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17β-ESTRADIOL IN CARBONDALE TREATED WASTEWATER EFFLUENT: A CROSS COMPARISON STUDY

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17β-ESTRADIOL IN CARBONDALE TREATED WASTEWATER EFFLUENT:

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By

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TITLE: 17β-ESTRADIOL IN CARBONDALE TREATED WASTEWATER EFFLUENT: A CROSS-COMPARISON STUDY

MAJOR PROFESSOR: Dr. Mary Kinsel

Natural estrogens are endocrine disrupting compounds and common pollutants in municipal wastewater. The concentration of 17β-estradiol was monitored in effluent from both the southeast and northwest Carbondale wastewater treatment plants (WWTPs) and their receiving waters for nine weeks. The analysis was performed using gas chromatography tandem mass spectrometry (GC/MS/MS) and the internal standard estrone 3-methyl ether. Recoveries were 60.0±3.9%, and significant loss of analyte was found after storage greater than one week. The northwest effluent (NWE) had higher 17β-estradiol levels of 7.1-76.2ng/L than the southeast effluent (SEE) between Below Detection Limits (BDL)-54.0ng/L, which suggests 17β-estradiol was carried with colloidal organic particulates. River water had very similar 17β-estradiol concentrations compared to the effluent despite dilution. The university exhibited no measurable effect on 17β-estradiol levels when samples from in-session were compared with samples from out-of-session. Future ecological studies are recommended to determine the effect of estrogenic pollution on fish populations of receiving waters.

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Chapter One

INTRODUCTION

Endocrine disrupting compounds (EDCs) are a large class of components that mimic hormones and include pesticides, phytoestrogens, alkylphenols, and synthetic estrogens. While such synthetic chemicals are a cause for concern, it was found that natural estrogens are nearly 1000 times more biologically potent.[1] Estrogens occur in the bloodstream of all mammals and are biosynthesized from cholesterol. Estrogens are necessary for health, but also exhibit mitogenic and mutagenic properties.[2] Mammals quickly "de-toxify" estrogens into conjugated forms by esterification with a sulfate or glucuronide functional group. Liu et al.[3] summarized that conjugation has two effects: 1) to increase polarity of the molecule and allow for excretion, and 2) to decrease the estrogenic potency of natural estrogens. The conjugated estrogens are excreted and found in wastewater.

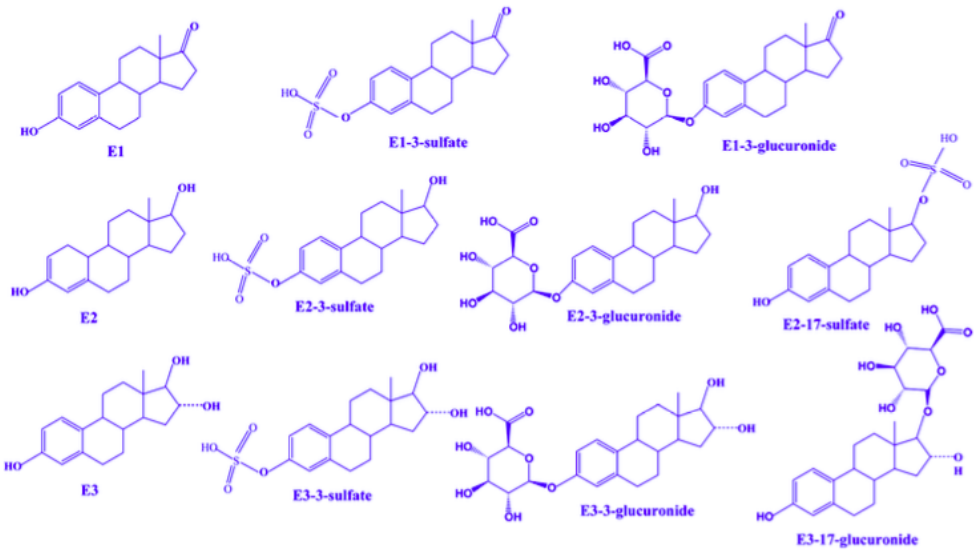

Figure 1. The three natural estrogens and their conjugates.[3]

There are three naturally occurring estrogens: 17β-estradiol (E2) and its metabolites, estrone (E1), and estriol (E3). Conjugated estrogens are predicted by *in vitro* batch studies to decompose into their natural (non-conjugated) forms during wastewater treatment, but studies on real WWTPs indicate presence of conjugates in some treated effluents.[3] De-conjugation is catalyzed by bacterial enzymes, particularly arylsulfatase and β-glucuronidase, both of which are found in *Escherichia Coli* (E. Coli).[3] After de-conjugation, estrogens can be interconverted. There is substantial evidence that 17β-estradiol and estriol are primarily decomposed into estrone.[4,5]

Studies show that estrogens are primarily released at the low ng/L level from wastewater treatment plant effluent[4], while elevated levels have been found in dairy farm effluent.[6] Research has documented environmental impacts of low-level estrogen releases. The earliest published impacts include the production of vitellogen, a female reproductive protein, found at elevated levels in male trout in estrogen-spiked water.[7] Vitellogen has now been so widely studied that it is considered an indicator of estrogenic disruption in surface water. Estrogenic disturbances have also been shown to decrease egg production in fish[8], and *in vivo* studies have even shown population collapse.[9] Anderson et. al.[10] generated a short-term predicted-no-effect concentration (PNEC) of 5ng/L in surface water, and a long-term PNEC of 2ng/L.

Table 1. Estrogens in the environment reported in ng/L. Adapted from Adeel and coworkers.[4]

Sample Type	E1	17α-E2	17β-E2	E3
Slurry in Swine pit	5900-150,000	4000-84,000	1800-49000	NDA
Swine Farm Effluent	5200-5400	650-680	1000-1500	2200-3000
Slurry in dairy pit	2500-80,000	2000-5000	800-27,000	NDA
Treated cattle feedlots	720	1100	1250	NDA
Dairy farm wastewater	370-2356	1750-3270	351-957	NDA
Lagoon Pond	650	NDA	NDA	NDA
Biogas digestate	593	50	24	NDA
Sow urine	416-490	NDA	85-97	127-193
Grazing land water	78	31	18	NDA
Swine manure	70	175	15	NDA
Swine manure leachate	68.1	2.5	NDA	NDA
1 m deep groundwater	68.1	NDA	2.5	NDA
STP/effluent	12-196	6.4-12.6	6.2-42.22	NDA
Sea Water	NDA	NDA	0.83	NDA

NDA: No data available.

Given the environmental impacts, concern exists about estrogen concentration in municipal drinking water. Elevated levels have been linked to increased incidence of breast cancer in females[11], but it is debatable as to whether elevated estrogen levels are cause or effect. Published studies have also correlated estrogen exposure and decline of sperm counts, as well as other reproductive disorders of men.[12] In a study on estrogens in sports drinks, Plotan et al.[13] used a no-observed-adverse-effect limit (NOEL) for humans of 0.3mg/d[13], but as Adeel and coworkers[4] pointed out, an acceptable daily limit is almost certainly lower than this level, and this subject requires more research. The concentrations in drinking water are significantly below this level; a Chinese study reported a maximum value of 1.7ng/L in headworks.[14] A

computational study on wastewater reuse concluded that estrogens in drinking water should not be of concern; although >50% of drinking water plants experienced unplanned wastewater reuse, the magnitude of reuse was very low (<1%).[15] It must be noted that all reported levels in effluent are significantly below NOELs, and immediate adverse effects for humans are unlikely. Future research is required to elucidate long-term effects.

This study focuses on 17β-estradiol in treated wastewater effluent from Carbondale, Illinois. The most studied and most potent of the natural estrogens, very low (ng/L) levels of 17β-estradiol are common in wastewater effluent. The levels detected tend to depend on the nature of the treatment, with the activated sludge process producing the lowest concentrations.[16] Three studies in the US reviewed by Liu et al.[3] reported 2.3, 6.4 and 0.5 ng/L of 17β-estradiol in treated effluent, but substantially higher values have been found. Considerably lower levels are reported in receiving river water due to the effects of dilution and degradation of the analyte. Lagana and coworkers[17] reported values in river water of 4ng/L, as lower than effluent concentrations of 3-8ng/L. Kumar et al.[18] reported receiving river water at 1.4ng/L. Despite the fact that sewage effluent has been more widely studied, the largest point source of estrogenic pollution by far is dairy farm effluent; Gadd and coworkers[6] reported values between 1-310ng/L (average of 24ng/L) for 17β-estradiol from dairy farm effluent and very high levels of estrone (10-580ng/L, average of 100ng/L). Livestock manure is frequently applied to farm fields; agriculture run off has been proposed as a very large source of estrogens, but very little is known about this potential source of pollution. The values anticipated in this study for treated sewage effluent were in the low ng/L range.

The city of Carbondale, Illinois, has two wastewater treatment plants, one in the northwest and one in the southeast, and their operating zones roughly bisect the city. Both plants have a rather conventional set-up for activated sludge process. The northwest plant has a shorter hydraulic retention time (~3-4 hours) compared to the southeast plant (~11-12 hours). The northwest plant also has a trickling filter installed as preliminary treatment of industrial waste from a Prairie Farms dairy processing plant. The northwest plant discharges effluent into the Big Muddy River, which eventually runs into the Mississippi River, while the southeast plant discharges into nearby Crab Orchard Creek, which subsequently joins the Big Muddy and Mississippi Rivers. It was anticipated that the effluent from the southeast plant would have higher levels of estrogen due to the municipal nature of the waste and Carbondale Memorial Hospital. Hospitals have been shown to be point sources for estrogenic waste.[19,20] Southern Illinois University also flows directly to the southeast plant, and may also elevate estrogenic levels when the university is in-session and the student population rises.

The anticipated low levels of 17β-estradiol pose an interesting question about estrogen transportation. As a highly hydrophobic molecule, 17β-estradiol may partition onto dissolved organic material and be carried much further distances than would be expected in clean water. If this were the case, it could be assumed that sorption to sewage sludge could account for a significant percentage of elimination during treatment; however, Muller et al.[21] cited that only 4-6% of estrogen was removed due to sorption during sludge removal. In a more comprehensive study Bowman et al.[22] calculated partition coefficients, K_d, of natural estrogens into different mediums and found the partition coefficient of colloidal solids to be two magnitudes higher than that of sediments. Thus, 17β-estradiol and estrone remain suspended

(as opposed to partitioning into sediment) while adhering to colloidal solids, and are carried much farther in rivers than previously anticipated.

Estrogen analysis is typically carried out by a form of chromatography coupled with a mass analyzer, such as liquid chromatography-mass spectrometry (LC/MS) or gas chromatography-mass spectrometry (GC/MS). Gomes and coworkers[23] reviewed the detection limits to be in increasing order of LC/MS/MS < GC/MS/MS < LC/MS < GC/MS. In general, there are higher detection limits in GC/MS due to risk of analyte loss during the extraction and derivatization steps. Gas chromatography requires that analytes be volatile; estrogens are not and must be derivatized. Derivatization usually involves an alcohol protection; the hydroxyl functional group participates in hydrogen bonding and the associated strong molecular interaction decreases volatility. Hence, protecting the alcohol increases volatility.

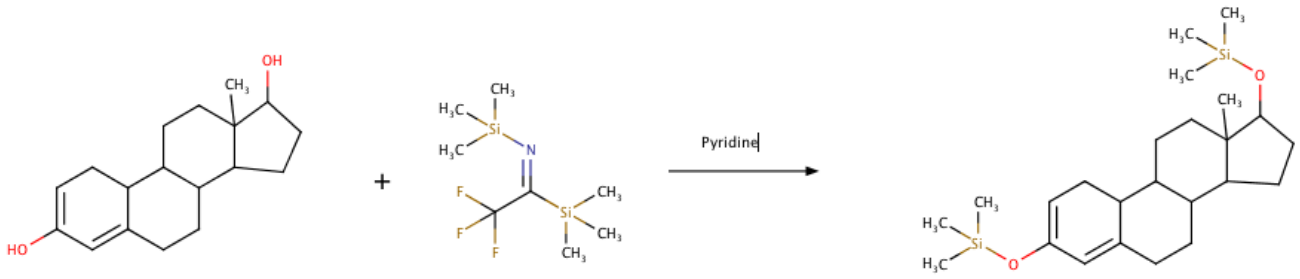

Figure 2. The bis(trimethylsilyl)trifluoroacetamide derivatization of 17β-estradiol

Liquid chromatography is preferred due to lower detection limits, as well as a lack of labor-intensive derivatization. Recently, solid phase micro extraction (SPME) techniques have been reported that avoid both extraction and derivatization steps[24], but that method was not pursued due to constraints imposed by manual fiber exposure. This study uses the GC/MS/MS

technique from Saravanabhavan et al.[25] Method detection limits reported were between 0.5-1.2ng/L for aqueous samples, which is more than adequate for 17β-estradiol analysis in wastewater effluent.[25]

The explicit purposes of this study are 1) to monitor estrogen levels in the treated effluent from the Carbondale wastewater treatment plants and interpret observed trends, especially the effect of a university on effluent, and 2) predict any possible adverse environmental impacts on aquatic and human health.

Chapter Two

MATERIALS AND METHODS

Solvents were purchased from Sigma-Aldrich or Fisher. Ethyl acetate (>99.9%, Sigma Aldrich, 650528), methanol (>99.9%, Sigma Aldrich, 34860-2L-R) and hexane (>95%, Sigma Aldrich, H306-4) were all either HPLC or GC grade, while cyclohexane (>99%, Sigma Aldrich, 179191-2.5L) was an ACS reagent. 17β-estradiol (>98%, Sigma Aldrich, E8875) was stored at ambient temperatures in the dark. Isopropanol (>99.5%, Fisher, 67-63-0) and acetone (>99.5%, Fisher, A18-4) were used for cleaning. Laboratory pure water (>18.0MΩ) was generated with an on-site Millipore Direct-Q™-5 system.

Glassware was washed twice with nonionic dish detergent (Decon™ Contrex™ CF-cation and phosphate free liquid detergent, Fisher, 0435826), rinsed once with acetone, again with isopropanol and a final rinse was completed with Millipore water.

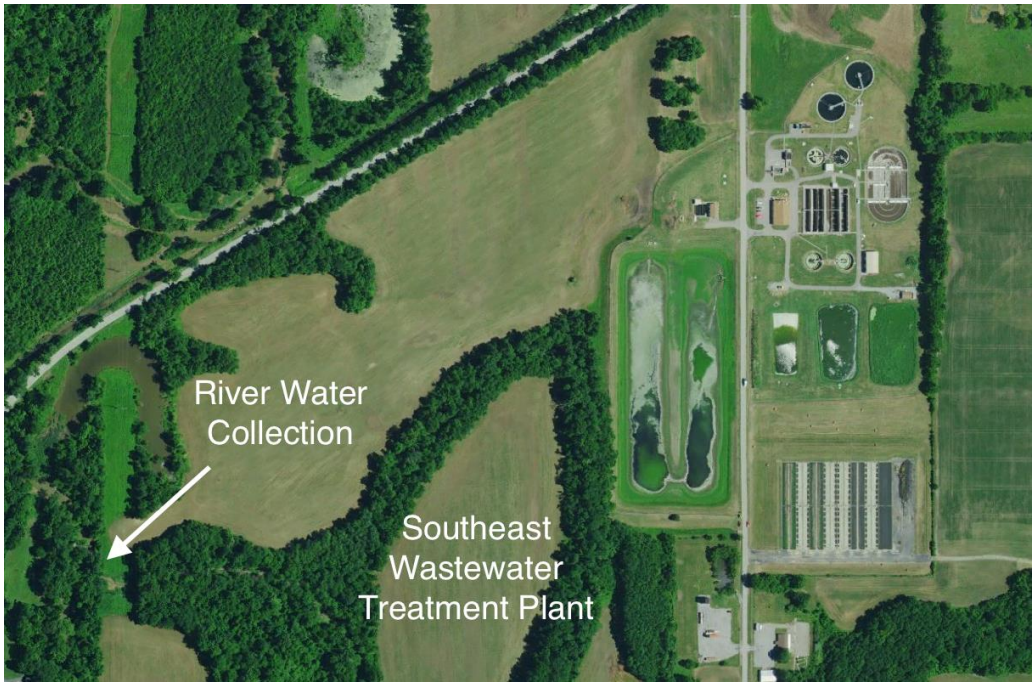

Figure 3. Aerial view of southeast WWTP and location of southeast river water collection.

Effluent samples were collected for nine weeks from both the southeast and northwest WWTPs. Toward the end of the study, four weeks of receiving river water was collected from both the Big Muddy River off of Old Route 13 bridge, and from Crab Orchard Creek close to the effluent outfall. Sample collection started on 11/3/2017 and ended the week of 2/9/2018. The collection period spanned the winter, as well as periods of Southern Illinois University being in and out of session. Grab samples were collected at 9:00am ±2hours, and transported to the laboratory the same morning.

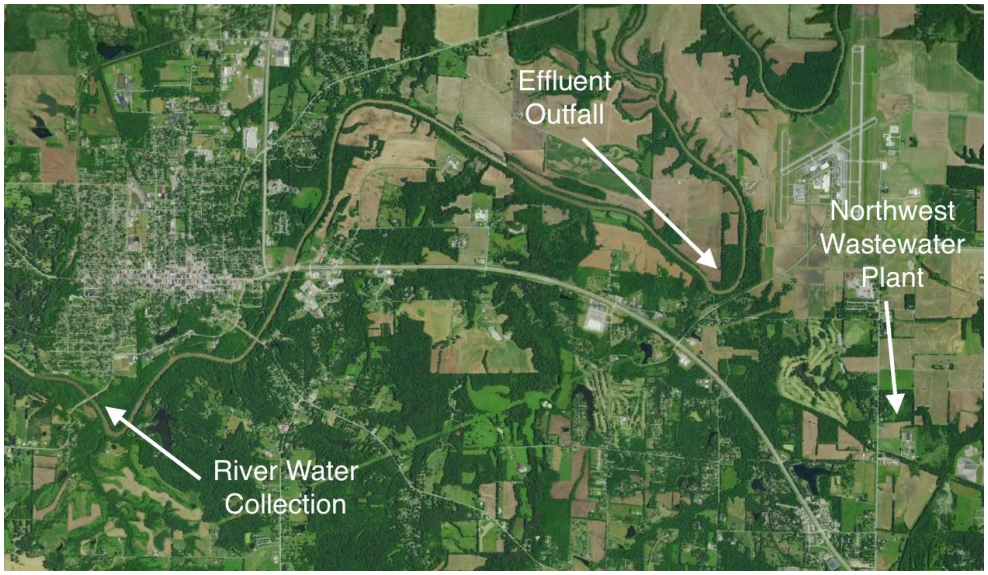

Figure 4. Aerial view of northwest WWTP, its effluent outfall, and the location of northwest river water collection.

Grab samples were collected in 250 and 500 mL high-density polyethylene (HDPE) bottles. Upon receipt, 4mL of 1% formaldehyde (>36.5%, Fluka, 47629) was added as a preservative, and samples were stored at 4°C until processing (no more than three days).

The analytical methods reported by Saravanabhavan et al.[25] were followed with minor modification. Samples were filtered (Whatman™, Fisher, 25mm, 1822-025) before extraction. Sample pH was adjusted to four using nitric acid (ACS grade, 15.8N, Fisher, A200-500) and 250 mL of effluent was extracted using manual solid phase extraction (SPE). SPE cartridges were purchased from Sigma (Supelco-Supelclean™ ENVI-18, 6mL, 1g, 505706), and were activated with 4mL of methanol followed by rinsing with 4mL of Millipore water. Vacuum was adjusted to zero to maintain a flowrate of approximately 2mL/min but had to be increased to -10 to -15mmHg for the northwest effluent (and northwest river water), which had significantly more colloidal solids than southeast effluent. Receiving river water was processed the same way but used 500mL of water because it was anticipated that 17β-estradiol would be lower.

SPE cartridges were dried for two minutes under vacuum and eluted with 5.5mL of ethyl acetate, and subsequently dried down under nitrogen stream. A silica column was prepared with 1g of activated silica (high purity grade, Sigma-Aldrich, pore size 30Å, 214477). The sample residues were re-suspended in 8% ethyl acetate: cyclohexane and applied to the column, before elution with 10mL of 50% ethyl acetate: cyclohexane. Solvents were then evaporated again using nitrogen.

Sample residues were now ready for derivatization. 250 µL of pyridine (>99.8%, anhydrous, Sigma-Aldrich, 275370) were added to each vessel with 100 µL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for GC derivatization with 1% trichloro(methyl)silane (Sigma Aldrich, 33148) and incubated in the oven for an hour at 60°C. Samples were then dried down under nitrogen before re-suspending them in 500µL of hexane with 0.26 µg/mL estrone 3-methyl ether (>97%, Sigma-Aldrich, E9875) as an internal standard.

Derivatized samples were stored in hexane for up to two weeks at 4°C, as they were found to be stable. External standards were prepared in HPLC grade methanol, dried down and derivatized without any further purification.

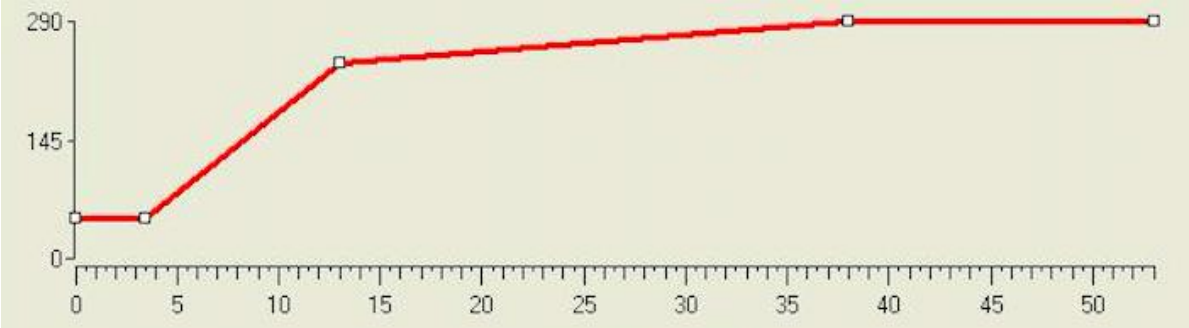

Figure 5. Visual of the GC oven temperature settings.

GC/MS/MS was conducted on a Thermo Trace GC Ultra with a PolarisQ ion trap mass analyzer. The column was a DB5-MS (J &W Scientific, 30m X 0.25mm i.d. X 0.25µm film thickness) with an internal coating of 5% diphenyl/95% polydimethylsiloxane. Briefly, the injection port was set to 250˚C. Initially, the column oven was 50˚C for 3.5 minutes, and then ramped to 240˚C at 20˚C/min. The temperature then increased to 290˚C at 2˚C/min, and was held for 15 minutes. The transfer line temperature was maintained at 275˚C, and the ion trap source was held at 240°C. The mass spectrometer was operated in electron impact mode (70-eV) with a mass range of 50-650 amu.

Five recovery studies were performed by spiking one liter of Millipore water with 60ng of 17β-estradiol dissolved in methanol, and then processed identically to treated sewage

effluent samples (250mL). In addition, three sets of stability studies were undertaken; samples were processed immediately after collection and either one or two weeks after storage at 4°C.

Temperature data for the days of sample collection was found on *Climate Data Online*, an online database published by the National Center for Environmental Information. The weather station used was Southern Illinois University Airport (GHCND:USW00093810).[26]

Chapter Three

ANALYTICAL PERFORMANCE

Ions used for quantitation were reported by Saravanabhavan et al.[25] and were verified to be accurate using standards. The internal standard, estrone 3-methyl ether included a precursor ion of m/z 284.0 and product ions used for quantitation of m/z 184.0, 199.00, and 284.0. 17β-estradiol had a precursor ion of m/z 416.00 and product ions used for quantitation were m/z 285.00, 298.00, and 326.00.

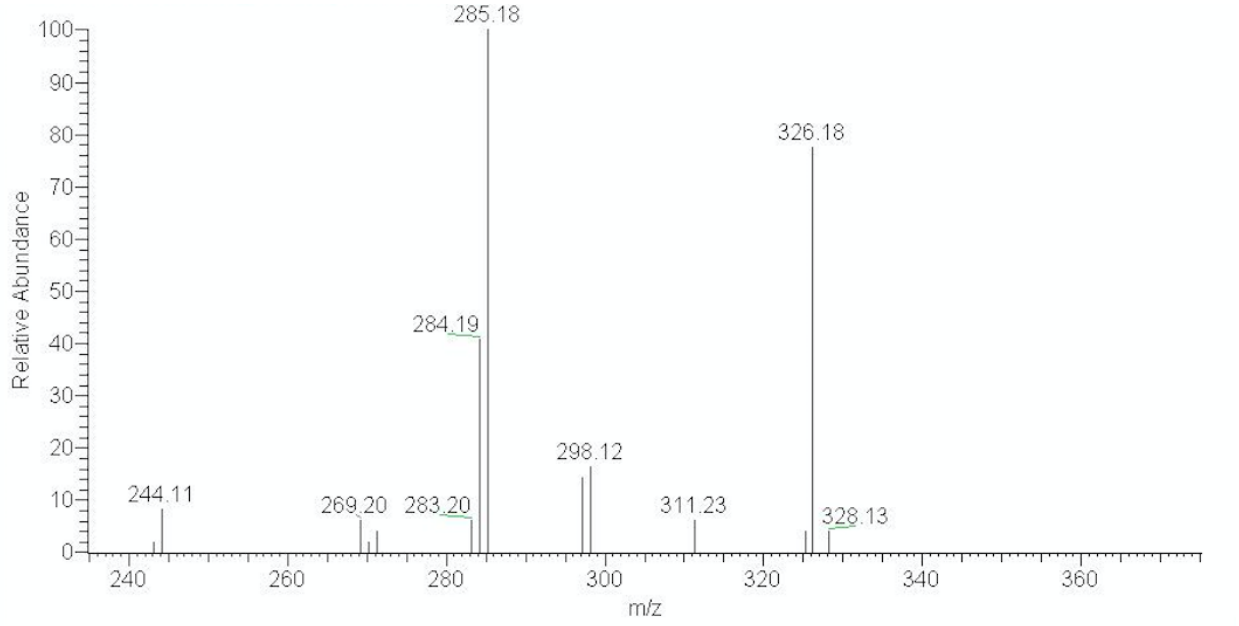

Figure 6. Product ion spectra of 17β-estradiol.

A chromatogram was recorded as the total ion current (TIC) as a function of time for standards to determine retention time of analytes (**Figure 7**). The internal standard (estrone 3-methyl ether) eluted first at ~19.7 minutes, and 17β-estradiol eluted at ~21.3 minutes, which were comparable to retention times reported by Saravanabhavan et al.[25]

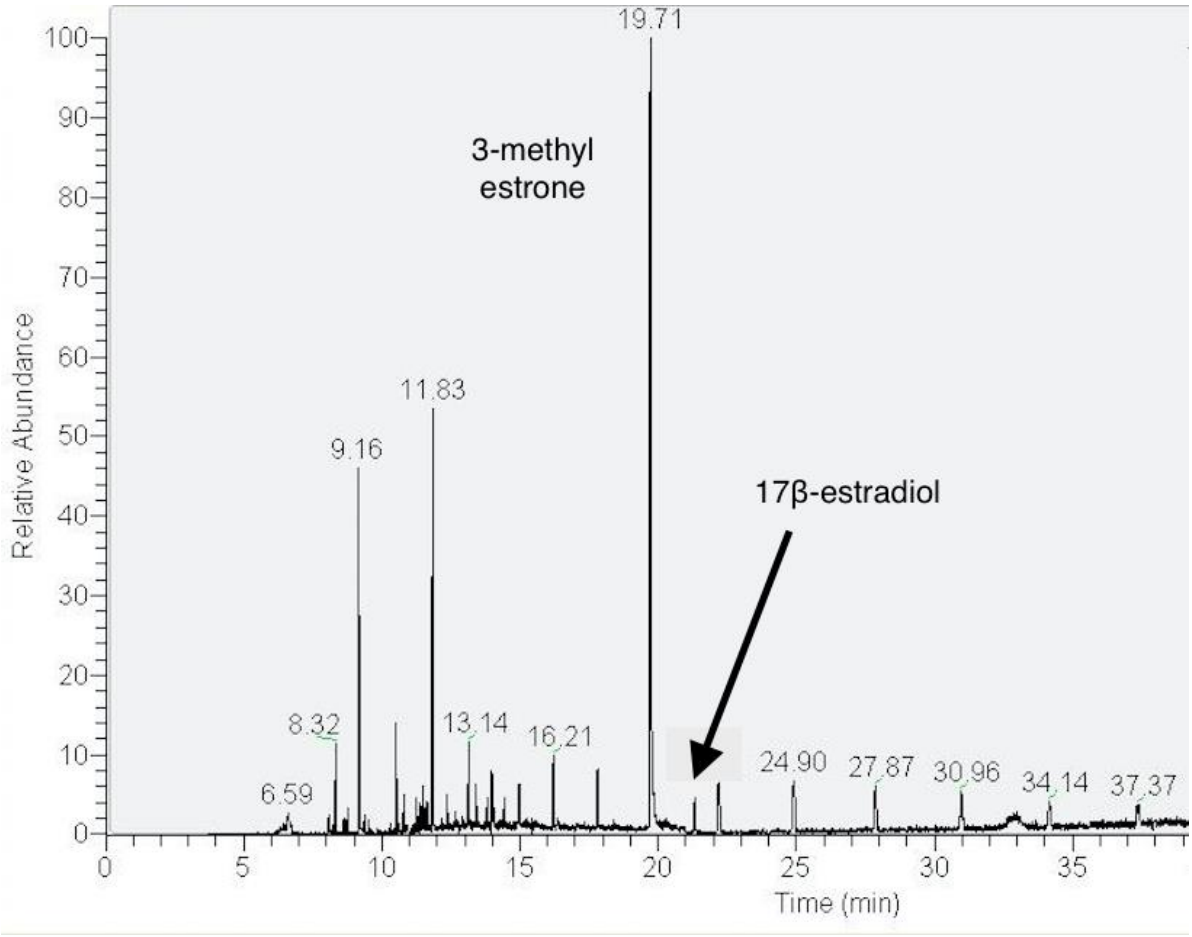

Figure 7. Chromatogram of standards.

GC/MS/MS is known for lowering detection limits because it records product ions rather than the parent ions of GC/MS. This can complicate finding limits of detection (LODs) and limits of quantitation (LOQs) because the only source of noise should be electronic noise. The instrument used in this study produced undetectable electronic noise in the mass spectrum which prevents calculation of an LOD or LOQ. Experimental determination of a detection limit would require diluting standard solutions until the chromatographic peak disappears. This experiment was not performed, but should be performed in future studies. Samples that do not

produce detectable 17β-estradiol signal will be reported as below detection limits (BDL) which is less than the lowest concentration standard (~0.75ng/mL).

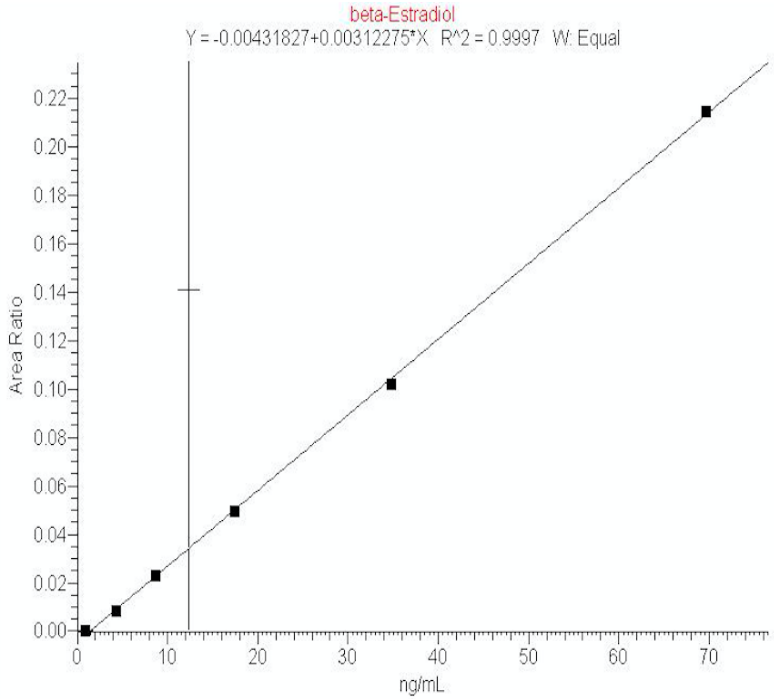

Figure 8. Example 17β-estradiol calibration curve (estrone 3-methyl ether, internal standard).

Six point calibration curves were used. External standards ranged from ~0.75-60.0 ng/mL and calibration curves would not be used unless R^2 values were greater than 0.995. Such an R^2 value is consistent with those published in the literature.[5] The ratio 17β-estradiol peak area to estrone 3-methyl ether peak area was plotted as a function of 17β-estradiol concentration to generate the calibration curve shown in **Figure 8**.

Duplicate samples were not analyzed in the present study. The 17β-estradiol levels reported are not averages and represent the preliminary data to support future research on the presence of 17β-estradiol in Carbondale treated wastewater effluent.

Recovery Studies

Table 2: Recovery studies of 17β-estradiol.

	Recovery Studies	
Theoretical (ng/mL)	Reported (ng/mL)	% Recovery
30	17.9	59.6%
-	18.1	60.3%
-	10.5	35.0%*
-	17.2	57.3%
-	18.9	63.0%

*Outlier (Grubbs Test, 95% confidence)

Recoveries were found to be 60.0% ± 3.9% RSD. Saravanabhavan et al[25] found 98% recovery for 17β-estradiol, and Jin et al.[5] reported recovery at 65.4±4.0%. The majority of 17β-estradiol in the present study was found to be lost in the silica column cleanup. An attempt was made to eliminate the silica column cleanup, and recoveries increased to 106%. When this shortened procedure was used to prepare treated wastewater effluent samples, the extract was dark brown in color (**Figure 9A**). **Figure 9B** shows another set of effluent samples after the silica column cleanup and the extract was colorless. Highly colored solutions are not routinely injected into the GC to reduce risk to overload and contaminate the inlet and column. Thus, the silica cleanup was retained as part of the sample preparation.

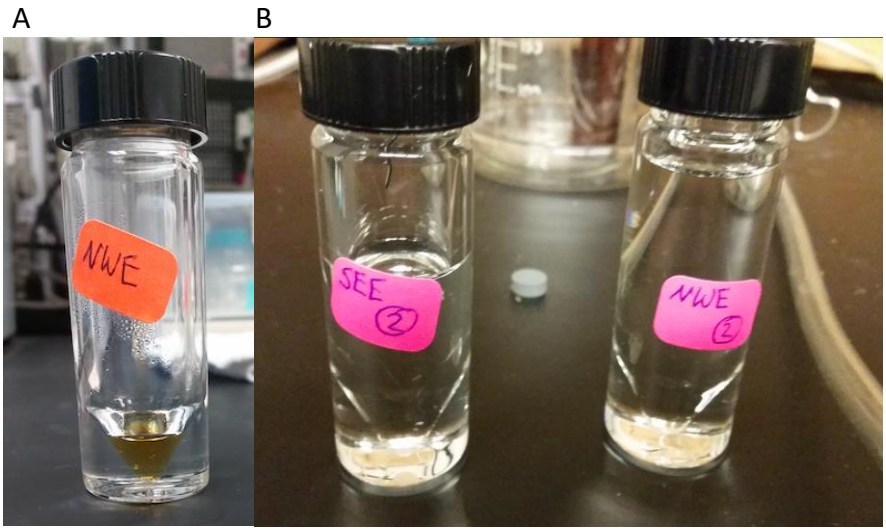

Figure 9. A) Treated sewage effluent extract without silica column cleanup B) Treated sewage effluent extract with silica column cleanup.

A second attempt to improve recoveries was made by changing the solvent composition used for elution in the silica column from 32% ethyl acetate:cyclohexane in Saravanabhavan et al.[25] to 50% ethyl acetate: cyclohexane, but recoveries did not improve.

Another possible explanation for a lower recovery is that this study used 60ng/L 17β-estradiol, which is more dilute than the 1000ng/L 17β-estradiol that Saravanabhavan et al.[25] used. The concentration of 60ng/L of 17β-estradiol was selected to produce a value in the middle of the calibration curve, and be more representative of actual concentration of 17β-estradiol expected in the wastewater effluent. 17β-estradiol concentrations reported herein are uncorrected for recovery and are underestimates of 17β-estradiol present in the samples.

Stability Studies

Table 3. Stability studies from treated sewage effluent. Analysis measured in ng/L 17β-estradiol.

Stability Studies					
Date Collected	Sample	First Analysis	Second Analysis	Time stored	% 17β-E2 loss
12/20/2017	SEE	54.0	7.1	Two weeks	86.8
	NWE	76.2	19.1		74.9
12/27/2017	SEE	20.0	8.9	Two weeks	55.5
	NWE	31.9	12.2		61.7
1/1/2018	SEE	25.1	17.4	One week	30.6
	NWE	31.5	6.7		78.7

SEE (Southeast effluent), NWE (Northwest effluent)

Baronti et al.[27] reported findings that 17β-estradiol had an 86% recovery over 28 days and a 56% recovery after 60 days under similar storage conditions (1% formaldehyde, 4°C). To establish 17β-estradiol stability in the present study, water samples were initially processed and analyzed the day they were collected. Unprocessed water samples were then stored for either one or two weeks, processed and analyzed again. 17β-estradiol loss was inconsistent for one week storage with a range of 30.6-78.7% analyte loss, while 17β-estradiol loss over two weeks ranged from 55.5-86.8%. The analyte may be lost due to decomposition or it may no longer be in solution due to adsorption to the hydrophobic HDPE bottle surfaces. Analyte loss was minimized in this study by storage no longer than three days before processing and derivatization.

Chapter Four

RESULTS AND DISCUSSION

Table 4. Concentrations of 17β-estradiol in treated effluent and river water.

River Water and Treated Effluent Samples					
Date Collected	Daily Temperature (Min-Max, °C)	SEE (ng/L)	NWE(ng/L)	SERW(ng/L)	NWRW(ng/L)
11/3/2017	10 - 17.7	6.9	12.3	-	-
11/8/2017	-0.5 - 12.2	28.5	14.1	-	-
12/13/2017	-6.6 - 11.6	6.1	7.4	-	-
12/20/2017	-1.1 - 11.6	54.0	76.2	18.4	25.2
12/27/2017	-13.8 – (-7.2)	20.0	31.9	16.0	32.3
1/1/2018	-18.3 – (-10.5)	25.1	31.5	*	*
1/28/2018	-5.5 – 13.3	-	-	16.9	3.2
2/5/2018	-15 – 0.5	BDL	9.7	4.7	9.8
2/9/2018	2.2 – 15.5	15.7	7.1	-	-

SEE (Southeast effluent), NWE (Northwest effluent), SERW (Southeast river water), NWRW (Northwest river water), BDL (Below detection limits), * Rivers were frozen

Concentrations of 17β-estradiol ranged from BDL-54.0 ng/L in SEE with an average of 22.3 ng/L. The NWE ranged from 7.1-76.2 ng/L with an average of 24.0 ng/L. The NWE 17β-estradiol concentrations were found to be higher than those measured in the SEE in seven out of the nine sample sets. The opposite outcome was expected because both the university and hospital flow to the southeast WWTP. One potential reason for these observations is the higher levels of colloidal particles in the NWE than the SEE. It is proposed that the dark brown solutions obtained when processing NWE are due to the presence of organic colloidal particles. 17β-Estradiol is the most hydrophobic of the natural estrogens[23], and therefore expected to

partition onto organic colloidal particles. It is possible the analyte adsorbed to and was carried by colloidal organic particles, leading to elevated levels in the NWE. Organic colloidal particles were not observed in the SEE.

The university exhibited no measurable effect on 17β-estradiol levels. Samples before 12/20, and after 1/28 were taken while the university was in session. The average 17β-estradiol level while not in session was 33.0ng/L for SEE and 46.5ng/L for NWE, which is higher than the average during session. This was not the expected trend as a higher student population was expected to lead to higher hormone levels in treated wastewater effluent. There was no conclusive evidence that elevated 17β-estradiol levels can be correlated to the university.

River water 17β-estradiol concentrations were also found to be elevated. Concentrations in SERW ranged from 4.7-18.4 ng/L and NWRW ranged from 3.2-32.3 ng/L. These 17β-estradiol concentrations were anticipated to be lower due to dilution. Previously reported values in river water were less than 6ng/L.[27,28] The measured river water 17β-estradiol concentrations followed a similar trend to effluent, with NWRW being higher in concentration than that observed in SERW. The notable outlier was week 1/28/2018 at 3.2ng/L, but this can also be explained by particulate matter. The NWRW sample from 1/28/2018 was noted by the analyst as the only sample that was brown with suspended (not colloidal) particulate matter. 17β-estradiol may adsorb onto the suspended organic matter and subsequently be removed in the microfiber filtration step before extraction, leading to a lower concentration of analyte.

The concentrations of 17β-estradiol in respective rivers are within ±6.0-8.0ng/L of the concentration in effluent from the wastewater treatment plants. Similarities are not surprising between the SEE and SERW given that river water was collected adjacent to the southeast

WWTP effluent outfall and less mixing and dilution would have occurred. However, in the Big Muddy River grab samples were collected approximately six miles downstream from the northwest WWTP effluent outfall. It was anticipated at this distance that significant mixing and dilution should occur and the resultant 17β-estradiol concentration would be lower. The possibility of unaccounted for sources of estrogenic pollution such as a feedlot, swine farm, or agriculture run off should be investigated to elucidate the sources resulting in elevated 17β-estradiol levels in the Big Muddy River.

The possible effects of temperature on the day of collection was also considered. Studies have argued about the influence of temperature on 17β-estradiol concentration in wastewater.[3] These studies suggest that higher temperature will lead to faster degradation, and lower temperature will lead to elevated levels in effluent because lower temperatures inhibit bacterial growth. Estrogen deconjugation and degradation is bacteria mediated.[5,16]

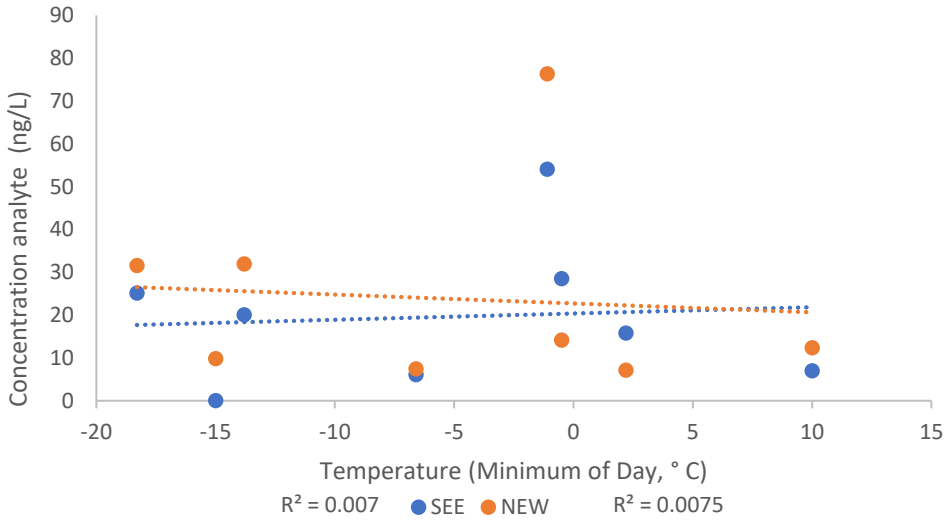

Figure 10. Concentration of 17β-estradiol (ng/L) against Temperature (min. of day, °C).

This study was conducted during the winter when temperatures ranged from -18 to 10°C. No influence of temperatures on 17β-estradiol concentrations was observed under these low temperatures. Perhaps it is not unexpected to find no observable dependence, because bacteria are not actively growing at temperatures approaching the freezing point of water.

Studies also argue about the effect that seasonal changes have on 17β-estradiol concentration. Jin et al.[5] found that 17β-estradiol concentrations do not change with season. This was explained by noting that estrone concentrations were affected seasonally; greater or lesser amounts of 17β-estradiol would decompose based on the season, but the final concentration in effluent was always the same. In contrast, Nie et al.[29] found that 17β-estradiol concentration was affected by seasonal changes; winter and spring had elevated levels. As noted earlier 17β-estradiol concentrations in the present study were somewhat elevated because the samples were collected in winter.

There was another possible source of bias from this study. All samples were grab samples taken in the morning. Past studies have emphasized the importance of 24-hour composite samples due to estrogenic fluctuations during the day.[5,16] In the morning estrogen is at higher concentrations due to the influx of morning urination. It is therefore possible that values reported here are slightly elevated due to sampling in the morning.

Several ecological effects would be anticipated from the 17β-estradiol levels reported in this study. Kidd et al.[9] reported that Fathead Minnow (*pimephales promelas*) populations collapsed after exposure to 5ng/L of a synthetic estrogen, 17α-ethinylestradiol (EE2). While 17α-ethinylestradiol is somewhat more potent than 17β-estradiol, significant reproductive disruption could still be anticipated such as those reported by Vajda and coworkers[8], including

elevated levels of vitellogen production, a biased female:male ratio, and the occurrence of intersex fish. The 17β-estradiol concentrations measured in this study would be expected to affect fish populations in the Big Muddy River and Crab Orchard Creek.

Physiologically, such low levels are not likely to cause effects in humans as the average NOEL (0.3mg/d)[13] is several orders of magnitude higher than those found in effluent. Even if this effluent is used downstream for another community's water supply, 17β-estradiol would decompose during drinking water treatment. There is little possibility of immediate negative human health effects from 17β-estradiol from Carbondale wastewater treatment plants, but it is important to note that the values reported here are only for 17β-estradiol, and are underestimates of estrogenic potency of the water, as wastewater is usually a mix of several endocrine disruptors.

CONCLUSIONS

17β-estradiol, a common municipal wastewater pollutant, was monitored for nine weeks in effluent and receiving waters from both the northwest and southeast Carbondale wastewater treatment plants. The analysis was performed by GC/MS/MS and an internal standard of estrone 3-methyl ether. Recoveries of 17β-estradiol were found to be 60.0±3.9%. There was shown to be significant decomposition after storage greater than one week. The SEE ranged from BDL-54.0ng/L and its receiving water, Crab Orchard Creek, ranged from 4.7-18.4ng/L. The NWE ranged from 7.1-76.2ng/L and its receiving water, the Big Muddy River, was found to be between 3.2-32.3ng/L. NWE 17β-estradiol levels were commonly higher, which suggests 17β-estradiol was partitioning onto and carried with colloidal organic particles observed in samples. Receiving water 17β-estradiol concentrations were found to be elevated despite expected dilution. There were no correlations between the university being in-session and concentration of 17β-estradiol during the study. Temperature on the day of collection did not exhibit a measurable effect either. The elevated level of 17β-estradiol suggest that fish populations may be effected in receiving waters. Future research is required to elucidate the link between colloidal organics as well as provide ecological data on fish populations in Carbondale rivers.

References

[1] Gomes, R.L.; Scrimshaw, M.D.; Lester, J.N. Determination of endocrine disrupters in sewage treatment and receiving waters. *Trends Anal. Chem.* **2003**, *22*, 697-707.

[2] Raftogianis, R.; Creveling, C.; Weinshilboum, R.; Weisz, J. Estrogen metabolism by conjugation. *J. Natl. Cancer Inst.* **2000,** *27,* 113-124.

[3] Liu, Z. H.; Lu, G. N.; Yin, H.; Dang, Z.; Rittmann, B. Removal of natural estrogens and their conjugates in municipal wastewater treatment plants: a critical review. *Environ. Sci. Technol.* **2015**, *49*, 5288-5300.

[4] Adeel, M.; Song, X.; Wang, Y.; Francis, D.; Yang, Y. Environmental impact of estrogens on human, animal and plant life: a critical review. *Environ. Int.* **2017**, *99*, 107-119.

[5] Jin, S.; Yang, F.; Liao, T.; Hui, Y.; Xu, Y. Seasonal variations of estrogenic compounds and their estrogenicities in influent and effluent from a municipal sewage treatment plant in china. *Environ. Toxicol. Chem.* **2008**, *27*, 146-153.

[6] Gadd, J.B.; Tremblay, L.A.; Northcott, G.L. Steroid estrogens, conjugated estrogens, and estrogenic activity in farm dairy shed effluents. *Environ. Pollut.* **2010**, *158*, 730-736.

[7] Sumpter, J.P.; Jobling, S. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ. Health Perspect.* **1995,** *103,* 173-179.

[8] Vajda, A.M.; Barber, L.B.; Gray, J.L.; Lopez, E.M.; Woodling, J.D.; Norris, D.O. Reproductive disruption in fish downstream from an estrogenic wastewater effluent. *Environ. Sci. Technol.* **2008**, *42*, 3407-3414.

[9] Kidd, K.A.; Blanchfield, P.J.; Mills, K.H.; Palace, V.P.; Evans, R.E.; Lazorchak, J.M.; Flick, R.W. Collapse of a fish population after exposure to a synthetic estrogen. *Environ. Sci.* **2007**, *104*, 8897-8901.

[10] Anderson, P.D.; Johnson, A.C.; Pfeiffer, D.; Caldwell, D.J.; Hannah, R.; Mastrocco, F.; Sumpter, J.P.; Williams, R.J. Endocrine disruption due to estrogens derived from humans predicted to be low in majority of U.S. surface waters. *Environ. Toxicol. Chem.* **2012**, *31*, 1407-1415.

[11] Moore, S.C.; Matthews, C.E.; Shu, X.O.; Yu, K.; Gail, M.H.; Xu, X.; Ji, B.; Chow, W.; Cai, Q.; Li, H.; Yang, G.; Ruggieri, D.; Boyd-Morin, J.; Rothman, N.; Hoover, R.N.; Gao, Y.T.; Zheng, W.; Ziegler, R.G. Endogenous estrogens, estrogen metabolites, and breast cancer risk in postmenopausal chinese women. *J. Natl. Cancer Inst.* **2016**, *108*, 1-12

[12] Sumpter, J.P.; Jobling, S. The occurrence, causes, and consequences of estrogens in the aquatic environment. *Environ. Toxicol. Chem.* **2013**, *32*, 249-251.

[13] Plotan, M.; Elliott, C.T.; Frizzell, C.; Connolly, L.; Estrogenic endocrine disruptors present in sports supplements: a risk assessment for human health. *Food Chem.* **2014**, *159*, 157-165.

[14] Fan, Z.; Hu, J.; An, W.; Yang, M.; Detection and occurrence of chlorinated byproducts of bisphenol A, nonylphenol, and estrogens in drinking water of China: comparison to the parent compounds. *Environ. Sci. Technol.* **2013**, *47*, 10841-10850.

[15] Rice, J.; Via, S.H.; Westerhoff, P. Extent and impacts of unplanned wastewater reuse in US rivers. *J. Am. Water Works Assoc.* **2015**, *107*, 571-581.

[16] Schlüsener, M.P.; Bester, K. Behavior of steroid hormones and conjugates during wastewater treatment- a comparison of three sewage treatment plants. *Clean*, **2008**, *36*, 25-33.

[17] Laganà, A.; Bacaloni, A.; De Leva, I.; Faberi, A.; Fago, G.; Marino, A.; Analytical methodologies for determining the occurrence of endocrine disrupting chemicals in sewage treatment plants and natural waters. *Anal. Chim. Acta.* **2004**, *501*, 79-88.

[18] Kuman, V.; Johnson, A.C.; Nakada, N.; Yamashita, N.; Tanaka, H. De-conjugation behavior of conjugated estrogens in raw sewage, activated sludge, and river water. *J. Hazard. Mater.* **2012**, *227*, 49-54.

[19] Maletz, S.; Floehr, T.; Beier, S.; Klümper, C.; Brouwer, A.; Behnisch, P.; Higley, E.; Giesy, J.P.; Hecker, M.; Gebhardt, W.; Linnemann, V.; Pinnekamp, J.; Hollert, H. In Vitro Characterization of the effectiveness of enhanced sewage treatment processes to eliminate endocrine activity of hospital effluents. *Water Res.* **2013**, *47*, 1545-1557.

[20] Escher, B.I.; Baumgartner, R.; Killer, M.; Lienert, J.; McArdell, C.S.; Environmental toxicology and risk assessment of pharmaceuticals from hospital wastewater. *Water Res.* **2011**, *45*, 75-92.

[21] Muller, M.; Combalbert, S.; Delgenès, N.; Bergheaud, V.; Rocher, V.; Benoît, p.; Delgenès, J.P.; Patureau, D.; Hernandez-Raquet, G. Occurrence of estrogen in sewage sludge and their fate during plant-scale anaerobic digestion. *Chemosphere.* **2010**, *81*, 65-71.

[22] Bowman, J.C.; Zhou, J.L.; Readman, J.W. Sediment-water interactions of natural oestrogens under estuarine conditions. *Mar. Chem.* **2002**, *77*, 4, 263-276.

[23] Gomes, R.L.; Scrimshaw, M.D.; Lester, J.N. Determination of endocrine disruptors in sewage treatment and receiving waters. *Trends Anal. Chem.* **2003**, *22*, 697-707.

[24] Chopra, S.; Gomes, P.; Dhandapani, R.; Snow, N. Analysis of steroids using solid phase microextraction-gas chromatography-mass spectrometry-mass spectrometry (SPME-GC-MS-MS) *Sci. Chroma.* **2014**, *6*, 105-115.

[25] Saravanabhavan, G.; Helleur, R.; Hellou, J. GC-MS/MS measurement of natural and synthetic estrogens in receiving waters and mussels close to a raw sewage ocean outfall. *Chemosphere.* **2009**, *76*, 1156-1162.

[26] *Climate Data Online*; National Centers for Environmental Information: Online, 3/31/2018, GHCND: USW00093810, https://www.ncdc.noaa.gov/cdo-web/datasets

[27] Baronti, C.; Curini, R.; D'Ascenzo, G.; Corcia, A.D.; Gentili, A.; Samperi, R. Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. *Environ. Sci. Technol.* **2000**, *34*, 5059-5066.

[28] Labadie, P.; Budzinski, H. Development of an analytical procedure for determination of selected estrogens and progestagens in water samples. *Anal. Bioanal. Chem.* **2005**, *381*, 1199-1205.

[29] Nie, Y.; Qiang, Z.; Zhang, H.; Ben, W. Fate and seasonal variation of endocrine disrupting chemicals in sewage treatment plant with A/A/O process. *Sep. Purif. Technol.* **2012**, *84*, 9-15