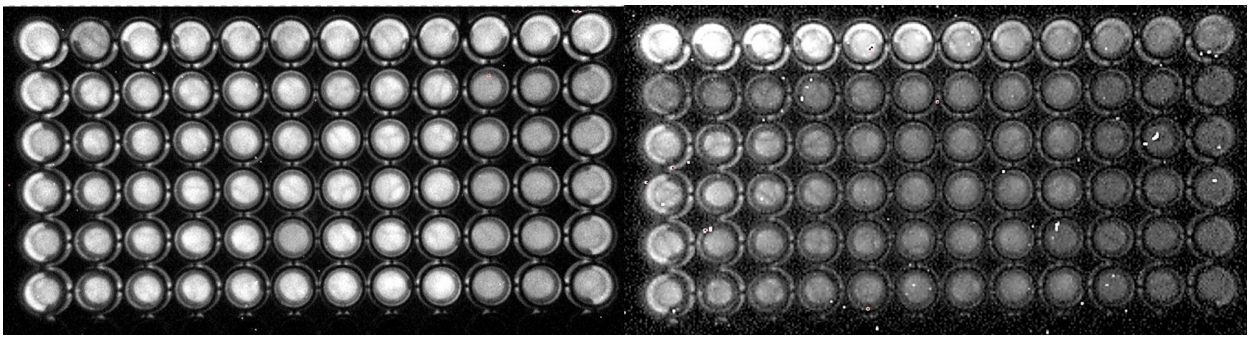

Figure 2. A comparison of methanol and DMSO used in the BLYES assay. The image on the left is of a 96-well plate using methanol as a solvent for the BLYES assay, whereas the image on the right shows the use of DMSO as a solvent instead. As you can see, the estrogenic activity of the methanol masks any estrogenic activity that may have been caused by the water samples, or the standard compound that was used in the top wells (17β-Estradiol).

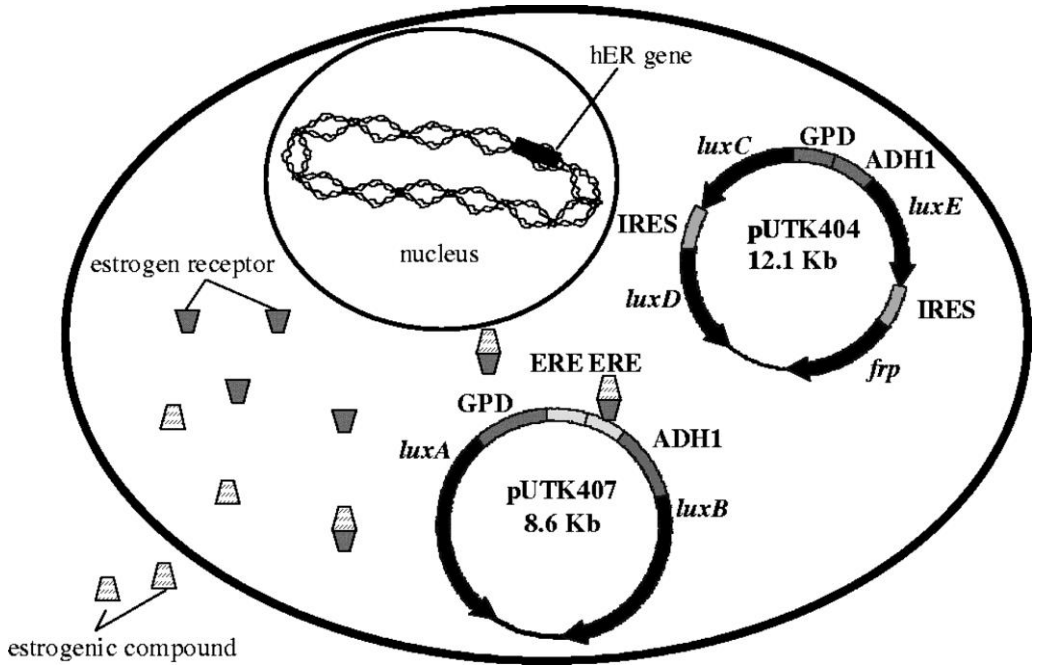

Figure 3. A depiction of the BLYES strain of *S. cerevisiae* used for this study. The human estrogen receptor gene (hER) is found on the chromosome, and the pUTK404 and pUTK407 plasmids have been added to the cell. When estrogenic compounds cross the cell membrane, they interact with the estrogen receptor which in turn binds to the estrogen response element (ERE) to start transcription of *lux*A and *lux*B. (John Sanseverino et al. Appl. Environ. Microbiol. 2005;71:4455-4460

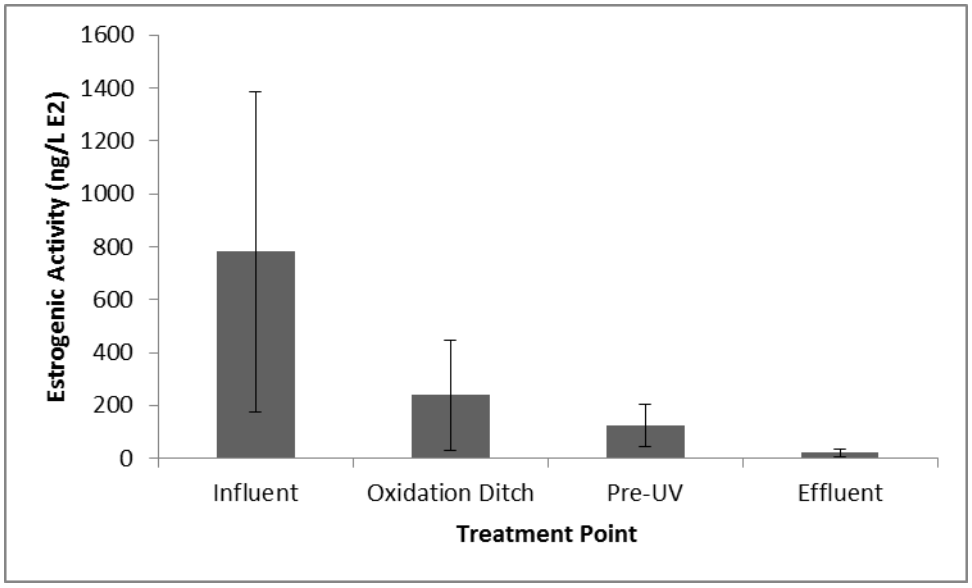

Figure 4. The mean concentration of estrogenic activity from the average of all three WWTPs tested, equated to ng/L of 17β-estradiol (E2) equivalents at each treatment point. There were no significant differences found among points in the wastewater treatment process. Error bars represent the mean ± 1 SE.

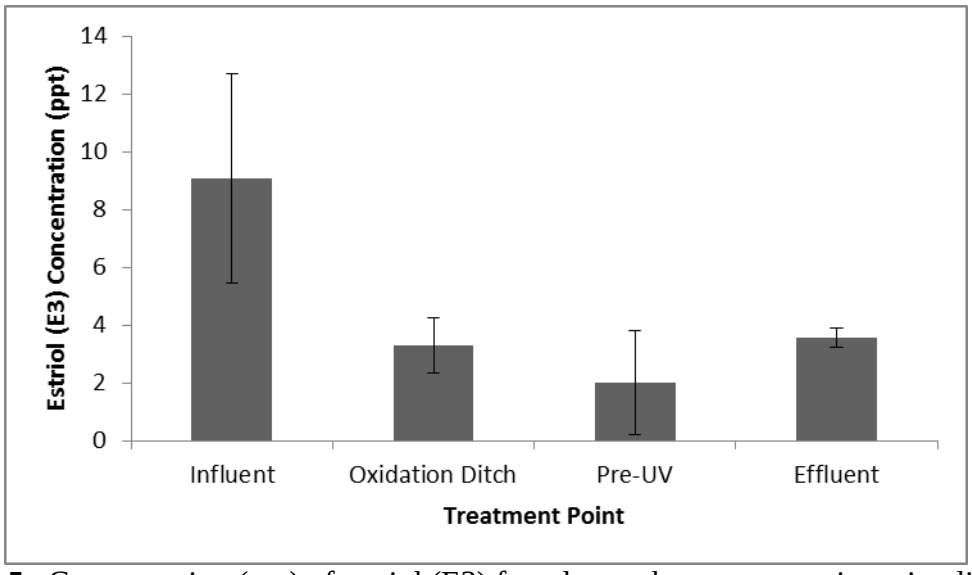

Figure 5. Concentration (ppt) of estriol (E3) found at each treatment point using liquid chromatography-mass spectrometry (LC-MS) analysis. Error bars represent the mean ± 1 SE. There were no significant differences found among points in the wastewater treatment process.