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Effects of Emerging Contaminants on Centrarchidae and Catostomidae in Midwestern Rivers: A Multiple Biomarker Approach

by

Camden Garret Nix

THEESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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2021

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Abstract

Natural habitats of fish are gradually declining due to land use and pollution caused by industrial wastes, intensive agriculture and contamination from sewage systems. The United States has 14,780 wastewater treatment facilities that discharge effluent into navigable waters, and in some cases these discharge waters represent a considerable proportion of the water system flow. These activities make it critical to discern the effects of pollutants that are entering our waterways at a consistent rate, such as 17 β -estradiol (E2) and nickel, on aquatic organisms. To determine the effects of 17 β -estradiol on endocrine disruption in fish, Bluegill Sunfish (*Lepomis macrochirus*) were exposed to 40 and 80 ng L⁻¹ of E2 for 21 days, and the change of vitellogenin levels was determined from caudal vein blood via semi-quantitative ELISA. The Kruskal-Wallis test indicated no statistical difference between treatment groups. The other portion of this study examined the consequences of the Sanitary District of Decatur (SDD) exceeding their monthly effluent permit limits of nickel (0.015 mg L⁻¹), with an average concentration of 0.0214 mg L⁻¹. To assess the impact this had on fish assemblages downstream, three Catostomidae populations (Shorthead Redhorses, Smallmouth Buffalo and River Carpsuckers) were captured from the Sangamon River using electrofishing and compared to the same species collected from the Embarras River, which has a lower concentration of nickel. Tissue nickel concentrations were analyzed via ICP-MS at the Savannah River Ecological Laboratory. The Mann-Whitney U Test indicated that River Carpsuckers had statistically higher nickel concentrations in the Sangamon River compared to the Embarras populations. When examining the potential for bioaccumulation using the bioaccumulation factor ($BAF = \frac{\{X\}_{organism}}{\{X\}_{water}}$), all populations indicated no potential for accumulation with values well below the critical threshold of 1000. To assess the potential for human risk the target hazard quotient ($THQ = \frac{EDI}{RfD}$) was calculated for all populations. Each population's THQ was well below 1; indicating no potential risk for human consumption.

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1. Introduction

The United States has approximately 14,780 wastewater treatment facilities, which is partially attributed to the Water Quality Act of 1972 (ASCE, 2003; USC, 1972). Increased population pressure necessitated the expansion of centralized wastewater treatment facilities to reduce primary sources of pollution (Moyle, 1992; Qasim, 1999). Inadvertently, this method has developed concentrated point sources that dominate localized lotic systems and present worst-case scenarios for exposure to aquatic life, with water systems heavily influenced by dam operations being particularly susceptible (Brooks et al., 2006; Diamond and Daley, 2000; Drury et al., 2013; Vajda et al., 2008). Past research has demonstrated that streams across the United States are heavily contaminated with pollutants, such as pharmaceuticals, heavy metals and endocrine disruptors (Brinton et al., 1995; Kolpin et al., 2002; Moyle and Leidy, 2002).

The conventional treatment of sedimentation, conjugation and chlorination for large-scale tertiary wastewater treatment plants (WWTPs) is ineffective at removing these contaminants, leading to potential risk for organisms inhabiting surface waters (Harries et al., 1996; Gunatilake et al., 2013). Although it is necessary to discern the interactive effects of these complex environmental mixtures, deciphering specific compound-species responses at environmental concentrations should be prioritized (Kolpin et al., 2002; Van der Oost et al., 2003). Typically, toxicological tests on model fish species have directed the regulations for all aquatic life (Ankely et al., 2006). This method of regulation ignores the dramatic diversity of teleost fishes, potentially leaving populations at risk (Elliot et al., 2014).

Among the most concerning group of chemicals in the effluent of WWTPs are estrogens, a major group of steroid hormones, which are involved in reproductive regulation within vertebrates (Sandor et al., 1993). The majority of estrogens are synthesized in the ovaries; however, production can occur in the testis, brain, hypothalamus, adipose tissue and placenta (Arcand-Hoy et al., 1998). These hormones are derived from cholesterol and pregnenolone, which are transformed into androstenedione and

testosterone before being synthesized into the three main forms of estrogen: estrone (E1), estradiol (E2) and estriol (E3) (Combalbert et al., 2010). These three-ring phenanthrene compounds differentiate on the position of the hydroxyl groups, with 17β -estradiol (E2) having two hydroxyl groups along with the strongest estrogenic activity (Bovee et al., 2004).

The primary therapeutic role of estrogen is for contraception, but has also been used for menopausal therapy, prostate cancer and breast cancer (Wright-Walters and Volz, 2007). These treatments along with naturally produced estrogens are responsible for humans excreting between 10 and 277 μg of estrogen daily, resulting in $30,000 \text{ kg yr}^{-1}$ of steroid estrogens entering our sewer systems via urine and feces (Adeel et al., 2017; Hamid et al., 2012; Kostich et al., 2013; Laurenson et al., 2014). This concept lead Stumm-Zollinger (1965) to raise concerns that estrogen loads were increasing with human population, and subsequently exacerbating the harmful effects downstream of WWTPs (Jones, 2009). For example, the effluent from the WWTP in Charleston, IL, has a concentration between 0-25.3 ng L^{-1} of E2 and a removal of 64% of the E2 entering the system (Heffron et al., 2016). Research has demonstrated that even at low dosages of E2 ($1\text{-}10 \text{ ng L}^{-1}$), aquatic organisms have disruptions in reproductive function (Guillette et al., 1994; Salla et al., 2006; Thorpe et al., 2001). Fish are particularly susceptible to environmental estrogens because their brain aromatase levels are 1000X higher compared to mammals; making them the only vertebrate with the capability of complete sexual plasticity (Diotel et al., 2010). Such evidence demanded that E2 be assigned to the US-EPA's Contaminant Candidate List to determine if future regulations are necessary (US EPA, 2009).

The endocrine system in vertebrates controls metabolism by responding to internal and external signals to achieve homeostasis (Kime, 1999). These messengers interact with receptors in cells to trigger responses and prompt normal biological functions such as growth, embryonic development and reproduction. Exposure to exogenous estrogens (*e.g.* E2) in fish can alter the gonadotrophin pathways and stimulate steroid synthesis in the gonads (Gunnarsson et al., 2007; Kime, 1999; Pandian, 2015).

Through normal endocrine pathways, gonadotrophin hormone (GTH-I) stimulates the ovary to produce estradiol, which induces production of vitellogenin (VTG), an egg-yolk protein that is normally synthesized by the liver (Hiramatsu et al., 2006). Vitellogenin is then rapidly incorporated into the growing oocytes throughout ovarian growth (Thomas, 2008). Concentrations of vitellogenin are significantly higher in female teleost fish, although the highly-conserved gene that codes for this protein is present in males and can activate when exposed to exogenous estrogens (Hiramatsu et al., 2006; Pandian, 2015). When vitellogenin is produced in males it can reduce fertilization rates, eliminate secondary sexual characteristics and lead to kidney failure by accumulation of vitellogenin around the gonads (Herman and Kincaid, 1988). Therefore, understanding biological response to varying environmental estrogen concentrations is critical for determining future WWTP regulations (Tabb et al., 2006).

Fish is an essential source of nutrients for the human diet, providing high protein content, omega-3 fatty acids, selenium, calcium and a plethora of other vitamins (Dural et al., 2007; Kalantzi et al., 2015). These natural benefits have directed the recommendation of two servings of fish per week by the American Heart Association for a healthy diet (Neff et al., 2009). Unfortunately, the high fat and protein content makes fish more susceptible to the accumulation of contaminants and a potential health risk, especially for human populations with high fish consumption (Usydus et al., 2009; Tao et al., 2012). This is of particular concern regarding heavy metals due to their toxicities, persistence and bioaccumulation potentials (Liao and Ling, 2003).

Nickel is the 24th most abundant naturally occurring element (ASTDR, 2005). Municipal wastewater effluents, industrial point sources (*e.g.* mine tailings), landfill leachates and soil disturbances are primary sources of Ni input into the aquatic environment (European Commission, 2008). Although essential metals, such as Ni, are necessary for growth and biomolecular synthesis, excessive exposure can lead to disease and abnormal conditions (Ali et al., 2019). In the United States, 69 metric tons of

nickel is annually discharged into surface waters, with municipal wastewater being the largest contributor (ASTDR, 2005; Rule et al., 2006). Global demand of Ni is expected to increase by 2,826% by 2040 due to new EV-battery demands (Fraser, 2021). Therefore, determining the rate of bioaccumulation in fish populations downstream of these facilities is imperative for protecting aquatic habitats as well as limiting nickel consumption in human populations.

Non-exposed healthy adult humans have an average body burden of $7.3 \mu\text{g Ni Kg}^{-1}$, with an absorption of 1-2% of ingested nickel (ASTDR, 2005). Nickel enters the food web through direct consumption and bioaccumulates in high trophic-level species, presenting the risk of entering the human diet. Acute nickel toxicity reports by ingestion of inorganic nickel are sparse within the literature (Szathmary and Daldrup, 1982). However, lung fibrosis, increase in spontaneous abortions, kidney disease and carcinogenic activity have been attributed to chronic nickel exposure in human case studies (Chashchin et al, 1994; Haugen et al., 1989). Therefore, determining sources of chronic nickel exposure is vital for human safety.

2. Objectives and Hypotheses

2.1. Bluegill Sunfish 17 β -estradiol Exposure (Mesocosm)

Endocrine disrupting chemicals (EDCs) have been detected in wastewater treatment plant effluents at concentrations from $1\text{-}80 \text{ ng L}^{-1}$ (Wright-Walters and Volz, 2009). The exposure to estradiol (E2) can induce vitellogenin production in males, a precursor protein of egg yolk, giving an early indication of feminization. Vitellogenin is normally synthesized by the liver in response to endogenous estrogen in females; however, males have the VTG gene that can be activated in the presence of this reproductive hormone. In the present study, we aimed to evaluate the effects of E2, a biogenic estrogen, on *Lepomis macrochirus* (bluegill sunfish). The specimens were dosed with estradiol over a 21-day period at various concentrations ($0, 40, 80 \text{ ng L}^{-1}$) and blood samples were collected from the caudal vasculature to quantify vitellogenin induction at the start and end of the experiment (Day 0, Day 21).

Plasma samples were then analyzed using ELISA to assess the concentration of vitellogenin at known concentrations of estradiol.

2.1.1 Hypotheses

The null hypotheses tested were: H_{01} : No difference in plasma vitellogenin concentrations for all *Lepomis macrochirus* based on estradiol treatment concentration (0, 40, 80 ng L⁻¹); H_{02} : No difference in plasma vitellogenin concentrations for male *Lepomis macrochirus* based on estradiol treatment concentration.

2.2. Catostomidae Nickel Exposure in Two Illinois Rivers

Recent monitoring efforts on the Sangamon River, downstream of the WWTP in Decatur, IL, have reported high levels of nickel that correspond to commercial activity upstream of the treatment facility. To determine the exposure and bioaccumulation of nickel to the fish assemblage downstream of Decatur's WWTP effluent, three Catostomidae species (*Ictiobus bubalus*, *Moxostoma macrolepidotum*, and *Carpio carpio*) were sampled via electrofishing. The health of the downstream assemblage was contrasted with specimens captured from the Embarras River in East Central Illinois. In the field, morphological measurements and tissue samples were obtained. Nickel concentrations were analyzed via inductively coupled plasma mass spectrometry (ICP-MS).

2.2.1. Hypotheses

The null hypotheses tested were H_{03} : No difference in nickel concentration (mg kg⁻¹) in the three Catostomidae species based on watershed (Embarrass or Sangamon). H_{04} : No difference in nickel concentration (mg kg⁻¹) in the three Catostomidae species within the Sangamon River. H_{05} : No probability of bioaccumulation of nickel in Catostomidae from both watersheds.

2.3. Risk Assessment

Heavy metals entering the aquatic environment not only pose a direct threat to organisms downstream, but also have the potential to indirectly effect human health due to consumption of

contaminated food. Daily consumption of fish in the Midwest is 0.0066 Kg of white fish (EPA, 2014), which is a potential risk if harvested fish populations are accumulating metals. Thus, the present study determined the estimated daily intake (EDI) of Ni of Catostomidae in two Illinois rivers (Embarrass and Sangamon), which was compared to the oral reference dose (RfD) established by the EPA to determine the Hazard quotient (HQ) for this population.

2.3.1 Hypothesis

The null hypothesis tested was H_0 : The hazard quotient for Catostomidae consumption in both Illinois watersheds will pose no risk to human health.

3. Methods

3.1. Bluegill Sunfish 17 β -estradiol Exposure (Mesocosm)

3.1.1. Study Species

To limit pre-experimental exposure to exogenous estrogens, all bluegill sunfish (*Lepomis macrochirus*) specimens were acquired from local impoundments in the Charleston area by 60 Hz pulsed-DC electrofishing gear (ETS Wisconsin, Madison, WI, US). Specimens were caught between March and September of 2016, with an average weight (g) and length (mm) of 125 and 188, respectively. Specimens were transported back to EIU immediately after capture in well-aerated coolers containing localized water. Upon arrival, fish were moved to individual tanks and acclimated for a minimum of 10 days prior to the start of dosing regiments.

3.1.2. Experimental Design

The mesocosm consisted of fifteen 10-gallon tanks at Eastern Illinois University in a secluded room with a 12 hour light/dark cycle. Tank conditions were kept consistent with temperatures of $21 \pm 1^\circ\text{C}$, salinity of approximately 1 ng L^{-1} , pH of 7.0 ± 0.2 and below detection limits of nitrogen derivatives (*i.e.* nitrates, nitrites and ammonia). Tanks were filled to 9 gallons with deionized water to limit the potential of additional contaminants. To limit variability of E2 concentrations during exposure, tanks were retrofitted with carbonless filters to limit sequestration, and fish were fed soy-free food (Dr. Tim's

Aquatics; Moorpark, CA, US) to limit additional sources of estrogens. These parameters have been overlooked in previous experiments (Elliot et al., 2014). Fish were examined daily for lesions and overall health. This protocol was approved by the Institutional Animal Care and Use Committee (IACUC Protocol approval number 15-004).

Three different E2 dosage schemes were used for this protocol (0, 40 and 80 ng L⁻¹) with 5 tanks representing each dosage scheme per trial. Each trial lasted 21 days. Tanks were dosed with 17 β -estradiol (Sigma-Aldrich, E8875-1G, St. Louis, MO, US) three times during each trial to correspond with weekly water changes (2/3 of volume) and the half-life of estradiol (6-8 days) (Jurgens et al., 2002). An 80 mg L⁻¹ stock solution was made prior to each dosage by dissolving 40 mg of E2 in 500 mL of methanol (Sigma-Aldrich, 322415, 99.8%, St. Louis, MO, US). This solution was diluted 1:9 (v:v; stock solution/DI water) to obtain a 8 mg L⁻¹ working solution. Initial dosages (Day 1) were 340 μ L and 170 μ L of working solution, respectively, to the 80 and 40 ng L⁻¹ tanks filled with 9 gallons of DI water. This corresponds to <0.001% of methanol present in the tank, and control tanks were given the same DI/methanol dosage. After day 7 and 14, 23 L of water was removed from each tank and replenished with DI water. The proper E2 concentrations were maintained by adding 285 μ L and 142.5 μ L of a fresh working solution to the 80 and 40 ng L⁻¹ tanks, respectively. This addition accounted for the degradation of E2 in the remaining 11 L and the amount displaced by the 23 L removed from the system, with the 0 ng L⁻¹ tank receiving a blank dosage. To ensure that actual E2 concentrations matched expected concentrations, water samples were acquired from tanks and stored at -40 °C. Treated tanks were sampled one day after dosage (Day 2, 8 and 15) to ensure the E2 homogenized. The control tank samples were taken on the first and last day of the trial. Aliquots of each working solution were also stored for future analysis.

Blood samples were taken Day 0 and Day 22 of the experiment. Blood samples from each specimen were collected via the caudal vein using a 0.75 inch, 23-gauge hypodermic needle (Fisher Scientific, 14-840-87, Waltham, MA, US) and 1 mL Luer-slip syringe coated with heparin solution (Sigma-

Aldrich, Z683531, St. Louis, MO, US). Blood samples were transferred to anticoagulant microcentrifuge tubes and centrifuged at 10,000 rpm for 3 minutes. Plasma was separated via pipette and stored at -80 °C until future analysis. Fish were euthanized after the experiment concluded and were dissected to determine sex.

3.1.3. Biochemical Analyses

Plasma samples were analyzed for vitellogenin using a semi-qualitative enzyme linked immunosorbent assay (ELISA) kit made for fish biomarkers (B00400402, Biosense Laboratories, Bergen, Norway). The manufactured protocol for the Vitellogenin kit was strictly followed. All samples were run in duplicate, yielding a coefficient of determination (R^2) of 0.9827 (Fig. 1). Absorbance values were measured using a 492 nm primary filter on an Abraxis 8-Channel Microplate Reader (Warminster, PA, US). The negative control absorbance values were subtracted from each well to allow for plate-to-plate comparisons, while a carp vitellogenin standard was used as a positive control (Biosense Laboratories, Bergen, Norway). Absorbance values prior to E2 exposure were subtracted from the post-E2 exposure absorbance values to determine relative change in vitellogenin levels ($\text{ABS}_{\text{After}} - \text{ABS}_{\text{Before}} = \Delta \text{ABS}_{\text{VIT}}$).

Samples for estradiol tank concentrations and working solutions were analyzed with an Ecologiena ELISA kit (ab108667, Tokyo, Japan) on an Abraxis 8-Channel Microplate Reader (Warminster, PA, US). Absorbance values were measured using a 450 nm primary filter and a 630 nm differential filter (*i.e.* 450 – 630). The quantitative range was between $0.025 \mu\text{g L}^{-1}$ to $1 \mu\text{g L}^{-1}$, using a 5-point calibration curve in duplicate. Samples were thawed and homogenized on a table shaker for 5 hours, and then filtered through a $1 \mu\text{m}$ pore diameter filter and adjusted to 10% methanol (v/v) prior to testing. Working solutions were diluted 1:1000 (v/v) prior to analysis due to their expected concentration. Each sample was tested in replicate to ensure quality assurance, with a coefficient of determination (R^2) of 0.9912 (Fig. 2). All samples from control tanks were below detection limits. Absorbance values were

converted to E2 concentrations using a 4-parameter logistics regression, $Y = \frac{(0.939 - 0.020)}{(1 + (\frac{X}{0.107})^{1.174})} + 0.020$, via

an Excel-problem-solver provided by Abraxis (Warminster, PA, US).

3.1.4. Statistical Analyses

All data was archived and statistically analyzed using Microsoft Excel. The change in vitellogenin for all treatments represented a non-normal distribution determined by the Shapiro-Wilk test and the D'Agostino-Pearson test. A Kruskal-Wallis one-way analysis of variance was used to determine if vitellogenin induction was correlated to estradiol exposure. Statistical significance was set to an alpha level of 0.05. One-way ANOVAs were also utilized to determine if E2 concentrations of each tank stabilized during the experiment, comparing day 2, 8 and 15 for the 80 and 40 ng L⁻¹ treatment groups.

3.2. Catostomidae Nickel Exposure in Two Illinois Rivers

3.2.1. Study Sites

A portion of the Sangamon River, approximately 5 km from the Lake Decatur Dam, is heavily influenced by the effluent from the Sanitary District of Decatur (SDD) (Fig. 3). This tertiary treatment facility serves Macon County, IL, and has a capacity of 41 million gallons per day. Sampling was conducted from the SDD effluent to 3.5 kilometers downstream. The Embarras River was used as the reference site because of similar fish assemblage, similar lake-river dynamics and minimal influence from a wastewater treatment effluent. The Embarras River sampling was conducted from the Lake Charleston impoundment to 2.5 kilometers downstream.

3.2.2. Study Species and Field Protocol

Three Catostomidae species, River Carpsucker (*Carpoides carpio*), Smallmouth Buffalo (*Ictiobus bubalus*) and Shorthead Redhorse (*Moxostoma macrolepidotum*) were sampled to assess nickel contamination. Fish specimens were sampled from the Sangamon (n = 85) and Embarras River (n = 60) using boat-mounted 60 Hz pulsed-DC electrofishing gear (ETS Wisconsin, Madison, WI, US) between March and June of 2016. Upon capture, fish were measured and weighed and then humanely

euthanized before being transported back to EIU. Fish were sexed, otoliths extracted for aging and a 10-gram tissue plug was taken from each specimen for metal analysis. Tissue samples were stored at -20°C until analysis. This protocol was approved by the Institutional Animal Care and Use Committee (IACUC Protocols 05-010 and 15-004).

3.2.3. Tissue Analyses

Tissue samples were dried at 80 °C in drying ovens upon arrival at the Savannah River Ecology Laboratory (SREL). Dried samples were then weighed to 0.50 ± 0.02 g in pure Teflon PFA vessels and hot-plate digested with 10 mL of 5 M HNO₃ and 3 mL of 5 M H₂O₂ (Fisher Scientific, Waltham, MA, US). Specific volumes of HNO₃ and H₂O₂ were recorded for correct back-calculations. All sample volumes were normalized at 50 mL with DI water. Nickel concentrations (mg kg⁻¹) were analyzed using inductively coupled plasma mass spectrometry (ICP-MS). Each batch of samples were conducted in conjunction with a HNO₃ blank and a U.S. National Institute of Standards and Technology (NIST) standard reference material (TORT). Digested tissue samples strictly followed the methodology outlined in USEPA method 3052B, while the approach to quality control followed USEPA method 6020. Indium-115 and Scandium-45 were used as internal standards.

3.2.4. Statistical Analyses

All data were archived and statistically analyzed using Microsoft Excel. Normality of nickel concentrations for each species was tested using the Shapiro-Wilk Test and the D'Agostino-Pearson Test. The Shorthead Redhorse Nickel data were log₁₀ transformed to normalize the data, while the River Carpsucker and Smallmouth Buffalo data had non-normal distributions that could not be transformed. Instead, the Mann-Whitney U Test was used to compare nickel-tissue concentrations based on tributary (Sangamon or Embarras) for the River Carpsucker and Smallmouth Buffalo. The difference in Shorthead Redhorse nickel-tissue concentrations based on tributary were determined with a pooled t-test. All statistical significance was set to the 0.05 alpha level.

The Bioaccumulation factor of nickel was also determined for each specimen based on the following equation:

$$BAF = \frac{\{X\}_{organism}}{\{X\}_{water}}$$

Where $\{X\}_{organism}$ is the concentration of nickel in that specimen and $\{X\}_{water}$ is the concentration of nickel in the watershed that specimen was sampled (Wang et al., 2017). The concentrations of nickel in the Sangamon and Embarras tributaries were previously determined to be 0.0214 and 0.005 mg L⁻¹, respectively (Illinois, 2018; Lin and Raman, 1991). BAF values below 1000 L kg⁻¹ have no probability of bioaccumulation, while anything above 1000 indicates bioaccumulation (Arnot and Gobas, 2006).

3.3. Human Risk Assessment

The estimated daily intake (EDI) of nickel through consumption of the Catostomidae examined in the present study was calculated using the following equation (Liu et al., 2018; Taghizadeh et al., 2017):

$$EDI = \frac{EF \times ED \times FIR \times C_m}{BW \times TA}$$

The constants used for this calculation were as follows: EF is exposure frequency (104 days year⁻¹; American Heart Association recommended); ED is the frequency duration (70 years, average human life-span (World Health Organization, 2010)); FIR is the white-fish ingestion rate for the Midwest (0.0066 kg person⁻¹day⁻¹); C_m is the concentration of Ni in the specimen (mg kg⁻¹); BW is the average body weight of an American, which is 90.6 and 77.5 kg for males and females, respectively; and TA is the average exposure time for non-carcinogens (365 days year⁻¹ x ED) (EPA, 2014; Fryar et al., 2021; Kris-Etherton, 2002). The units of EDI are mg kg⁻¹day⁻¹.

The target hazard quotient (THQ) is a ratio that expresses the potential hazard of consuming based off the oral reference dosage (RfD) for nickel, which is 0.02 mg kg⁻¹day⁻¹ (ATSDR, 2005; Liu et al., 2018). The equation is the following:

$$THQ = \frac{EDI}{RfD}$$

THQ values that are less than 1 indicate no potential risk for the exposed consumer, while THQ values greater than 1 suggest potential health risks for the exposed individual.

4. Results and Discussion

4.1. Bluegill Sunfish 17 β -estradiol Exposure (Mesocosm)

Wastewater treatment plants are the primary point source of endocrine disruptors, with 113,000 kg yr⁻¹ of natural steroid estrogens (E1, E2, E3) entering our surface waters each year from human and cattle excrement (Adeel, 2017). The common tertiary wastewater treatment methods are inefficient at removing exogenous estrogens (*i.e.* 64%). Past research has indicated that the effluent from the WWTP in Charleston, IL, has an average estradiol concentration of 25.3 ng L⁻¹, with other facilities detecting concentrations up to 80 ng L⁻¹ (Heffron et al., 2016; Wright-Walters and Volz, 2009). This presents a potential conundrum for fish populations downstream since low dose concentrations of 10 ng L⁻¹ of estradiol have been shown to disrupt normal reproductive function (Routledge and Sheahan, 1998). Therefore, to discern if these environmental relevant concentrations impact fish reproduction, we exposed Bluegill Sunfish (*Lepomis macrochirus*) to 40 and 80 ng L⁻¹ of estradiol for 21 days and took blood samples pre- and post-experiment to determine vitellogenin levels. Vitellogenin is the primary biomarker used to assess the impact of exogenous estradiol loads have on aquatic organisms and can be an early indicator of male-feminization (Rodgers-Gray et al., 2000; Xu et al., 2008).

Bluegill sunfish (*Lepomis macrochirus*) are generalist predators with small home-ranges that hold significant importance for Midwest fisheries based on the characteristics of rapid growth, acceptance in commercial diets and low water quality requirements (Elinger and Wilson, 1988; Gao et al., 2009). This species is a vital tool for comparing ecosystem health due to their spatial range in the Midwest, allowing monitoring efforts to pinpoint emerging contaminants. Previous experiments have demonstrated varying responses of E2 exposure by Centrarchidae in terms of vitellogenin production

(Elliot et al., 2014; Fentress et al., 2006; Gao et al., 2009). However, no previous experiments have analyzed the change in vitellogenin levels in Bluegill Sunfish pre- and post-exposure at the maximum E2 concentrations (80 ng L^{-1}) measured in the wild (Wright-Walters and Volz, 2009).

The average concentration of the estradiol working solution was 8.1 mg L^{-1} , with a recovery of 101.4%. The average estradiol concentrations throughout the experiment for the 80 and 40 ng L^{-1} tanks were 80.38 and 42.51 ng L^{-1} , respectively (Fig. 4). No statistical difference of estradiol tank concentration based on sample day (Day 2, 8 and 15) was detected for either treatment groups (80 ng L^{-1} : $F(2,18) = 0.012$, $p = 0.988$; 40 ng L^{-1} : $F(2,15) = 0.032$, $p = 0.968$), which suggests that the E2 concentrations were consistent throughout the trial. A total of 44 Bluegill Sunfish were used in the present study, although one specimen died during the first trial that was part of the 80 ng L^{-1} treatment group.

The average change of vitellogenin absorbance for the 0, 40 and 80 ng L^{-1} treatment groups was 0.257, 0.624 and 0.594, respectively (Fig. 5). The range of vitellogenin induction for each treatment group can be found in Table 1. No statistical difference in vitellogenin levels was detected when including both sexes in the analyses ($H(2) = 1.98$, $p = 0.372$). To discern if there were statistical differences between treatment groups when the data were separated by sex, both male and female groups were run independently. There were 26 individuals identified as males post experiment, with 10, 9 and 7 individuals in the 0, 40 and 80 ng L^{-1} treatment groups, respectively. The average change of vitellogenin absorbance for the males in the 0, 40 and 80 ng L^{-1} treatment groups was 0.364, 0.370 and 0.645, respectively (Fig. 6). The ranges and standard errors of vitellogenin induction for the male treatment groups can be found in Table 2. No statistical difference in vitellogenin levels was detected for the males ($H(2) = 0.40$, $p = 0.819$). There were 18 individuals identified as females post experiment, with 5, 6 and 7 individuals in the 0, 40 and 80 ng L^{-1} treatment groups, respectively. The average change of vitellogenin absorbance for the females in the 0, 40 and 80 ng L^{-1} treatment groups was 0.043, 1.006 and 0.544 respectively (Fig. 7). The ranges and standard errors of vitellogenin induction for the female

treatment groups can be found in Table 3. As with the male group, no statistical difference in vitellogenin levels was detected for the females ($H(2) = 1.926$, $p = 0.382$).

The severity of exogenous estrogens on fish reproduction is highly dependent on length of exposure, age during exposure (immature/mature) and species type (Folmar et al., 1996; Nam et al., 1998; Writer et al., 2010). Accordingly, previous studies examining the induction of vitellogenin in Bluegill Sunfish showed varying results (Jorgenson et al., 2014; Schultz et al., 2013; Wang et al., 2008). For example, Wang et al. (2008) exposed bluegill fry to an E2 diet of 50-200 mg kg⁻¹ over a 120-day period that resulted in a 100% monosex female population. While other studies that examined mature bluegill sunfish wild populations downstream of WWTPs indicated no change in vitellogenin levels (Fentress et al., 2006; Schultz et al., 2013). A non-correlated response was also recognized by Jorgenson et al. (2014) in a laboratory study that exposed Bluegills to 10 and 30 ng L⁻¹ for a 21 day period, while silvery minnows from that same study exhibited an increase in vitellogenin concentration. These previous experiments along with the data from the present study may indicate that Centrarchidae are more tolerant of exogenous estrogen than other species (Teather and Parrott; 2006).

Another potential explanation for no significant induction of vitellogenin among treatments in the present study is that these fish were taken from local impounds that had limited predation and an abundance of food, thereby creating a healthier population than would typically be seen immediately downstream of wastewater effluent (Jorgenson, 2014). Past research has detected 10-fold variation of vitellogenin levels in wild Bluegill Sunfish populations, with some variation being explained by seasonal changes and difference in water temperature (Cheek et al., 2014). Also, the bioavailability of estradiol has been correlated to the amount of endogenous compounds already bound to plasma proteins, which can limit the number of estrogen receptor sites available to exogenous estrogen (Kohn and Melnick, 2002). This could be a possible explanation for these results since all individuals selected had distinct secondary-sexual characteristics, which suggests an abundance of steroid hormones already present

within their plasma. It should also be noted that polyclonal antibodies were used for the ELISA analyses, potentially misrepresenting the amount of vitellogenin within an individual due to the presence of nine distinct vitellogenin mRNAs in Bluegill sunfish (Hutchinson et al, 2005). Future studies that target specific proteins or examine the levels of individual transcripts may be necessary to elucidate changes in expression within this protein family.

Fish are capable of liver biotransformation of lipophilic xenobiotics (*e.g.* E2) into hydrophilic metabolites that can be more readily excreted to avoid toxic burden on the organism (Thomas, 2008). However, biotransforming these metabolites places an energy constraint on cellular function and can further increase energy demand and inhibit growth. For example, a previous study indicated that E2 exposure on Bluegill Sunfish can alter basal metabolism, indicating that multiple biological responses could be induced by exposure to environmental estrogens (Karki, 2017). Therefore, understanding varying biological responses to environmental estrogens is critical for determining future regulations (Tabb et al., 2006). Although the present study did not examine the effects of E2 exposure on basal metabolism of the fish, subsequent projects should consider energy constraints in addition to endocrine disruption.

The ability of steroid estrogens to disrupt endocrine pathways has been shown in a variety of animal taxa (Nikaido et al., 2004; Oehlmann et al., 2000; Setchell et al., 1987). These scenarios should provide foresight into the potential danger to human populations, especially if estrogen loads in water systems continue to increase, which has been highlighted in a wide range of academic publications (Caldwell et al., 2009; Janfaza et al., 2006). For example, E2 exposure in pre-pubertal and pubertal children may lead to excessive rapid growth, as well as the early onset of puberty in females and the late onset of puberty in males (Andersson and Skakkebaek, 1999). In addition, post-puberty exposure to high concentrations of E2 can induce testicular and ovarian cancer, as well as stimulate cardiovascular disease, hypertension, metabolic disorders (such as obesity and diabetes) and immune disorders

(Wright-Walters and Volz, 2009; Prossnitz & Barton, 2011). Consequently, examining biological responses to active environmental contaminants in sentinel species is vital to protecting human health.

4.2. Catostomidae Nickel Exposure in Two Illinois Rivers

The increasing utilization of nickel in modern industries has exacerbated the environmental burden, with domestic wastewater being the largest anthropogenic source of nickel entering surface waters in the United States (Nriagu and Pacyna; 1988). Therefore, it is critical to assess potential impacts on aquatic organisms downstream of these systems, particularly for subsistence fish populations that provide essential nutrients, such as omega-3 fatty acids, proteins and micronutrients to local human populations. Systems that regularly surpass their permit limits are presenting situations of chronic exposure and should be monitored accordingly. As an example, the Sanitary District of Decatur (SDD; Central Illinois) WWTP has consistently exceeded their nickel effluent concentration permit limit with an average concentration of 21 ng L^{-1} , largely due to upstream river-dam dynamics and industrial effluents.

Lake Decatur Dam is a primary water supply for local municipalities and industry, and water is only discharged downstream in periods of high precipitation or during dam operations. This river-dam-dynamic results in a portion of the Sangamon River being 100% composed of SDD effluent during significant portions of the year. This creates a dilemma for the SDD because diluting effluent contaminants (*i.e.* heavy metals) for local and national permit requirements depends on the 7Q10, which is the lowest 7-day average flow that occurs (on average) once every 10 years. Without the ability to dilute the effluent, the monthly average Ni effluent concentration of the SDD WWTP consistently exceeds their permit limit (0.015 mg/L). The background concentration of nickel in the Embarras River is below 0.005 mg L^{-1} (Lin and Raman, 1991).

To assess the effects of nickel exposure on fish populations in the Sangamon River, a total of 81 Catostomidae were collected in the spring of 2016, with 24, 25 and 32 specimens of River Carpsucker, Shorthead Redhorse and Smallmouth Buffalo, respectively. A total of 60 Catostomidae were collected

from the Embarras River (East Central Illinois) consisting of 20 River Carpsuckers, 23 Shorthead Redhorses and 17 Smallmouth Buffalos. The nickel concentration of the Embarras River has been shown to be lower than the Sangamon River (Lin & Raman, 1991; SDD, 2018), making the Embarras River a useful comparison system in the region. These species were chosen due to their abundance in both tributaries, small home ranges (5 km) and foraging habits (Scott, 2009). River Carpsuckers and Smallmouth Buffalo are primarily detritus & algae foragers while Shorthead Redhorses primarily feed on benthic macroinvertebrates (Gildo, 2001; Scott, 2009). These long-living species also have commercial and recreation fishing value throughout the Midwest; presenting a potential risk for human consumption.

The average nickel concentration found in River Carpsuckers in the Sangamon was 0.164 mg kg^{-1} of tissue, while the Embarras population had an average of 0.094 mg kg^{-1} (Fig. 8). A statistical difference was detected in nickel-tissue concentration for River Carpsuckers between the Sangamon and Embarras River using the Mann-Whitney test ($U(135) = 2.463$, $p = 0.0138$). The average nickel concentration found in Smallmouth Buffalo in the Sangamon River was 0.143 mg kg^{-1} compared to 0.111 mg kg^{-1} in the Embarras River (Fig. 9). While the average nickel concentration found in Shorthead Redhorses was 0.144 mg kg^{-1} for the Sangamon River and 0.112 mg kg^{-1} for the Embarras River (Fig. 10). No statistical difference of nickel concentration between tributaries was detected for Shorthead Redhorses ($t(46) = 1.217$, $p= 0.23$) or Smallmouth Buffalo ($U(209) = 1.839$, $p = 0.066$).

The average bioaccumulation factor for the River Carpsuckers, Smallmouth Buffalo and Shorthead Redhorses populations of the Sangamon River were 7.66 , 6.70 , 6.74 L kg^{-1} respectively; while the Embarras River's River Carpsuckers, Smallmouth Buffalo and Shorthead Redhorses populations had a bioaccumulation factor of 18.78 , 21.12 and 22.48 L kg^{-1} respectively. The bioaccumulation factors for all species in both tributaries indicated no probability of accumulation (Table 4). However, all species from the Embarras River demonstrated higher bioaccumulation compared to the Sangamon River, with the

Shorthead Redhorses from the Embarras having the largest factor of 22.48 L kg^{-1} . Nevertheless, this value is much less than the suggested minimum factor of 100 L kg^{-1} used to indicate bio-accumulation (Arnot and Gobas, 2006). This suggests that the chronic nickel exposure in the Sangamon River is not resulting to biomagnification in Catostomidae and should not place risk on subsistence fishing. A previous study on the Chinese Tapetail Anchovy (*Coilia nasus*) from Taihu Lake had a BAF (nickel) of 120 L kg^{-1} and resulted in the government placing massive restrictions on fishing for this species, presenting a large burden for local fishermen. Therefore, it is crucial to examine fish assemblages that are exposed to permit-limit violations and discern if there is a potential for risk.

The estimated human daily intake (mg of Ni per kg of body weight $^{-1}\text{day}^{-1}$) for males and females was calculated for all three species in both tributaries (Table 5). These values were used to calculate the Target Hazard Quotient (THQ) for each sampling group (Table 6). No potential risk for male or female human consumption was indicated for River Carpsuckers, Smallmouth Buffalo or Shorthead Redhorses in either tributary ($\text{THQ} \leq 1$). With the River Carpsuckers from the Sangamon River presenting the most risk with an average Target Hazard Quotient of 1.7E^{-3} . Although these populations do not present risk, previous studies have used the THQ to alter fishing regulations. For example, Zig-zag eel (*Mastacembelus armatus*) from the Kasimpur canal in India had a THQ of 1.008 and resulted in the government banning fishing for this species for the foreseeable future (Javed and Usmani, 2016).

Normal extracellular metabolism of nickel is facilitated by ligand exchange reactions (Sarkar, 1984). Within the aquatic environment, studies have demonstrated that Ni toxicity can induce multiple endpoints in a variety of organisms, but the mechanism of Ni toxicity is still poorly understood (Blewett and Leonard, 2017; Brix et al., 2007; Murray et al., 2010). Evidence has suggested that oxidative stress could be induced by Ni toxicity, stimulating the production of reactive oxygen species within fish tissue (Kubrak et al., 2012; Zheng et al., 2014). These by-products readily interact with DNA, proteins and other biomolecules, in turn, inflicting cellular and molecular damage (Palermo et al., 2015). It has also

been suggested that exposure to nickel induces symptoms similar to hypoxia, altering intracellular metabolism and glucose transport (Denkhuas and Salnikow, 2002). It has also been demonstrated that nickel can influence homeostasis of $\text{Fe}^{2+}/\text{3+}$, Ca^{2+} and Mg^{2+} within aquatic organisms (Brix et al., 2007). Varying fish species accumulate Ni at various rates and deposit in different tissues, presenting a problem for identifying correct populations to examine (Bosch et al., 2016). Therefore, identifying aquatic populations with Ni bioaccumulation will guide future studies in determining the mechanism of toxicity and facilitate more accurate consumption rates for fish in Central Illinois.

5. Conclusions and Future Work

The present study evaluated the effects of emerging contaminants, nickel and 17β -estradiol, on fish assemblages in Central Illinois. To discern the effects of 17β -estradiol, Bluegill Sunfish were exposed to 40 and 80 ng L⁻¹ for 21 days in a controlled mesocosm and the change of vitellogenin was calculated. The findings showed that acute E2 exposure did not induce vitellogenin in mature Bluegill Sunfish. This may be explained by limited accepting steroid-receptors due to competition with endogenous steroid hormones; potentially explaining the disparity of vitellogenin induction that is noticed in Bluegill Sunfish fry studies (Wang et al., 2008). It should also be noted that most laboratory specimens, such as Fathead Minnows, are fractional spawners compared to Bluegill Sunfish, which are annual spawners. The sensitivity differences between these reproduction strategies may indicate that fractional spawners are more sensitive to physicochemical environmental cues.

Future studies should expand from 15 individuals per treatment group to determine if low sample sizes were at least partially responsible for the lack of statistical difference in vitellogenin levels. The exclusion of females from subsequent studies may also improve the statistical analysis by allowing for greater numbers of male fish to be examined, which may have more pronounced changes in vitellogenin as a result to estrogen levels. Another suggestion is to acquire monoclonal antibodies that are specific for Bluegill Sunfish for the ELISA analysis; potentially limiting the variation among samples

and replicates. This study used polyclonal antibodies that were applicable to a variety of fish species and more specificity may have altered the results. It would also be beneficial to acquire farm-raised Bluegill Sunfish that were guaranteed to have no previous EDC exposure and were relatively the same age.

The other portion of this study examined nickel exposure to three Catostomidae populations in the Sangamon River downstream of the Sanitary District of Decatur compared to Embarras River populations. The population of River Carpsuckers from the Sangamon River had significantly higher nickel concentration ($\text{mg kg of tissue}^{-1}$) relative to the Embarras River. A better understanding of Catostomidae home ranges would discern whether these elevated nickel concentrations in River Carpsuckers can be associated with acute or chronic exposure.

Future studies with nickel exposure should sample the Sangamon River during the periods of lowest flow (August – November) since that is when the river's composition is largely comprised of WWTP effluent. Nickel concentrations would be expected to be highest during this period. It would also be beneficial to assess other biomarkers in River Carpsuckers, such as metallothionein and DNA-strand-breakage, to decipher if these elevated nickel concentrations are placing extra energy burdens on a cellular level. Another suggestion is collecting gut content within these Catostomidae to determine how much nickel is entering via their diets. Correlating nickel content in tissue compared to dietary nickel could provide insight to the rate of uptake but also shed light on other aquatic organisms that are at potential risk from nickel exposure.

Finally, it is vital to examine the effects of these and additional emerging contaminants in Central Illinois rivers on fish populations to better inform fisheries managers and governmental agencies when determining environmental limits. With wastewater treatment facilities being the primary point source of pollution in the U.S., they provide unique opportunities to study the effects of emerging contaminants on downstream aquatic organisms. These studies are critical for determining what contaminants are of primary concern and limiting widespread irreversible environmental consequences.

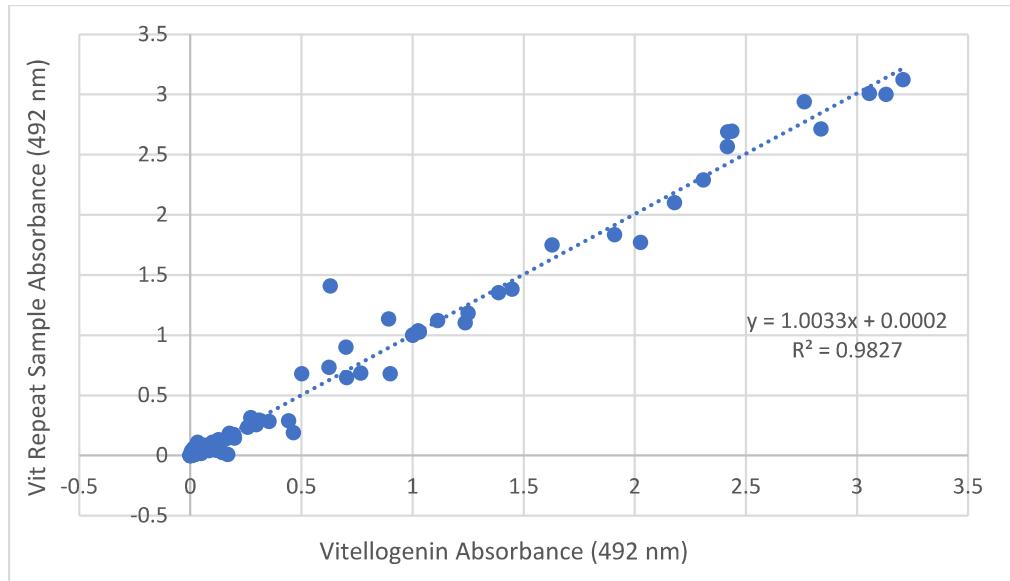


Figure 1: Repeatability of sample replication of vitellogenin absorbance at 492 nm via ELISA. All samples were run in duplicate, totaling 116 samples.

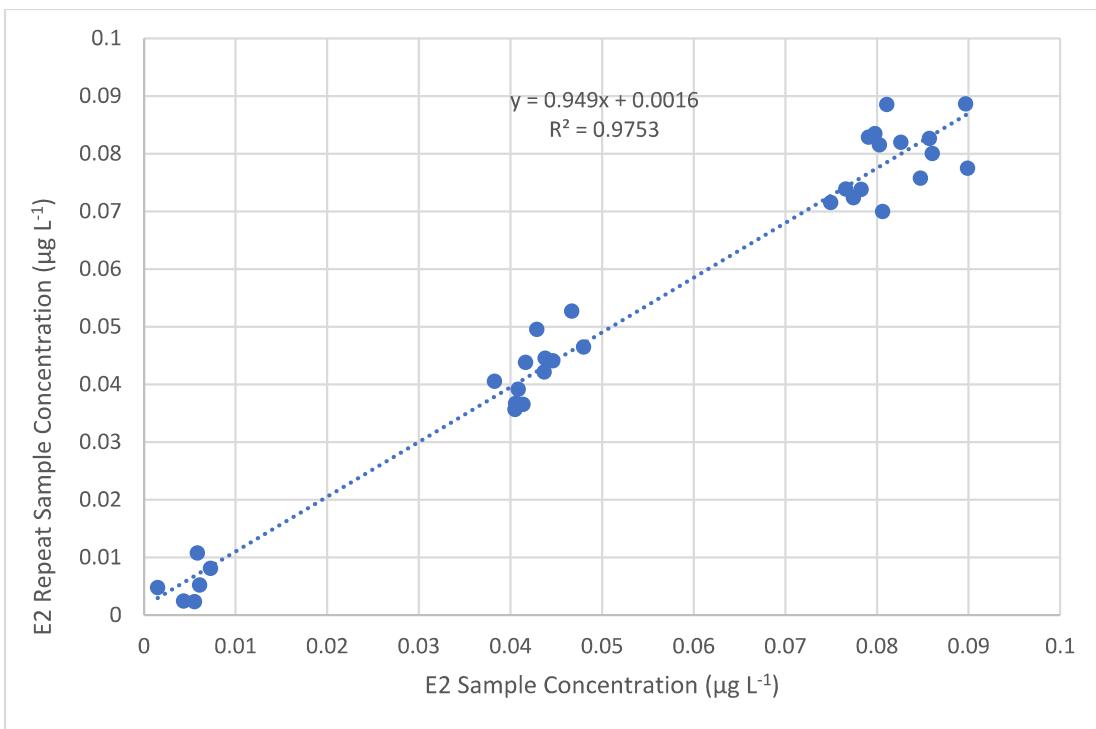


Figure 2: Repeatability of sample replication of 17β -estradiol concentration ($\mu\text{g L}^{-1}$) via ELISA. All samples were run in duplicates, totaling 42 samples.

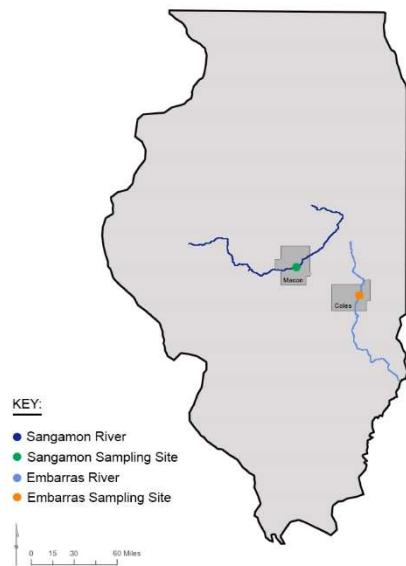


Figure 3: A map of the Sangamon River, located in Macon County of Central Illinois, and the Embarras River, located in Coles County of East Central Illinois. The sampling locations for the present study are indicated.

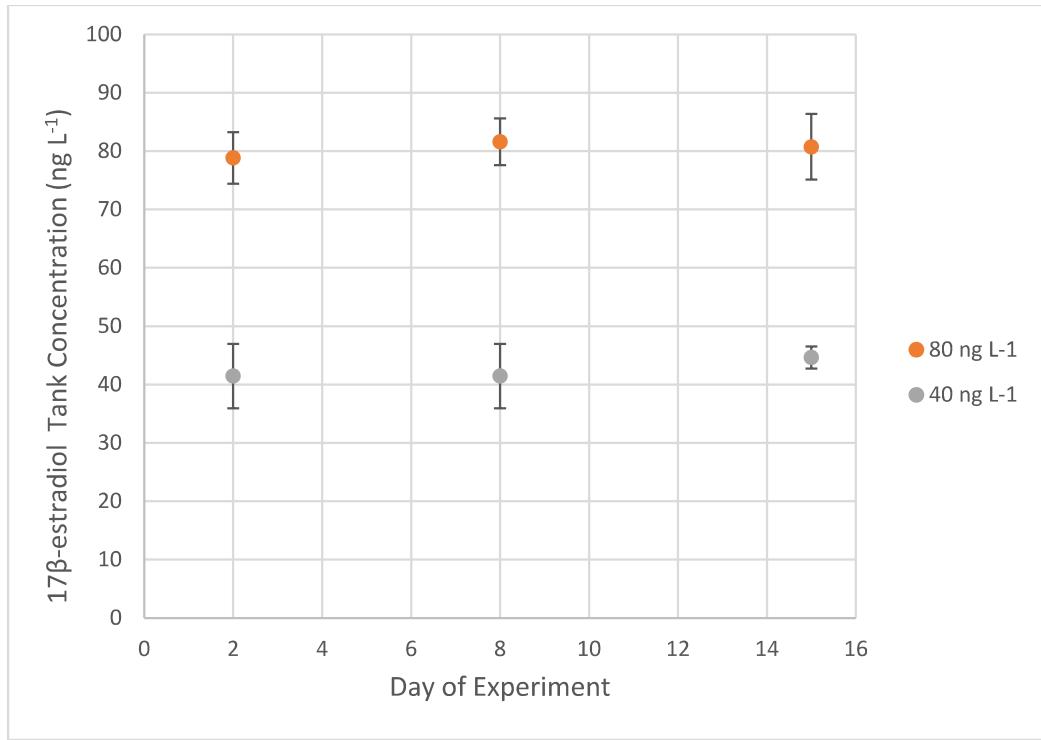


Figure 4: Average 17β -estradiol tank concentrations (ng L⁻¹) from day 2, 8 and 15 of mesocosm experiment.

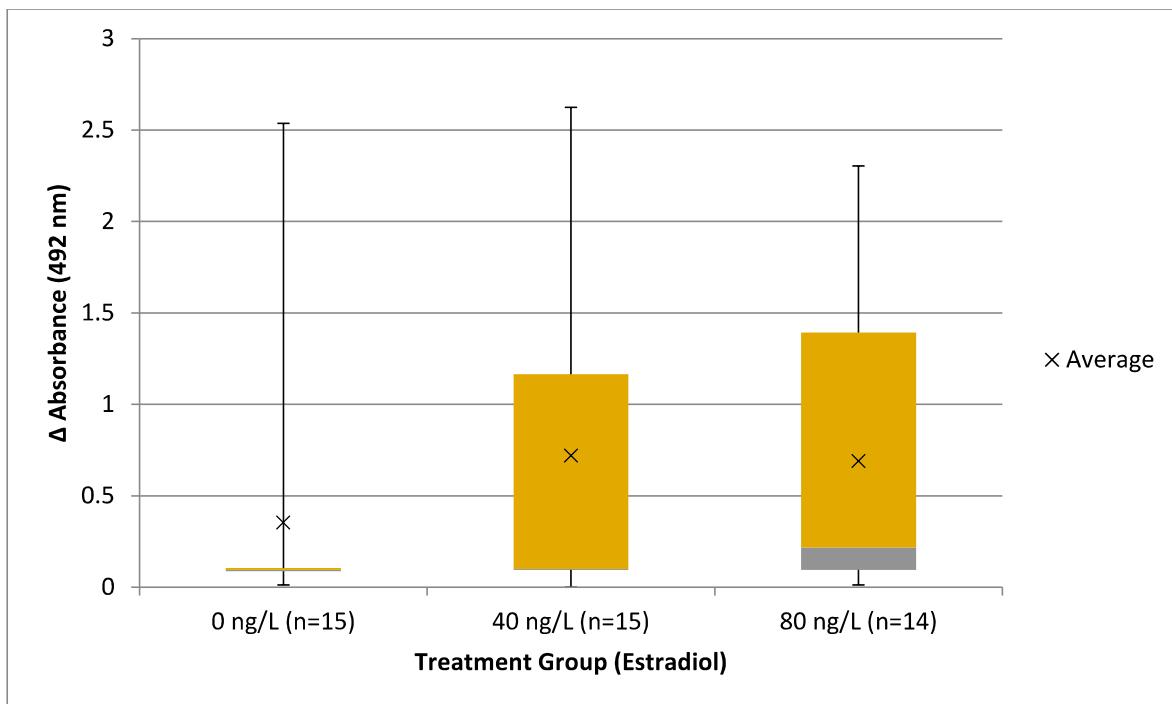


Figure 5: Boxplots showing the change of vitellogenin absorbance (492 nm) from before and after exposure to 17 β -estradiol for all (male and female) specimens in control and treatment groups.

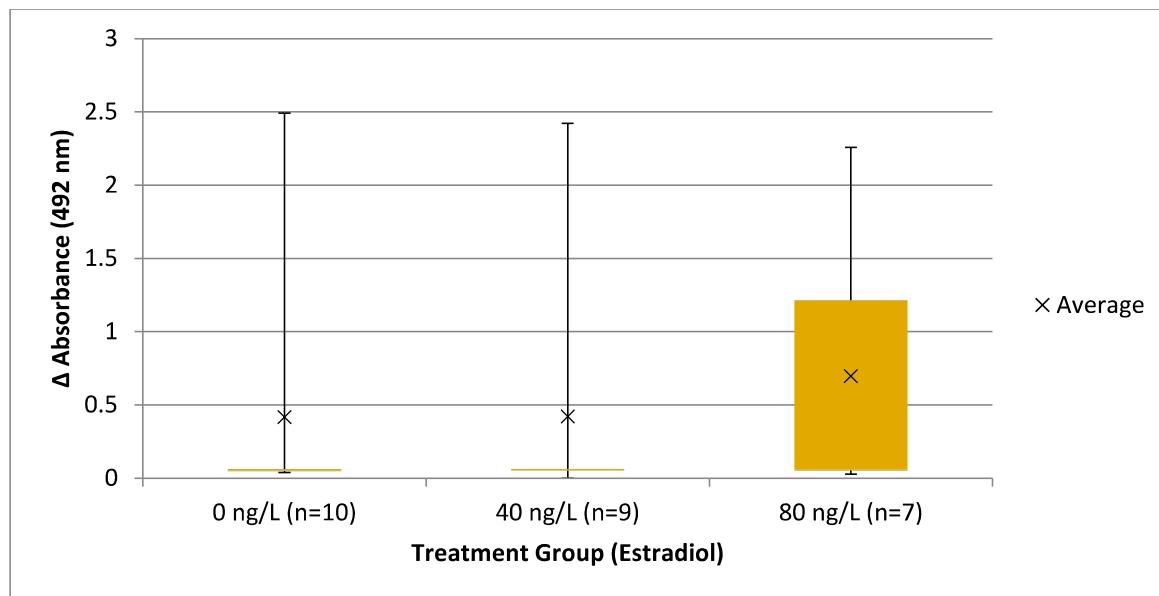


Figure 6: Boxplots showing the change of vitellogenin absorbance (492 nm) from before and after exposure to 17β -estradiol for all males in control and treatment groups.

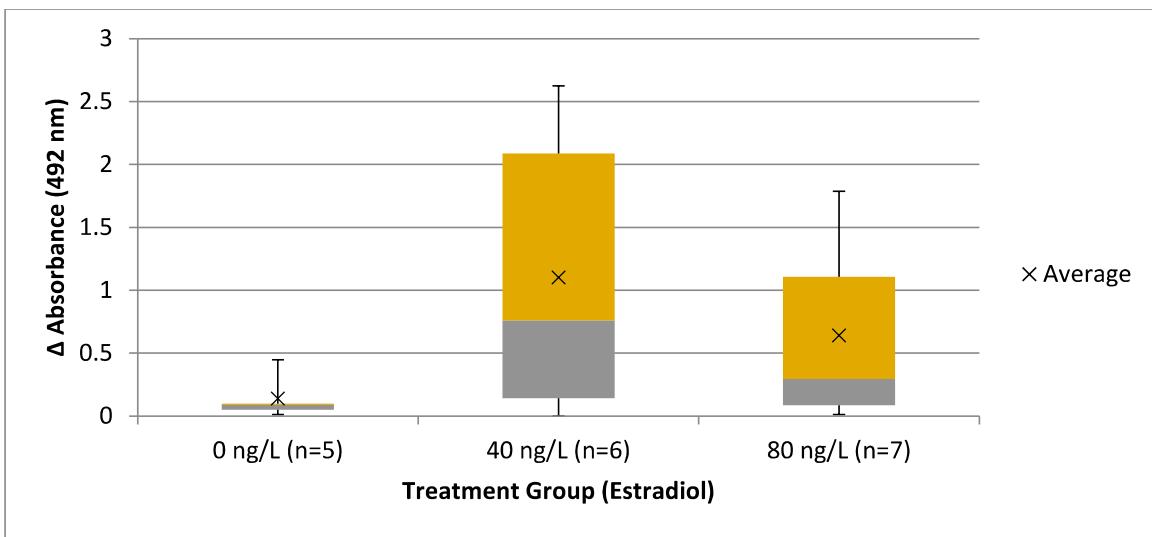


Figure 7: Boxplots showing the change of vitellogenin absorbance (492 nm) from before and after exposure to 17 β -estradiol for all females in control and treatment groups.

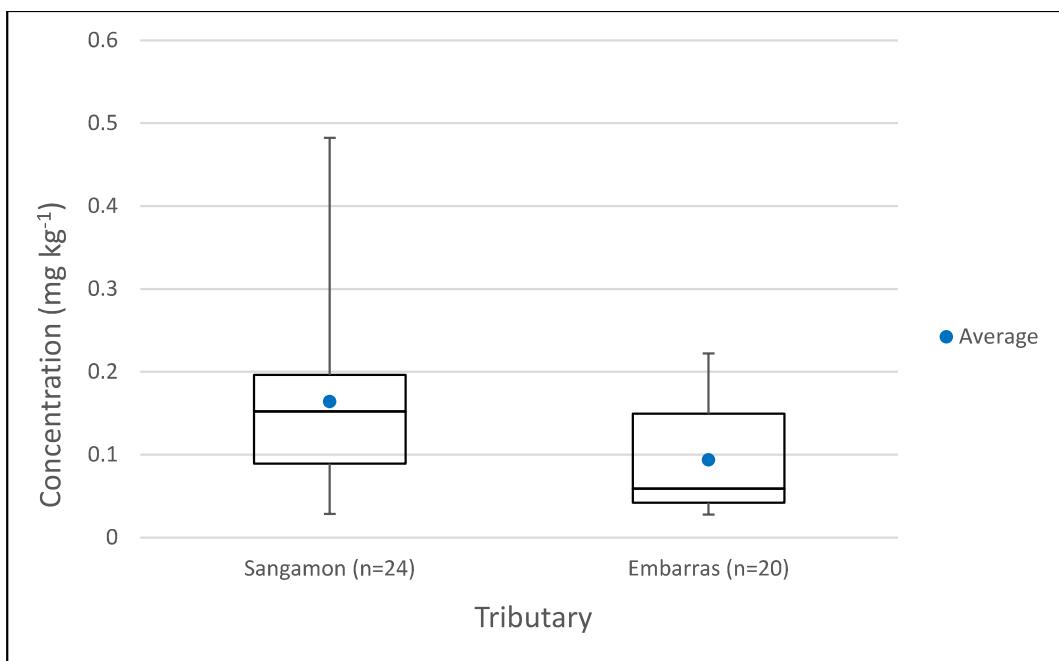


Figure 8: Boxplot showing nickel concentration (mg kg^{-1}) in tissue for River Carpsuckers for the Sangamon and Embarras Rivers.

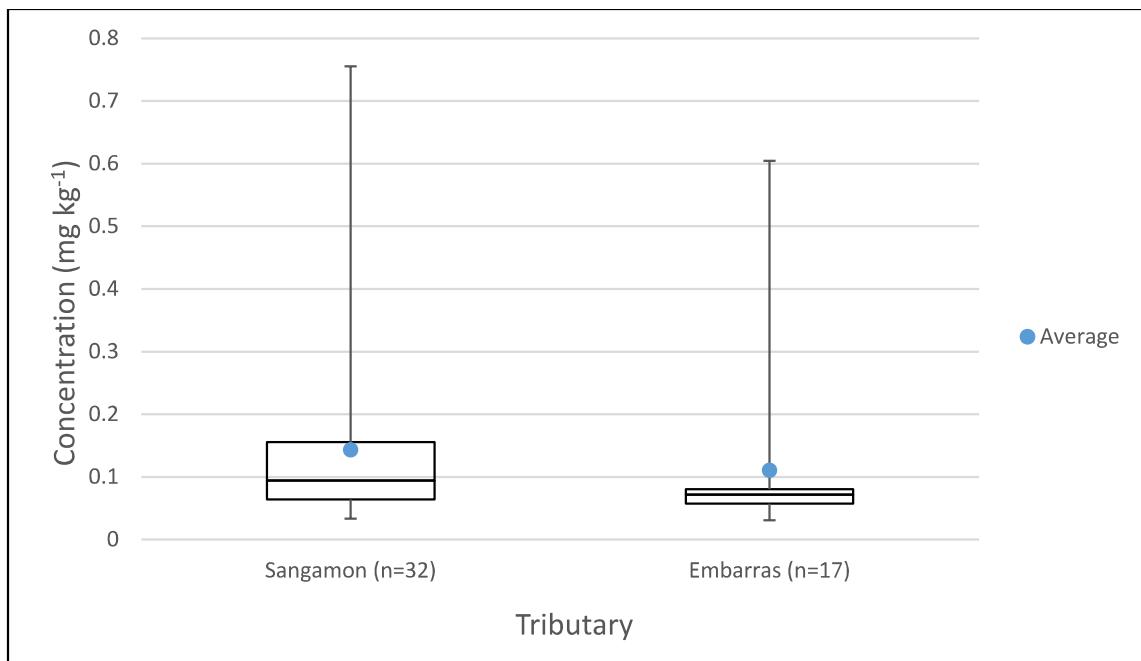


Figure 9: Boxplot showing nickel concentration (mg kg^{-1}) in tissue for Smallmouth Buffalo for the Sangamon and Embarras Rivers.

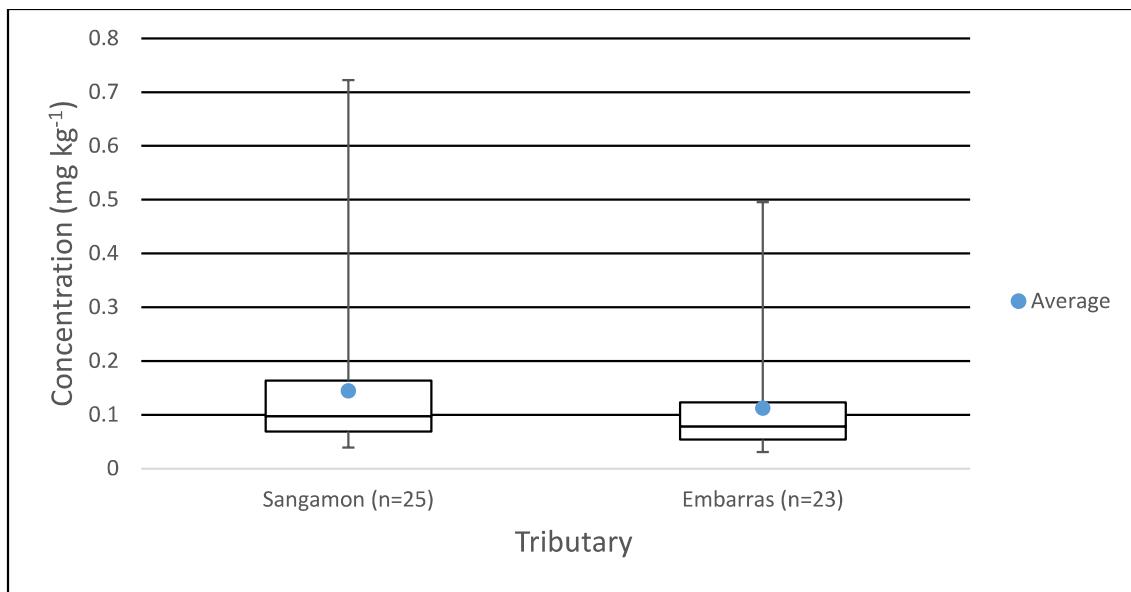


Figure 10: Boxplot showing nickel concentration (mg kg^{-1}) in tissue for Shorthead Redhorse for the Sangamon and Embarras Rivers.

Table 1: Mean (standard error in parentheses) and range of vitellogenin absorbance (492 nm) change from before and after exposure to 17 β -estradiol for all specimens in control and treatment groups.

Treatment Group	Range: Δ in Vitellogenin absorbance (492 nm)	Average (\pm SE) Vitellogenin (492 nm)
0 ng/L	-0.084 – 2.441	0.257 (0.177)
40 ng/L	-0.097 – 2.529	0.624 (0.255)
80 ng/L	-0.085 – 2.207	0.594 (0.220)

Table 2: Mean (standard error in parentheses) and range of vitellogenin absorbance (492 nm) change from before and after exposure to 17 β -estradiol for all males in control and treatment groups.

Treatment Group	Range: Δ in Vitellogenin absorbance (492 nm)	Average (\pm SE)
0 ng/L	-0.013 – 2.441	0.364 (0.260)
40 ng/L	-0.052 – 2.371	0.370 (0.274)
80 ng/L	-0.025 – 2.207	0.645 (0.356)

Table 3: Mean (standard error in parentheses) and range of vitellogenin absorbance (492 nm) change from before and after exposure to 17 β -estradiol for all females in control and treatment groups.

Treatment Group	Range: Δ in Vitellogenin absorbance (492 nm)	Average (\pm SE)
0 ng/L	-0.084 – 0.352	0.043 (0.079)
40 ng/L	-0.097 – 2.529	1.006 (0.478)
80 ng/L	-0.085 – 1.692	0.544 (0.286)

Table 4: Mean bioaccumulation factor for each study species and river with standard deviation.

Bioaccumulation Factor	Embarras River (Average \pm SD)	Sangamon River (Average \pm SD)
River Carpsuckers	18.78 \pm 2.87	7.66 \pm 1.02
Smallmouth Buffalo	22.12 \pm 6.46	6.70 \pm 1.08
Shorthead Redhorses	22.48 \pm 4.25	6.74 \pm 1.32

Table 5: Mean estimated daily intake (EDI) for female and male human consumption for each study species and river, including standard deviation.

EDI: Males	Embarras River (Average ± SD)	Sangamon River (Average ± SD)
River Carpsuckers	$2.0E^{-0.5} \pm 3.0E^{-06}$	$3.4 E^{-0.5} \pm 4.5E^{-06}$
Smallmouth Buffalo	$2.3E^{-0.5} \pm 4.4E^{-06}$	$3E^{-0.5} \pm 5.9E^{-06}$
Shorthead Redhorses	$2.3E^{-0.5} \pm 4.4E^{-06}$	$3E^{-0.5} \pm 5.9E^{-06}$

EDI: Females	Embarras River (Average ± SD)	Sangamon River (Average ± SD)
River Carpsuckers	$2.3E^{-0.5} \pm 3.7E^{-06}$	$4.0E^{-0.5} \pm 5.4E^{-06}$
Smallmouth Buffalo	$2.7E^{-0.5} \pm 7.8E^{-06}$	$3.5E^{-0.5} \pm 5.6E^{-06}$
Shorthead Redhorses	$2.7E^{-0.5} \pm 5.2E^{-06}$	$3.5E^{-0.5} \pm 6.9E^{-06}$

Table 6: Mean target hazard quotient (THQ) for female and male human consumption for each study species and river, including standard deviation.

THQ: Males	Embarras River (Average ± SD)	Sangamon River (Average ± SD)
River Carpsuckers	$9.7E^{-05} \pm 1.5E^{-5}$	$1.7E^{-3} \pm 2.1e^{-4}$
Smallmouth Buffalo	$1.4E^{-4} \pm 4.7E^{-5}$	$1.5E^{-3} \pm 3.8e^{-4}$
Shorthead Redhorses	$1.2E^{-4} \pm 2.2E^{-5}$	$1.5E^{-3} \pm 3.3e^{-4}$

THQ: Females	Embarras River (Average ± SD)	Sangamon River (Average ± SD)
River Carpsuckers	$1.2e^{-3} \pm 1.7e^{-4}$	$2e^{-3} \pm 2.7e^{-4}$
Smallmouth Buffalo	$1.4e^{-3} \pm 2.4e^{-4}$	$1.8e^{-3} \pm 3.7e^{-4}$
Shorthead Redhorses	$1.5e^{-3} \pm 5.1e^{-4}$	$1.7e^{-3} \pm 2.8e^{-4}$

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