APPLICATION OF THE TRIFISHER ASSAY FOR ESTROGENIC ACTIVITY FROM WASTEWATER TREATMENT PLANT (WWTP) EFFLUENTS

A Thesis
by
Charles Brandon Tate

Submitted to the Graduate School
at Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

August 2018
Department of Biology

Application of the TriFISHer Assay for Estrogenic Activity from Wastewater Treatment Plant (WWTP) Effluents

A Thesis
by
Charles Brandon Tate
August 2018

APPROVED BY:

Dr. Shea R. Tuberty
Chairperson, Thesis Committee

Dr. Mike Madritch
Member, Thesis Committee

Dr. Mary Beth Hawkins
Member, Thesis Committee

Dr. Carol Babyak
Member, Thesis Committee

Dr. Zack E. Murrell
Chairperson, Department of Biology

Michael J. McKenzie, Ph.D
Dean, Cratis D. Williams School of Graduate Studies

Abstract

APPLICATION OF THE TRIFISHER ASSAY FOR ESTROGENIC ACTVITY FROM WASTEWATER TREAMTMENT PLANT (WWTP) EFFLUENTS

Charles Brandon Tate
B.A., North Carolina State University
M.A., Appalachian State University

Chairperson: Dr. Shea R. Tuberty

Endocrine disrupting compounds (EDCs) that mimic natural estrogens (e.g.17ß-estradiol) have been found in aquatic environments and disturb reproduction and development of aquatic wildlife. This project provided an application of the TriFISHer assay and evaluated the risk from wastewater treatment plant (WWTP) effluent EDC effects to the citizens of Boone (on the S Fork New River, SFNR) and Blowing Rock (on the Middle Fork of the New River, MFNR) as well as the aquatic vertebrate populations. Water samples were taken along the SFNR ($n = 16$) and the MFNR ($n = 15$) from 2014 to 2016. Previous research suggests EDCs have potential impact at sub-ng/L concentrations, interfering with fish reproduction. Estrogen equivalent concentrations (EEQs) were negatively correlated with distance from the WWTPs along both rivers analyzed ($p < 0.0001$). EEQ concentrations from Atlantic Croaker Estrogen Receptor (acERα) were not significantly different between rivers analyzed but had a significant amount of variation (26-83%) due to

seasonal effects ($p = 0.0002$). acERβa and acERβb EEQ from SFNR and MFNR sewage effluent were different across all seasons analyzed ($p =< 0.0001$). Seasonal differences in ACER activity along the rivers can be attributed to temperature influences on bacterial metabolic rates in WWTPs, variation in UV irradiation levels, or precipitation effects on dilution rates. Differences between effluents entering the rivers are likely associated with differences in employed WWTP treatment technology and differences in pharmaceutical use in populations with median ages of 21 (Boone) and 60 (Blowing Rock) years according to 2010 census data.

Keywords: environmental estrogens, estrogen receptors, wastewater treatment, TriFISHer

Acknowledgments

The author would like to express his deepest gratitude to the committee chair, DR. Shea Tuberty, for providing guidance and displaying patience throughout the graduate program, and for never giving up on the author or the project; Dr. Mary Beth Hawkins, for all the advice in about every aspect of the project; Dr. Carl Babyak for bring such a unique prospective to the project and for all the direction her advice produced. The author is grateful for his various funding sources including Appalachian state university office of student research and the cratis D. Williams Graduate School. Without these funds, a large portion of the work would not have been possible. The author would also like to think all his family and friends for their continued support and badgering.

Dedication

I would like to dedicate this thesis to my unborn daughter (Charlotte), my wife (Ashley), my aunt (Betty Gail), and my grandma (Dorothy). Without the support and dedication you have shown me during my education this would not have been possible. To my daughter, I hope that one day this work will inspire you to always reach for the stars no matter how far you must climb.

Table of Contents

List of Tables

List of Figures

Foreword

Chapter 1 of this thesis manuscript will be submitted to the journal, *Environmetnal Monitoring and Assesment.* The thesis has been formatted according to the style guide for this journal for rapid acceptance with minimal revisions.

Application of the TriFISHer Assay for Estrogenic Activity from Wastewater Treatment Plant (WWTP) Effluents Released into the New River

Brandon Tate
Appalachian State University
572 Rivers Street, Boone, NC 28608

Dr. Mary Beth Hawkins
North Carolina State University
2727 Bostian Hall, Raleigh, NC 27695

Dr. Shea Tuberty
Appalachian State University
572 Rivers Street, Boone, NC 28608

Corresponding Author
Dr. Shea Tuberty
(828) 263-2443
tubertysr@appstate.edu

Abstract

Endocrine disrupting compounds (EDCs) that mimic natural estrogens (e.g.17ß-estradiol) have been found in aquatic environments and disturb reproduction and development of aquatic wildlife. This project provided an application of the TriFISHer assay and evaluated the risk from wastewater treatment plant (WWTP) effluent EDC effects to the citizens of Boone (on the S Fork New River, SFNR) and Blowing Rock (on the Middle Fork of the New River, MFNR) as well as the aquatic vertebrate populations. Water samples were taken along the SFNR ($n = 16$) and the MFNR ($n = 15$) from 2014 to 2016. Previous research suggests EDCs have potential impact at sub-ng/L concentrations, interfering with fish reproduction (Jobling & Tyler 2003). Estrogen equivalent concentrations (EEQs) were negatively correlated with distance from the WWTPs along both rivers analyzed ($p < 0.0001$). EEQ concentrations from Atlantic Croaker Estrogen Receptor (acERα) were not significantly different between rivers analyzed but had a significant amount of variation (26-83%) due to seasonal effects ($p = 0.0002$). acERβa and acERβb EEQ from SFNR and MFNR sewage effluent were different across all seasons analyzed ($p =< 0.0001$). Seasonal differences in ACER activity along the rivers can be attributed to temperature influences on bacterial metabolic rates in WWTPs, variation in UV irradiation levels, or precipitation effects on dilution rates. Differences between effluents entering the rivers are likely associated with differences in employed WWTP treatment technology and differences in pharmaceutical use in populations with median ages of 21 (Boone) and 60 (Blowing Rock) years according to 2010 census data.

Keywords: environmental estrogens, estrogen receptors, wastewater treatment, TriFISHer

Introduction

Wastewater in the United States is treated at different treatment levels through wastewater treatment plants (WWTP). Once treated, effluents are discharged back into the environment into adjacent surface waters. Due to the persistence of some classes of industrial compounds and inefficient removal by current water treatment systems, many chemicals are not completely removed and are discharged into surface and ground waters, including sources used for drinking water (Caldwell et al. 2010; Touraud et al. 2011). Pharmaceuticals are a wide spread group of chemicals designed to be biochemical active at low concentrations in order to treat diseases and improve health. However, pervasive use of these compounds has led to their occurrence in surface and ground waters (Norris and Carr 2006). Among the wide variety of pharmaceuticals released to the environment, estrogenic compounds (both estrogen hormones and compound that mimic estrogens) have emerged as a concern in the last few decades (Jobling and Tyler 2003) due in part to their biological activity at concentrations in the sub ng/L level (Norris and Carr 2006). Approximately 40% of all oral contraceptives consumed arrive at WWTP before entering streams in the form of active synthetic estrogen (Wise et al.2011). Khanal et al. (2006) reported that 90% of the estrogen load in the environment comes from animal manure that contains steroidal hormones used in herd health programs. Roughly 30 tons of livestock natural steroid compounds are released into the environment surrounding Sydney, Australia every year (Fisher and Borland 2003). The presence of estrogens in the environment has generated concern related to the effects that these compounds may have on non-target species, including humans.

During the last 20 years, the scientific community have established evidence and discussed the ecological significance of endocrine disrupting compounds (EDCs). The majority of the discussion on endocrine disruption in regard to a compound's ability to cause reproductive and/or developmental anomalies in a wide range of wildlife species, from invertebrates to mammals, through their effects on the endocrine system (Jobling and Tyler 2003). The endocrine system is responsible for the regulation and secretion of hormones that influence various functions such as metabolism, growth, development, and reproduction. The current testing on chemicals that elicit EDC have been found to have the ability to interfere with the endocrine system through the mimicking of naturally found hormones, a topic first discussed by E. C. Dodds and others (1938) when he discovered a synthetic compound that had the ability to mimic natural estrogen (Dodds et al. 1938).

Endocrine disruption were initially recognized as an environmental issue in 1993 with the publication of Theo Colborn's *Developmental Effects of Endocrine-Disrupting Chemicals in Wildlife and Human*, which discussed the known effects of EDCs (Colborn et al. 1993). While it is unclear how the majority of endocrine disrupting compounds have made it into the environment, many compounds are released directly into the environment through pest management practices, such as the case of DDT (Carson 1962). Compounds can also be released indirectly and make their way into the environment via effluents from wastewater treatment plants, runoff, and private wells. Many compounds can remain in the environment for many generations, being stored in fat tissue within animals or within aquatic sediments (Pinto et al. 2014). EDCs have been identified in many aquatic environments and are known to mimic natural estrogens (e.g.17ß-estradiol)

and disturb reproduction and development of aquatic wildlife (Carson 1962; Norris and Carr 2006).

Unfortunately, the removal of estrogens and/or estrogenic activity from water is not an easy task. In several cases, conventional treatment processes are not sufficient to remove these micropollutants from water (Jelic et al. 2010) and, under recharge conditions, residues of these compounds may leach into groundwater aquifers. Thus, estrogenic compounds have been reported to occur in ground and drinking water samples downstream from municipal wastewater that use bank filtration or artificial groundwater recharge (Koplin et al. 2002). In natural waters, the main removal processes for estrogens are biodegradation, sorption and photolysis (Xu et al. 2010). Nevertheless, many of these compounds are highly biorefractory and the ability of sediments to adsorb these materials is highly dependent on their nature and composition (Scheytt et al. 2005), resulting in variable and limitations in the efficiency of the most important elimination pathways (Xu et al. 2010).

The identification of specific EDCs that interact with estrogen receptors (ERs) is a complex issue due to the diversity of endocrine disrupting chemicals found in the environment. Even more uncertain is the number of the ~70,000 chemicals not yet tested for EDC activity (USEPA, 2013). Therefore, it remains unknown if these unidentified chemicals contribute to the combined effects of EDCs. The US EPA has developed a two-tier system of standardized testing to determine if a compound is likely to affect humans and wildlife through an estrogen, androgen, or thyroid hormone (EAT) mode of action (Escher et al. 2008; USEPA 2013). The routes of uptake, patterns of bioactivation, biotransformation, excretion, and metabolism of these compounds have yet to be fully

studied. Furthermore, the role of endocrine disrupting compounds in different matrices and mixtures have not been well studied to understand how the components act additively, synergistically or antagonistically (Luna et al. 2015).

Although vertebrates possess multiple ER subtypes with different ligand binding and transactivation properties, most screening is done using a single ER subtype, the human ERα (Hawkins and Thomas 2004). This approach may result in an underreporting of estrogenic contamination, particularly for non-mammalian and aquatic species. Although there is a high percentage of conservation in the sequences between ERs of many vertebrates groups studied to date (Hawkins et al. 2004), teleost fish express three ER subtypes (ERα, ERβa, and ERβb), with ligand binding profiles distinct both from each other and from mammalian ERs (ERα and ERβ) (Hawkins and Thomas 2004). Currently, the most adopted protocol for determining an estrogenic mode of action is the human α-estrogen receptor-based yeast estrogen screen (YES) assay, which may not identify EDCs that are environmentally relevant to aquatic species due to differential binding of fish ERs (Hawkins and Thomas 2004; USEPA 2013).

Mode of action assays such as the YES assay, LYES assay, E-Screen-assay, and the receptor binding-assay with hER-α and hER-β, are conventional assays that have been used to quantify estrogenicity of compounds (Soto et al. 1995; Andersen et al. 1999; Schultis and Metzger 2004; Escher et al. 2008). The yeast estrogen screen (YES) is based on a recombinant strain of *Saccharomyces cerevisiae* yeasts DNA which contains the human estrogen receptor and a reporter gene *lac-Z* (Escher et al. 2008*;* Sanfilippo et al. 2010). The YES assay is a cheap assay that takes 3 to 5 days to run and has a working sensitivity of $1.4 \pm 0.3 \text{ X } 10^{-10}$ (Andersen et al. 1999; Schultis and Metzger 2004). An

alternative version of the YES assay is the optimized LYES assay which adds a step during the digestion stage of the YES assay for faster conversion of CPRG to chlorophenol red (Routledge and Sumpter 1996) and therefore takes only 7 hours to complete (Schultis and Metzger 2004). The E-SCREEN assay uses human breast cancer cells (MCF-7), which proliferate quickly in the presence of estrogens (Soto et al. 1995; Andersen et al. 1999). The E-SCREEN assay is the most expensive assay of the 4 described, takes 6 days to complete, and has the highest sensitivity $1.2 \pm 0.2 \text{ X } 10^{-12}$ (Schultis and Metzger, 2004). The receptor binding-assay with hER-α and hER-β uses human estrogen receptors ER-α and ER-β in a receptor-fluorescent ligand complex to test for estrogenicity (Schultis and Metzger, 2004; Scheytt et al. 2005). This assay was the least costly and fastest out of the 4 assays described with a working time of 2 hours, but has the least sensitivity working in a range of $7.5 \text{ to } 3.9 \pm 0.5 \text{ X } 10^{-9}$ (Schultis and Metzger 2004).

This project will provide an application of the TriFISHer assay by screening WWTP effluents as they enter the river to determine presence and activity of xenoestrogens. Results of the screening will allow evaluation of risk from wastewater treatment plant (WWTP) effluent EDC effects to the citizens of Boone (on the S Fork New River, SFNR) and Blowing Rock (on the Middle Fork of the New River, MFNR) as well as the aquatic vertebrate populations. The triFISHer assay was employed because of its unique ability to measure the individual activity of each of the three ER that is common among teleost fish making it more environmentally relevant as compared to traditional yeast assays that employ different vertebrate receptors. Having the ability to measure activity from all three ERs has the advantage of giving the total activity of all estrogens in the matrix.

Material and methods

Water collection and sample sites

Water samples were collected using 500mL acid washed amber vials with PTFE-lined lids. Water grab samples were taken from mid-depth below the water surface from each site and kept on ice until transported to the Appalachian State Aquatic Ecophysiology and Toxicology lab. Samples were then filtered through a Whatman P05 11-centimeter quantitative filter paper and kept at 4°C until processing. The 500mL amber vials were shaken for 30 seconds and triplicate samples of 5mL were dried down via centrifugal evaporation (speedvac). All samples were analyzed using the TriFISHer binding assay for total binding. Water chemistry measurements (temperature, conductivity, pH, dissolved oxygen) were taken using a YSI multi-meter before each water sample is collected. Discharge was determined using a flow meter (Global Water) and tape measure at each pre-determined location from the sewage effluent site to the end of the study reach.

The Town of Boone is located in North West North Carolina in Watauga County, the town is home to 17,122 full time residents according to the 2010 US Census (City Data 2016) and approximately 18,000 students who attend Appalachian State University. The headwaters of the South Fork of the New River (SFNR) originate in Boone, NC and combine to create the SFNR which flows north to combine with the North Fork of the New River becoming the New River proper thereafter. The Jimmy Smith Waste Water Treatment plant has been in operation since 1966 and is permitted to a release a maximum of 4.82 million gallons per day (MGD) of sewage into the SFNR (Jimmy Smith 2018). The SFNR samples were collected adjacent to the Jimmy Smith Wastewater

Treatment plant in distance 0 increasing by factor of 2 for 24 miles terminating at a proposed location for an additional Town of Boone drinking water intake facility. Reference water samples were taken from a pristine SFNR headwater, Winkler's Creek, that flows from the forested drainage of the Blue Ridge Parkway. Water samples ($n = 16$) were taken from the SFNR from 2014 to 2016 samples were taken upstream at Winkler's Creek (Reference Stream), Sewage Effluent (0 meters), 50 meters, 100 meters, 150 meters, 300 meters, 500 meters, 725 meters, 1609 meters, 6437 meters, 7242 meters, 8046 meters, 9656 meters, 20921 meters, and 38624 meters.

The Town of Blowing Rock is located in North West North Carolina between Watauga and Caldwell Counties and is home to 1250 full time residents and tourists that fluctuate with the season. The Middle Fork of the New River (MFNR) originates from a weir dam that holds back a 45-million-gallon pond on Brick house creek. The MFNR flows north to Boone, NC, where is combines with several head waters and the East Fork of the New river to create the SFNR. The Town of Blowing Rocks Wastewater Treatment Plant was built in 1978 and operates at 1.0 MGD and is expandable to 2 MGD. The MFNR samples were collected adjacent to the Town of Blowing Rock's Wastewater Treatment plant and from increasing distances for 8 miles until the MFNR joins the East Fork to become the SFNR. Water samples ($n = 15$) were taken from the MFNR from 2014 to 2016 samples were taken upstream at Winkler's Creek (Reference Stream), Sewage Effluent (0 meters), 50 meters, 100 meters, 150 meters, 300 meters, 500 meters, 1609 meters, 3400 meters, 5000 meters, 6437 meters, 7242 meters, 8046 meters, 10500 meters, and 12000 meters. Locations for flow measurements were chosen based on

accessibility of site location, proximity of sampling locations, and channel cross section conditions.

TriFISHer assay

In order to express Atlantic Croaker Estrogen Receptor (acER) proteins, ligand binding domains of each of the three acER cDNA were subcloned into the pET-27b^{+}vector. BL21(DE3)-competent cells were then transformed with these constructs for bacterial expression. *Escherichia coli* cells containing acER constructs were grown in Luria-Bertoni media (pH 7.6; 30 μg/ml kanamycin, 37°C) to an optical density at 600 nm of 0.6-0.8. Cell cultures were placed on ice and protein translation induced with 1 mM isopropyl-b-d-thiogalactopyranoside. Induced cells were incubated at 25°C for 16–20 hours and then harvested via centrifugation. Cell pellets were stored at -80°C until they were weighed and resuspended in GUS buffer (20 mM HEPES, 150 mm NaCl, 10% (wt/vol) glycerol, 1.5 mM EDTA, 6 mM monothioglycerol, and 10 mM $NaMoO_4$) at a dilution factor of 3.5 mL/g pellet. Lysozyme (at a final concentration of 1 mg/mL) was added to the resuspended cells and the cell/lysozyme mixture was placed on ice for 5 minutes and sonicated (with twelve 1-sec bursts at 30% power) using a sonic horn. Protease inhibitor cocktail set III (Calbiochem, San Diego, CA) was added after sonication at a concentration of 2.5% by volume and the crude bacterial lysate was centrifuged (12,000 x *g* at 4°C for 30 min) and the supernatant collected.

This lysate was diluted in GUS buffer at 4°C and incubated with varying concentrations of tritiated estradiol, [^{3}H]E2 (Hawkins and Thomas 2004), in a final concentration range of 0.12 – 9.6 nM. The concentration of [^{3}H]E2 resulting in saturation of the ERs was determined for each lysate preparation. Nonspecific binding at each

concentration of [^{3}H]E2 was determined by adding 4µl of 1M diethylstilbestrol to duplicate tubes. 4µl of Ethanol was added to duplicate tubes to measure total binding. Tubes were incubated overnight at 4°C. Free [^{3}H]E2 was removed by incubating each tube with an equal volume of dextran coated charcoal (0.1% dextran and 0.5% charcoal) for 10 min at 4°C, followed by centrifugation (3400 x *g* at 4°C for 10 min). The supernatant was transferred to scintillation vials and 5 ml CytoScint scintillation cocktail added. A liquid scintillation counter was then used to determine the total bound [^{3}H]E2. Nonlinear regression analysis determined the equilibrium K_d and binding capacity using a one-site binding model. Specific binding was determined by subtracting nonspecific binding from total binding. Reconstituted water samples (1 mL) added to each tube before adding lysate and saturating amounts of [^{3}H]E2 (~ 2–3 nM). Total binding and nonspecific binding determined as noted above, and the estrogenic activity of the water sample determined by comparison of scintillation counts between experimental and standard samples. All assays were performed in triplicate.

Data analysis

Data were analyzed using GraphPad PRISM v. 7.03 (GraphPad, 2017). This study utilized estradiol (E2) equivalent concentrations (EEQ) determined by three estrogen receptors ERα, ERβ_a, and ERβ_b from water samples collected along the SFNR and MFNR between October 2014, to May 2016. E2 equivalent concentrations were paired with the distance below sewage effluent where the samples were collected. Repeated measures ANOVAs were used to analyze E2 equivalent concentrations at increasing distances from sewage effluent. Geisser-Greenhouse Epsilon correction was employed when the assumption of sphericity was violated to produce a more valid critical F-value.

Sphericity is violated when the variances of the differences between all combinations of related groups are not equal. Post hoc comparisons using Dunnett's multiple comparison test were used to compare stream samples to the upstream reference. Post hoc comparisons using Tukey's test were used to compare samples with each other to determine significant differences. Two-way ANOVA analyses were employed to determine significance between samples collected from the SFNR and the MFNR during spring, summer, and fall seasons. Pearson correlation coefficient was used to determine the strength of the linear regression between distance from sewage effluent and mean EEQ from the three estrogen receptors acERα, acERβa, and acERβb during Spring, Summer, and Fall.

Results

Association between downstream distance and E2 equivalent concentrations (EEQ)

Sewage effluent (distance from source = 0M) to 500m samples were examined to determine if samples downstream of the sewage effluent correlated with E2 equivalent concentrations (EEQs) in both the SFNR and the MFNR. EEQs from samples taken along the SFNR had a strong and negative correlation that ranged from -0.909 to -0.9892 (Table 1) across all seasons among the three estrogen receptors acERα, acERβa, and acERβb. SFNR correlations were stronger during the fall season (Average -0.9696) as compared to spring (-0.9691) and summer (-0.9407). All correlations with SFNR data were significant ($P < 0.005$, two tailed). EEQs from samples taken along the MFNR also had a negative correlation with distance that ranged from -0.8698 to -0.9948 (Table 1) between seasons among the three estrogen receptors acERα, acERβa, and acERβb. MFNR correlations were stronger during the spring season (Average -0.9909) as compared to fall (-0.9142)

and summer (-0.9130). Correlations with the SFNR data were significant ($P < 0.05$, two tailed).

Seasonal effects on EEQs in the South Fork of the New River

During the fall season there was an effect of EEQ from acERα by distance [F (1.215, 3.644) = 33.42, $p = 0.0054$]. The assumption of sphericity was violated, so the Geisser-Greenhouse's epsilon correction (0.08677) was used. Post hoc comparison using the Dunnett's multiple comparison test indicated that the mean EEQs of sewage effluent ($p = 0.03$), 50m ($p = 0.0367$), 100m ($p = 0.046$), and 150m ($p = 0.024$) were significantly different than the upstream reference (M = 0.8596, SD = 0.2085), but found no other significant differences between the upstream reference and distance past 150 meters downstream of the sewage effluent to 38624 meters(**Fig 1**). EEQs from the acERβa estrogen receptor had a significant effect on distance at the $p < 0.05$ level [F (1.704, 5.113) = 58.35, $p = 0.0003$]. The assumption of sphericity was violated so the Geisser-Greenhouse's epsilon correction (0.1217) was used. Post hoc comparison using the Dunnett's multiple comparison test indicated that the mean concentrations of sewage effluent ($p = 0.0034$) was significantly different than the upstream reference (M = nd, SD = nd) (**Fig 1**). Dunnett's multiple comparison test found no other significant differences among samples downstream of the sewage effluent discharge point in the SFNR. E2 equivalent concentration from the acERβb did not have a significant effect on distance at the $p < 0.05$ level [F (1.043, 3.129) = 4.27, $p = 0.1271$]. The assumption of sphericity was violated so the Geisser-Greenhouse's epsilon correction (0.07451) was used. Post hoc comparison using the Dunnett's multiple comparison test indicated that

the mean concentrations analyzed were not significantly different from the concentration of upstream reference (**Fig 1**).

During the spring season there was an effect of acERα EEQs by distance at the $p<0.05$ [F (1.618, 3.236) = 18.48, $p = 0.0178$]. EEQs from the acERβa assay did not have a significant effect by distance downstream at the $p < 0.05$ level [F (1.007, 2.014) = 14.79, $p = 0.0608$] (**Fig 2**). However, post hoc comparison using the Dunnett's multiple comparison test indicated that the mean concentrations of sewage effluent ($p = 0.0121$) was significantly different than the upstream reference (M = nd, SD = nd) but found no other significant differences between the reference and other sample sites.

There was not an effect during the summer season of acERα EEQs by distance at the $p < 0.05$ level [F (1, 1) = 52.46, $p = 0.0873$]. Post hoc comparisons using the Dunnett's multiple comparison test indicated that the mean concentrations of sewage effluent ($p = 0.0339$) was significantly different than the upstream reference (M = 0.4405, SD = 0.3623) (**Fig 3**). However, no other significant differences between the upstream reference and sites downstream of the sewage effluent were determined. There were no other significant effects during the summer season between EEQs of acERβa or acERβb along the SFNR.

Middle Fork of the New River

There was an effect of acERα EEQs during the summer season by distance at the $p < 0.05$ level [F (1, 1) = 984.2, $p = 0.0203$]. The assumption of sphericity was violated so the Geisser-Greenhouse's epsilon correction (0.07143) was used. Post hoc comparison using the Dunnett's multiple comparison test indicated that the mean concentrations of

Sewage Effluent ($p = 0.0057$), 100m ($p = 0.0291$), 150m ($p = 0.00359$), and 300m ($p = 0.0162$) was significantly different than the upstream reference (M = 0.2347, SD = 0.1352) (**Fig 4**). There were no other significant differences between the upstream reference and distance past 300m downstream of the sewage effluent revealed by Dunnetts multiple comparison test to 12000 meters. EEQs from the acERβa had a significant effect on distance at the $p < 0.05$ level [F (1, 1) = 194.1, $p = 0.0456$]. The assumption of sphericity was violated so the Geisser-Greenhouse's epsilon correction (0.07143) was used. Post hoc comparison using the Dunnett's multiple comparison test indicated that the mean concentrations of Sewage Effluent ($p = 0.0308$) was significantly different than the upstream reference (M = 0.5089, SD = 0.08886) (**Fig 4**). acERβb EEQ had no significant effect on distance at the $p < 0.05$ level [F (1, 1) = 106.8, $p = 0.0614$]. Post hoc comparison using the Dunnett's multiple comparison test indicated that only the mean concentrations of Sewage Effluent ($p = 0.0223$) was significantly different than the upstream reference (M = nd, SD = nd) (**Fig 4**).

During the spring season there was not an effect of acERα EEQs by distance at the $p < 0.05$ level [F (1, 1) = 8.147, $p = 0.2145$]. The assumption of sphericity was violated so the Geisser-Greenhouse's epsilon correction (0.07143) was used. Post hoc comparison using the Dunnett's multiple comparison test indicated that the mean concentrations of Sewage Effluent ($p = 0.0316$) was significantly different than the upstream reference (M = 0.1896, SD = 0.2682) (**Fig 5**). Dunnetts multiple comparison test found no other significant differences once the effluent mixed with stream water of the MFNR. There were no other significant effects during the summer season between EEQs of acERβa or acERβb along the MFNR. Mean concentrations of Sewage Effluent

($p = 0.0363$) was significantly different than the upstream reference (M = 0.1429, SD = 0.2021) (**Fig 5**).

During the summer season there was not an effect of acERα EEQs by distance at the $p < 0.05$ level [F (1, 1) = 107.2, $p = 0.0613$]. The assumption of sphericity was violated so the Geisser-Greenhouse's epsilon correction (0.07143) was used. Post hoc comparison using the Dunnett's multiple comparison test indicated that the mean concentrations of Sewage Effluent ($p = 0.0497$) was significantly different than the upstream reference (M = 0.03261, SD = 0.04612) (**Fig 6**). Dunnetts multiple comparison test found no other differences past sewage effluent downstream of the Middle Fork of the New River. There was not a significant effect of acERβa EEQ during the summer season on distance at the $p < 0.05$ level [F (1, 1) = 144.5, $p = 0.0528$]. Post hoc comparison using the Dunnett's multiple comparison test indicated that the mean concentrations of Sewage Effluent ($p = 0.0076$) was different than the upstream reference (M = 0.004364, SD = 0.0006172) (**Fig 6**). There was a significant effect of acERβb EEQ during the summer season by distance at the $p < 0.05$ level [F (1, 1) = 410.1, $p = 0.0314$]. Post hoc comparison using the Dunnett's multiple comparison test indicated that the mean concentrations of Sewage Effluent ($p = 0.0463$) and 50m ($p = 0.0119$) was different than the upstream reference (M = nd, SD = nd).

Difference between rivers and seasonal differences

Two way anova was used to determine if E2 concentrations from acERα, acERβa, and acERβb were significantly different between the South Fork and Middle Fork of the New River at increasing differences from the sewage effluent. Sewage Effluent acERα EEQ between the South Fork and the Middle Fork of the New River had 63.08% ($p < 0.0001$)

variation from seasonal effects, acERβa EEQ had 73.16% ($p < 0.0001$) variation from river effects, and Sewage Effluent acERβb EEQ had 72.77% ($p < 0.0001$) variation from river effects (Table 2). 50m downstream of the sewage effluent samples at the South Fork and the Middle Fork of the New River had 78.458% ($p < 0.0001$) variation from seasonal effects from the acERα, acERβa and acERβb EEQ had 61.34% ($p < 0.0001$) and 67.3% ($p < 0.0001$) variation from river effects, respectively (Table 2). At 100m downstream of the sewage effluent, acERα EEQs between the South Fork and the Middle Fork of the New River had 82.66% ($p < 0.0001$) from seasonal effects, acERβa and acERβb EEQ had 60.1% ($p = 0.0003$) and 43.83% ($p = 0.0193$) variation from river effects, respectively. 150m downstream of the sewage effluent acERα EEQ between the South Fork and the Middle Fork of the New River had 78.89% ($p < 0.0002$) variation from seasonal effects, acERβa and acERβb had 62.93% ($p = 0.0005$) and 33.43% ($p = 0.0401$) variation from river effects (Table 2). 300m downstream of the sewage effluent acERα EEQ between the South Fork and the Middle Fork of the New River had 48.18% ($p = 0.0060$) variation from seasonal effects, acERβa and acERβb had 52.64% ($p = 0.0010$) and 32.52% ($p = 0.0452$) variation from river effects (Table 2). 500m downstream of the sewage effluent acERα EEQ between the South Fork and the Middle Fork of the New River had 56.41% ($p = 0.0037$) variation from seasonal effects, acERβa and acERβb had 51.45% ($p = 0.0006$) and 23.06% ($p = 0.0452$) variation from river effects (Table 2). 1600m downstream of the sewage effluent alpha E2 equivalent concentration between the South Fork and the Middle Fork of the New River had 26.28% ($p = 0.0463$) variation from seasonal effects while acERβa and acERβb had no significant effects from river or season (Table 2).

Discussion

The purpose of this study was to determine estrogenic EDC activity of municipal wastewater effluent and persistence of the extent of activity in water downstream from the WWTPs on the South Fork and Middle Fork of the New River. Monitoring the EDC activity of effluent downstream from WWTPs is important so that any variation can be correlated with changes to the river ecosystem and observe new sources of EDC activity along the river. The triFISHer assay was employed because of its unique ability to measure the individual activity of each of the three ER that is common among teleost fish making it more environmentally relevant as compared to traditional yeast assays that employ different vertebrate receptors. Having the ability to measure activity from all three ERs has the advantage of estimating the total activity of all estrogens in the matrix. However, this technique has the disadvantage as compared to more direct methods (LC/MS) because it is unable to identify specific compounds. Water samples were taken from the South Fork and Middle Fork at increasing distances downstream of the WWTP discharge point for twenty-four and eight miles, respectively, over a two-year period. 56%-82% of the of the variability among alpha EEQ concentrations were due to seasonal effects ($p < 0.0001$). Seasonal effects such as higher estrogenic activity during colder months (mean temperatures 26-56 F) as compared to warmer months (mean temperatures 60+ F) as well as changes in activity during periods of low (mean discharge below 30 cfs SFNR and 7cfs MFNR) and high (mean discharge above 40 cfs SFNR and 10cfs MFNR) stream discharge. EEQ concentrations for all three estrogen receptors from fall samples were 2-10 fold higher when compared to summer samples. Samples taken during the spring months were highly variable and generally had low concentrations due to dilution

from seasonally increased rainfall and elevated river discharge. Discharge levels and concentration patterns observed in this study (Fig 2) are consistent with previous studies that found concentration of chemicals were higher during dry or drought conditions as compared to samples taken after a precipitation event (Benotti and Brownawell 2007; Benotti et al. 2009). The seasonal differences can also be explained in part by temperature effects on the rate of microbial degradation of compounds in the WWTPs which Rodgers-Gray et al. (2000) was able to show for both natural estrogens and synthetic compounds. A review of the microbial degradation of compounds concluded that while several microbial strains were able to degrade estrogen, the degradation resulted in metabolites that could also be estrogenic (Combalbert and Hernandez-Raquet 2010). Estrogenic compounds usually follow the accepted estriol E3 degradation pathway (Lee and Liu 2002) which results in estradiol E2, and eventually estrone (E1). Hawkins and Thomas (2004) found that the EEQ activity of the chemicals in that degradation process for acERα is E2>E1>E3, acERβa E2>E3>E1, and acERβb E2>E1>E3. Microbial degradation rates of estrogenic compounds also depend on environmental conditions such as nutrient availability, retention time, and access to carbon and energy sources (Combalbert & Hernandez-Raquet 2010). Estrogenic activity varys according to differences in physiochemical properties of individual chemicals and the environment they are being introduced (Jobling & Tyler 2003). Estrogenic chemicals usually present relatively high hydrophobic properties causing them to be sorbed to organic carbon and other byproducts of wastewater (Lai et al. 2000). However, sewage effluent specific studies have found that estrogen sorption is very low (0-14%) during primary treatment attributed to the low sorption to suspended solids and colloid interactions with estrogenic

compounds that are only present in the primary treatment step in contemporary WWTP process (Lai et al. 2000, Yu and Huang 2005, Muller et al. 2008).

This study examined estrogenic activity of the SFNR for twenty-four miles downstream at a location on the river proposed to become a new water intake for the town of Boone. Boone has a growth rate in excess of 15% a year putting pressure on current drinking water resources (Appalachian Voice, 2018). The location of the new town of Boone raw water intake caused much debate about effects on water quality, risk to recreational use, and increased estrogenicity of the water downstream of the munciple WWTP, farms, septic tank drain fields, and landfill leachate. Results displayed in Fig 1-3 detail the findings of mean EEQ concentrations of neat sewage effluent which ranged from 0.77 ng/L to 7.5 µg/L and samples within the river ranged from 0.76 ng/L to 4.06 µg/L. Physiological effects due to EDC have been well documented in freshwater fisheries, most notably the synthesis of the female specific egg yolk protein precursor in male fish, inhibited gonad development, abnormal hormones levels in blood, intersex (condition where genital from both sexes are present in an individual), impaired reproductivity, complications of maturity, and altered behavior (Vos et al. 2000). Results of measurable river EEQ levels many kilometers downstream of sewage plants are not uncommon as Harries et al. (1997) found measurable EDCs at distances of over 5km which caused a response in the synthesis of vitellogenin (VTG, the yolk protein precursor) in male fish. Cellular mechanisms such as the lipid cell membrane, targeted binding proteins, and negative feedback loops are in place to deter the expected outcome of EDCs, research has shown these events still occur both in nature and in controlled laboratory experiments. One of the first novel experiments to determine if male fish could

produce vitellogenin was conducted in the laboratory by C.E. Purdom et al. in 1994. The experiment was designed to assess if treated sewage effluent being introduced into the river could induce the protein vitellogenin in male rainbow trout. Purdom et al. found plasma levels of vitellogenin in male rainbow trout increased 1000-fold over a period of 3 weeks (Purdom et al. 1994). They also determined that 17B-estradiol, estrone, estriol, and 17a-ethynylestradiol at levels as low as 1 ng/l in exposed male trout were enough to cause vitellogenin production (Purdom et al. 1994). Purdom et al. placed trout directly below WWTP sites in steel cages for several weeks, for the duration of this study the lowest concentration observed below the WWTP from acERα was 7 ng/l which is seven times more than the low level observed during Purdom's experiment. In the same location directly below the SFNR WWTP male white suckers were found to have synthesized VTG (unpublished data). Environmentally relevant concentrations are difficult to express in a single concentration for individual chemicals because the physiological effects on fish vary by species and life stage. Sappington et al. (2001) found slight differences between surrogate fish (Fathead Minnow and Rainbow Trout) and fish that are endangered or federally listed (Bonytail Chub, Colorado Pikeminnow, Lahontan Cutthroat Trout, Greenback Cutthroat Trout, and Apache Trout) from both warm and cold waters when exposed to known EDCs. One other study found that estrogen responsive gene transcription was higher in juvenile and male adult medaka as compared to the larval stage or female adult medaka (Yuanxiang et al. 2011). Asimilar study that exposed fish in early life stages to ethinylestradiol induced VTG synthesis and disrupted the development in sex cell development in male fathead minnow (Van Aerle et al 2002). Despite well-documented effects of single compounds there are several

confounding variables that limit the understanding of mixted EDC effects from point source solutions such as wastewater (Thorpe et al. 2001).

Sewage effluent is a mixture of chemicals from public waste, agricultural waste, runoff, and commercial waste with many chemicals. A report published in 2013 from the US EPA advisory committee approximated that ~70,000 chemicals that are in use have not had little to no testing for EDC activity (USEPA 2013). Therefore, it remains unknown if compounds present in sewage effluent respond additively, synergistically, or antagonistically (Jobling and Tyler 2003). The triFISHer assay does not require any additional filtering or column step to prepare the sample, instead uses whole water samples to measure the combined risk of all compounds in the water sample rather than attempting to determine presence and concentration of individual compounds.

The MFNR was selected as the other river sampled during this study due to similarities in watersheds but also because the WWTP is located roughly 2000m upstream of a municipal drinking water treatment plant. Results of this study show EEQ concentrations just upstream of the drinking water treatment plant of 13 ng/L at the lowest and to 211 ng/L at their highest. These observations bring into question the potential human impacts from tap water drawn from this source. A recent study in Las Vegas, Nevada on drinking water frequently found 11 EDC such as atrazine, carbamazepine, estrone, gemfibrozil, meprobamate, naproxen, phenytoin, sulfamethoxazole, TCIP, and trimethoprim in source water samples (Benotti & Brownawell 2007). The same study discovered that compounds were detected less frequently in tap waters compared to source or finished water samples attributing the reduction to drinking water treatment plants employment of chlorination or ozone as a

means of oxidization (Benotti & Brownawell 2007). This information would lead one to conclude that potential human impact from drinking water should be minimal despite elevated levels of EDC concentrations present in surface waters receiving sewage effluent.

Differences in samples taken from the South Fork of the New River and the Middle Fork of the New River are not very apparent from EEQ concentrations of the alpha receptor which only accounted for 0.6% to 15% of the variation ($p>0.1$) (Table 1). However, data generated from the acERβa or acERβb showed a significant difference in EEQ concentrations ($p<0.0001$) between rivers which accounted for 20-70% of the total variation (Table 1). EEQ concentration differences between rivers is the result of the WWTP effluent each river receives, which directly reflect WWTP technology efficiency and differences in populations of the area served by their prospective WWTP. The WWTP on the South Fork of the New River was newly renovated in 1998, is permitted to discharge up to 4.82 million gallons per day and takes advantage of an advanced system with ultraviolet irradiation tertiary treatment of sand filters for solid removal (Jimmy Smith wastewater treatment plant 2018). Wastewater on the Middle Fork of the New River is processed through a smaller facility that was refurbished in 1989, has a permitted discharge of up to 1.2 MGD, and uses conventional aeration treatments (Vison 2018). The South Fork WWTP services the college town of Boone, NC which includes Appalachian State University (~20,000 students) a population of 18130 residents where the median age is 21.4 according to 2014 census data (City Data 2018). The Middle Fork WWTP services Blowing Rock, NC which has a permanent population of 1,233 with the median age at 60.7 years old in 2014 (City Data 2018). Data collected on the South fork

has lower levels of EEQ concentration form Ba and Bb receptors (0.98 to 343.7 ng/L) when compared to EEQ concentrations from the Middle Fork (13 to 3499 ng/L). These distinct differences are in part due to the age of the population of each community and that age groups use of pharmaceutical and personal care products. The CDC report on Health prepared by Zhong et al. found that among adults age 18-44 36% had used at least one prescription drug compared to finding of 70% in the population range of 45-64. A recent study in 2013 found that the most frequently prescribed drugs in Americans age 19-29 were systemic contraceptives, antibiotics, and antidepressants, while adults age 50-64 most commonly used antilipemic, antidepressants, and opioid analgesics (Zhong et al. 2013).

In summary, EDC activity was negatively correlated with distance along the SFNR and the MFNR showing a decrease in EDC activity with distance from the original source. Water samples were taken along the SFNR (n=16) and the MFNR (n= 15) at increasing distances from 2014 to 2016 during different spring, summer, and fall seasons. Seasonal effects such as higher estrogenic activity during colder months (mean temperatures 26-56 F) as compared to warmer months (mean temperatures 60+ F) as well as changes in activity during periods of low (mean discharge below 30 cfs SFNR and 7cfs MFNR) and high (mean discharge above 40 cfs SFNR and 10cfs MFNR) stream discharge. EEQ concentrations for all three estrogen receptors from fall samples were 2-10 fold higher when compared to summer samples. Samples taken during the spring months were highly variable and generally had low concentrations due to dilution from seasonally increased rainfall and elevated river discharge. The use of the triFISHer assay demonstrated that it had the ability to distinguish EDC activity due to difference in

WWTP effluent inputs between SFNR and MFNR across all three estrogen receptors acERα, acERβa, and acERβb. Differences in WWTP effluent can be associated with the composition of the population that generates the waste entering the WWTP.

This project has demonstrated that there is sufficient EEQ EDC activity that could influence the river ecosystem. In the future all water samples that have been tested with the triFISHer assay would benefit from being tested for common estrogenic compounds with a more quantitative method such as LC/MS to confirm the level of risk associated with the concentration of chemicals found. Having EDC activity data and quantitative results of chemicals of the water samples should build a stronger case of risk and warrant a survey of the aquatic ecosystem and the histology of the fish populations to determine effects of the WWTP effluents.

References

Andersen, H. R., A. Andersson, S. F. Arnold, H. Autrup, M. Barfoed, N. A. Beresford, P. Bjerregaard, L. B. Christiansen, B. Gissel, R. Hummel, E. B. Jorgensen, B. Korsgaard, R. Le Guevel, h. Leffers, J. McLachlan, A. Moller, j. Bo Nielsen, Nl Olea, A. Oles-Karasko, F. Pakdel, K. L. Pendersen, P. Perez, N. E. Skakkeboek, C. Sonnenschein, A. Soto, J. P. Sumpter, S. M. Thorpe, and P. Grandjean. (1999). Comparison of Short-Term Estrogenicity Tests for Identification of Hormone-Disrupting Chemicals. *Environmental Health* Perspectives 107:89-108.

Appalachin Voice. (2018, April 5). Retrieved from http://appvoices.org/2018/02/19/water-systems-the-invisible-infrastructure/.

Benotti, M. J., & Brownawell, B. J. (2007) Distributions of Pharmaceuticals in an Urban Estuary during both Dry and Wet- Weather Conditions. Environmental Science and Technology, 41, 5795-5802.

Benotti, M. J., Trenholm, R. A., Vanderford, B. J., Stanford, B. D. & Snyder, S. A. (2009) Pharmaceuticals and Endocrine Disrupting Compounds in U.S. Drinking Water. Environmental Science and Technology, 43, 597-603.

Caldwell, D.J., Mastrocco, F., Nowak, E., Johnston, J., Yekel, H., Pfeiffer, D., Hoyt M., DuPlessie, B.M., Anderson, P.M. (2010) An Assessment of potential Exposure and Risk from Estrogens in Drinking Water. Environmental Health Perspectives 118(3): 338-344.

Carson, R. (1962) Silent Spring. Boston: Houghton Mifflin, Boston, MA

City Data (2018, April 5) Retrieved from http://www.city-data.com/city/Boone-North-Carolina.html.

Combalbert, S., & Hernandez-Raquet, G. (2010) Occurrence, Fate, and Biodegradation of Estrogens in Sewage and Manure. Applied Microbiology and Biotechnology, 86, 1671-1692.

Colborn, T., F. Vomsaal, and A. Soto. (1993) Developmental Effects of Endocrine-disrupting Chemicals in Wildlife and Humans. Environmental Health Perspectives 101(5):378-84.

Dodds, E. C., Lawson, W., & Campbell, N. R. (1938). Estrogenic Activity of Anol; A highly Active Phenol Isolated from the By- Products. Nature, 12(142), 3608.

Escher, B.I., N. Bramaz, P. Quayle, S. Rutishauser, and E. L. M. Vermeirssen. (2008) Monitoring of the ecotoxicological hazard potential by polar organic micropollutants in sewage treatment plants and surface waters using a mode-of-action based test battery. Journal of Environmental Monitoring 10: 622-631.

Fisher, P.M.J., Borland, R. (2003) Gauging the Pharmaceutical Burden on Sydney's Environment: A Preventative Response. Journal of Cleaner Production 11: 315-320.

Harries, J.E., Sheahan, D.A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J.P., Tylor, T., and Tylor, T., and Zaman, N., (1997) Estrogenic Activity in Five United Kingdon Rivers Detected by Measurement of Vitellogenesis in Caged Male Trout. Environmental Toxicology and Chemistry, 16: 534-542

Hawkins, M.B., Thomas, P. (2004) The Unusual Binding Properties of the Third District Teleost Estrogen Receptor Subtype ERBa are Accompanied by Highly Conserved Amino Acid Changes in the Ligand Binding Domain. Endocrinology 145(6): 2968-2977

Jelic A., Gros M., Ginebreda A., Céspedes-Sánchez R., Ventura F., Petrovic M., Barcelo D. (2010) Occurrence, Partition and Removal of Pharmaceuticals in Sewage Water and Sludge During Wastewater Treatment. Water Research 45(3): 1165-1173.

Jimmy Smith Wastewater Treatment Plant. (2018, April 5). Retrieved from https://www.water-technology.net/projects/boone.

Jobling, S., & Tyler, C. R. (2003) Endocrine Disruption in Wild Freshwater Fish. Pure and Applied Chemistry. 75(11-12), 2219-2234.

Khanal, S.K., Xie, B., Thompson, M.L., Sung, S., Ong, S., van Leeuwen, J. (2006) Fate, Transport and Biodegradation of Natural Estrogens in the Environment and Engineered Systems-A review. Environmental Science and Technology 40(21): 6537-6546.

Koplin D.W., Furlong E.T., Meyer M.T., Thurman E.M., Zaugg S.D., Barber L.B., Buxton H.T. (2002) Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999-2000: A National Reconnaissance. Environmental Science and Technology 36: 1202-1211.

Lai, K.M., Johnson, K.L., Scrimshaw, M.D., Lester, J.N. (2000) Binding of waterborne steroid estrogens to solid phases in river and estuarine systems. Enviornmental Science and Technology 34: 3890-3894

Lee, H. B., & Liu, D. (2002) Degradation of 17ß-Estradiol and its Metabolites by Sewage Bacteria. Water, Air, & Soil Pollution, 134, 353-368.

Luna, T. O, Plautz, S. C., & Salice, C. J. (2013) Chronic Effects of 17α-Ethinylestradiol, Fluoxetine, and the Mixture on Individual and Population-Level End Points in Daphnia magna. Archives of Environmental Contamination and Toxicology, 68, 603-611.

Muller, M., Rabenoelina F., Balaguer, P., Patureau, D., Lemenach, K., Budzinski, H., Barcelo, D., De Alda, M.L., Kuster, M., Delgenes, J.P., Hernandez-Raquet, G. (2008) Chemical and Biological Analysis of Endocrine Disrupting Hormones and Estrogenic Activity in an Advanced Sewage Treatment Plant. Environmental Toxicology and Chemistry 27: 1649-1658.

Norris, D. O., & Carr, J. A. (2006) Endocrine Disruption: Biological Bases for Health Effects in Wildlife and Humans. New York: Oxford University Press

Pinto, P. I. S., Estevao, M. D. & Power, D. M. (2014). Effects of Estrogens and Estrogenic Disrupting Compounds on Fish Mineralized Tissues. Marine Drugs, 12, 4474-4494.

Purdom, C. E., Hardiman, P. A., Bye, V. V. J., Eno, N. C., Tyler, C. R. & Sumpter, J. P. (1994) Estrogenic Effects of Effluents from Sewage Treatment Works. Chemistry and Ecology, 8(4), 275-285.

Rodgers-Gray, T. P., Jobling, S., Morris, S., Kelly, C., Kirby, S., Janbakhsh, A., Harries, J.E., Waldock, M.J., Sumpter, J.P., Tyler, C.R., (2000) Long-Term Temporal Changes in the Estrogenic Composition of Treated Sewage Effluent and Its Biological Effects on Fish. Environmental Science and Technology 34: 1521-1528.

Routledge, E. J., & Sumpter, J. P.. (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environmental Toxicology and Chemistry, 15, 241-248.

Sanfilippo, K., Pinto, B., Colombini, M. P., Bartolucci, U., & Reali, D. (2010) Determination of trace endocrine disruptors in ultrapure water from laboratory use by the yeast estrogen screen (YES) and chemical analysis (GC/MS). Journal of Chromatography *B*, 878, 1190-1194.

Sappington, L.C., Mayer, F.L., Dwyer, J., Buckler, D.R., Jones, J.R., Ellersieck, M.R. (2001) Contaminant Sensitivity of Threatened and Endangered Fishes Compared to Standard Surrogate Species. Environmental Toxicology and Chemistry 20(12): 2869-2876.

Schultis, T., & Metzger, J.W. (2004) Determination of estrogenic activity by LYES-assay (yeast estrogen screen-assay assisted by enzymatic digestion and lyticase). Chemosphere, 57, 1649-1655.

Soto, A. M., C. Sonnenschein, K. L. Chung, M. R. Fernandez, N. Olea, and F. O. Serrano. (1995) The E-Screen Assay as a Tool to Identify Estrogens: An update on Estrogenic Environmental Pollutants. Environmental Health Perspectives 103:133-122.

Thorpe, K.L., Hutchinson, T.H, Hetheridge, M.J., Scholze, M., Sumpter, J.P, Tyler, C.R. (2001) Assessing the Biological Potency of Binary Mixtures of Environmental Estrogens using Vitellogenin Induction in Juvenile Rainbow Trout (Oncorchynchus Mykiss). Environmental Science and Technology 35: 2476-2481.

Tourad, E., Roig, B., Sumpter, J.P., Coetsier, C. (2011) Drug Residues and Endocrine Disruptors in Drinking Water: Risk for Humans? International Journal of Hygiene and Environmental Health 214: 437-441.

United States Environmental Protection Agency (USEPA) Office of Chemical Safety and Pollution Prevention. (2013) Proposed Endocrine Disruptor Screening Program (EDSP) Tier 2 Ecotoxicity Tests, FIFRA Scientific Advisory Panel Meeting. 151

Van Aerle, R., Pounds, N. Hutchinson, T. H., Maddix, S., & tyler, C. R. (2002) Window of Sensitivity for the Estrogenic Effects of Ethinylestradiol in Early Life-Stages of Fathead Minnow, Pimephales promelas. Ecotoxicology, 11, 423-434.

Vos, J. G., Dybing, E., Greim, H. A., Ladefoged, O., Lambre, C., Tarazona, J. V., Brandt, I., & Vethaak., A. (2000) Health Effects of Endocrine-Disrupting Chemicals on Wildlife, with Special Reference to the European Situation. Critical Reviews in Toxicology 30(1):71

Wise, A., Brien, K.O., Woodruff, T., (2011) Are Oral Contraceptives a Significant Contributor to the Estrogenicity of Drinking Water? Environmental Science and Technology 45(1): 51-60.

Xu H., Cooper W.J., Jung J., Song W. (2010) Photosensitized Degradation of Amoxicillin in Natural Organic Matter Isolate. Water Research 45(2): 632-638.

Yuanxiang, J., Shu, L., Huang, F., Cao, L., Sun., L., & Fu, Z. (2011) Environmental Cues Influence EDC-Mediated Endocrine Disruption effects in Different Developmental Stages of Japanese Medaka (Oryzias Latipes). Aquatic Toxicology 101: 254-260.

Yu, Z.Q., Huang, W.L., (2005) Competitive Sorption Between 17α-Ethinylestradiol and Naphthalene/Phenanthrene by Sediments. Environmental Science and Technology 39:4878-4885

Zhong, W., Maradit-Kremers, H., St. Sauver, J.L., Barbara, P.Y., Ebbert, J.O., Roger, V.L., Jacobson, D.J., McGree, M.E., Brue, S.M., Rocca, W.A. (2013) Age and Sex Patterns of Drug Prescribing in a Defined American Population. Mayo Clinic Proceedings 88: 697-707.

Tables and Figures

*Table 1. Correlation Data examining the relationship between the distance from sewage effluent and E2 equivalent concentration of the South Fork and Middle Fork of the New River. Significance (≤0.05) of the correlation is indicated by an * beside the P-value.*

River	Season	Estrogen Receptor	N	Pearson R	P-Value (2-tailed)
South Fork of the New River	Fall	Alpha	7	-0.9892	<0.0001***
		Beta a	7	-0.9799	0.0001**
		Beta b	7	-0.9399	0.0016**
	Spring	Alpha	7	-0.9682	0.0003**
		Beta a	7	-0.9699	0.0003**
		Beta b	7	nd	nd
	Summer	Alpha	7	-0.9602	0.0006**
		Beta a	7	-0.953	0.0009**
		Beta b	7	-0.909	0.0046**
Middle Fork of the New River	Fall	Alpha	6	-0.8736	0.0230*
		Beta a	6	-0.9045	0.0132*
		Beta b	6	-0.9646	0.0019**
	Spring	Alpha	6	-0.9823	0.0005**
		Beta a	6	-0.9957	<0.0001***
		Beta b	6	-0.9948	<0.0001***
	Summer	Alpha	6	-0.9364	0.0059**
		Beta a	6	-0.8698	0.0243*
		Beta b	6	-0.933	0.0066**

*. Significant at 0.05 Level
**. Significant at 0.01 Level
***. Significant at >0.0001 Level

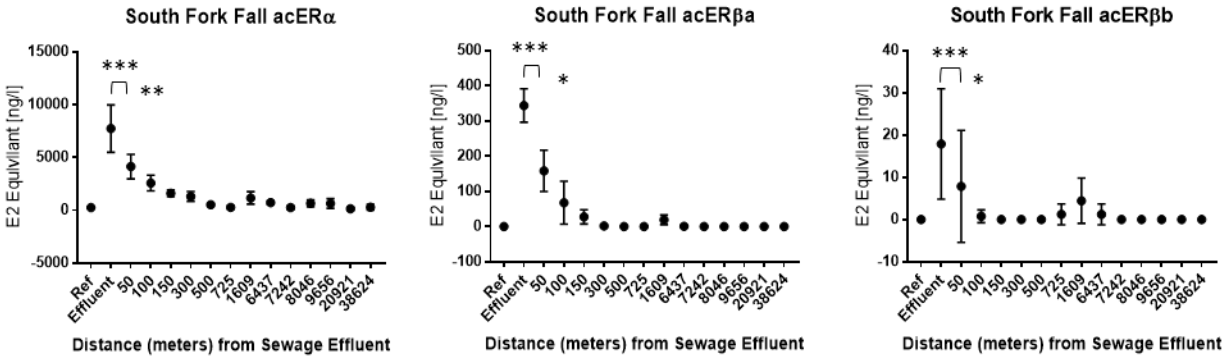

*Figure 1. South Fork of the New River E2 equivalent concentrations at increasing distances from sewage effluent taken during the Fall Season. E2 equivalent concentrations displayed from the three estrogen receptors acERα, acERβa, and acERβb. *P<0.05, **P<0.01, ***P<0.0001.*

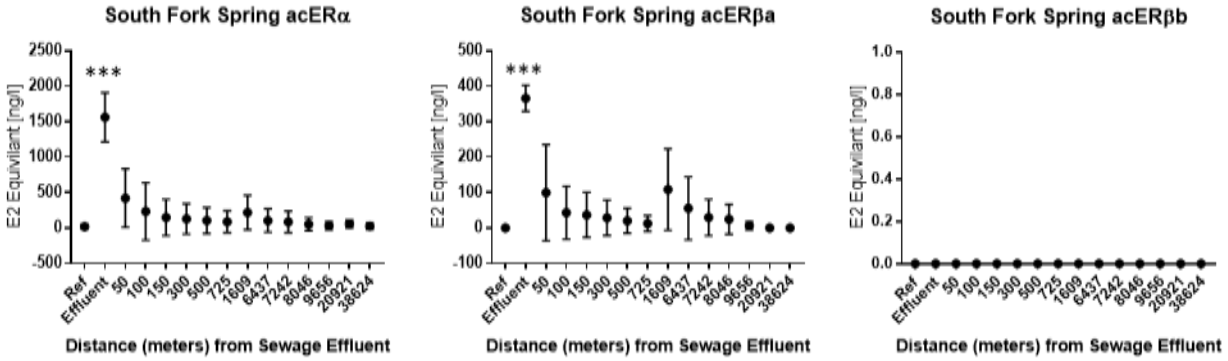

*Figure 2. South Fork of the New River E2 equivalent concentrations at increasing distances from sewage effluent taken during the Spring Season. E2 equivalent concentrations displayed from the three estrogen receptors acERα, acERβa, and acERβb. *P<0.05, **P<0.01, ***P<0.0001.*

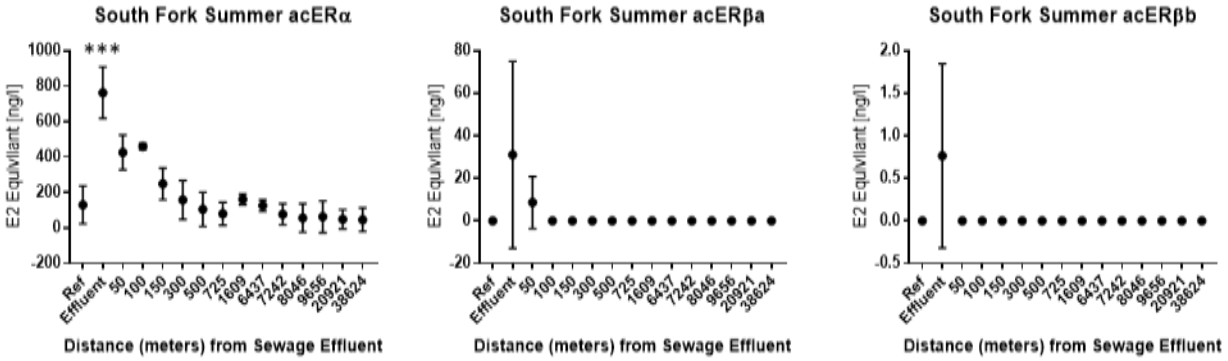

*Figure 3. South Fork of the New River E2 equivalent concentrations at increasing distances from sewage effluent taken during the Summer Season. E2 equivalent concentrations displayed from the three estrogen receptors acERα, acERβa, and acERβb. *P<0.05, **P<0.01, ***P<0.0001.*

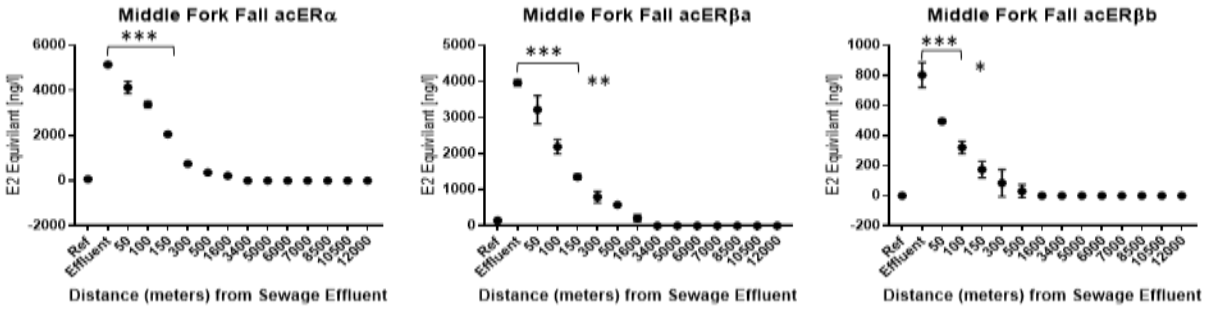

*Figure 4. Middle Fork of the New River E2 equivalent concentrations at increasing distances from sewage effluent taken during the Fall season. E2 equivalent concentrations displayed from the three estrogen receptors acERα, acERβa, and acERβb. *P<0.05, **P<0.01, ***P<0.0001.*

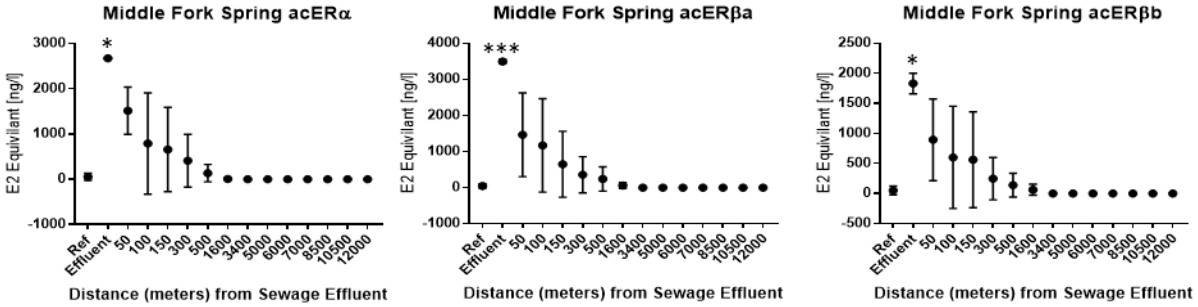

*Figure 5. Middle Fork of the New River E2 equivalent concentrations at increasing distances from sewage effluent taken during the spring season. E2 equivalent concentrations displayed from the three estrogen receptors acERα, acERβa, and acERβb. *P<0.05, **P<0.01, ***P<0.0001.*

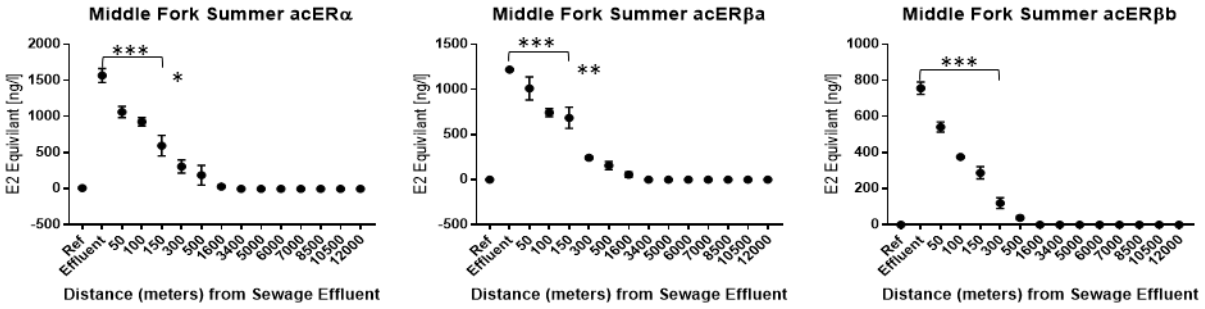

*Figure 6. Middle Fork of the New River E2 equivalent concentrations at increasing distances from sewage effluent taken during the summer season. E2 equivalent concentrations displayed from the three estrogen receptors acERα, acERβa, and acERβb. *P<0.05, **P<0.01, ***P<0.0001.*

Table 2. Percentage variation of three estrogen receptors calculated from two-way ANOVA between river and season. South Fork and Middle Fork were both analyzed against Spring, Summer, and Winter.

	%Variation acERα	P value	%Variation acERβa	P value	%Variation acERβb	P value	df
Sewage Effluent							
Interaction	6.763	0.1217	10.84	<0.0001***	14.7	<0.0001***	2
Season	63.08	0.0002*	17.77	<0.0001***	14.15	<0.0001***	2
River	0.6309	0.4969	73.16	<0.0001***	72.77	<0.0001***	1
50m							
Interaction	1.774	0.4698	15.8	0.0087	5.554	0.3586	2
Season	78.45	<0.0001***	19.78	0.0044	5.261	0.3764	2
River	1.153	0.3281	61.34	<0.0001***	67.3	0.0005**	1
100m							
Interaction	1.824	0.5494	11.86	0.0923	3.651	0.7226	2
Season	82.66	0.0001**	13.98	0.0671	3.621	0.7245	2
River	2.482	0.2193	60.1	0.0003**	43.83	0.0193*	1
150m							
Interaction	1.505	0.6306	9.212	0.1874	8.114	0.5229	2
Season	78.89	0.0002**	9.539	0.1783	8.114	0.5229	2
River	4.13	0.1371	62.93	0.0005**	33.43	0.0401*	1
300m							
Interaction	9.774	0.1999	15.81	0.0769	7.474	0.5593	2
Season	48.18	0.0060**	14.64	0.0891	7.474	0.5593	2
River	0.8288	0.5807	52.64	0.0010**	32.52	0.0452*	1
500m							
Interaction	3.333	0.5428	18.72	0.0367	12.15	0.4129	2
Season	56.41	0.0037**	17.21	0.0445	12.15	0.4129	2
River	2.151	0.3820	51.45	0.0006**	23.06	0.0862	1
1600m							
Interaction	10.87	0.2165	34.54	0.0608	23.26	0.2030	2
Season	26.28	0.0463*	16.12	0.2180	19.86	0.2481	2
River	15.19	0.0504	13.87	0.1112	9.077	0.2527	1

*. Significant at 0.05 Level
**. Significant at 0.05 Level
***. Significant at >0.0001 Level

Vita

Charles Brandon Tate was Born in Mount. Airy, North Carolina to Charles Lee Tate and Lisa Ann Tate. He graduated from North Surry High School with Honors in Mount Airy, North Carolina in May 2009. The following August, he entered North Carolina State University to study Environmental Technology and Chemistry, and in August of 2013 he was awarded the Bachelor of Science degree. In August of 2013 he enrolled in Appalachian State University to begin study toward a Master of Science in Cellular and Molecular Biology. During his degree he took a job with BMG LABTECH, Inc. as a Service Technician and was awarded his M.S. in August 2018.

Mr. Charles Brandon Tate is currently still employed at BMG LABTECH, Inc. and resides in Raleigh, North Carolina with his wife, dogs, and daughter.