ARE TISSUES OF CHANNEL CATFISH MORE ESTROGENIC IN AREAS WITH HIGH DENSITIES OF COMBINED SEWAGE OVERFLOWS?

by

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The Three Rivers area of Pittsburgh, Pennsylvania has more combined sewer overflow (CSO) release points than any other city in the United States. CSOs and sanitary sewer overflows (SSOs) release untreated waste directly into receiving water during wet weather events such as rain or snow. A wide range of estrogenic agents is contained in municipal wastewater, including pharmaceutical estrogens, plastic additives, pesticides and detergent breakdown products such as nonyl-phenol.

The goal of this analysis was to examine estrogenicity of channel catfish fillet tissue in areas significantly impaired by CSO/SSOs compared to store-bought catfish and catfish from up-river areas on the Allegheny River that are less impacted. Estrogenicity was based on the ability of catfish fillet tissue to proliferate MCF7 human breast cancer cells. Cell proliferation was quantified using a serial dilution assay. Replicate values for each fish at each dilution were analyzed using a random intercept model. Area effects were quantified in terms of absolute and relative differences, controlling for background. In this study, cell proliferation is higher for catfish sampled from the most contaminated CSO/SSO sites than for catfish sampled from areas on the Allegheny with fewer CSOs/SSOs.

The risk information concerning cumulative estrogenicity in channel catfish, in this study may provide a linkage between the ecological compounds contained in wastewaters and human health. Estradiol equivalents could be constructed from the estrogenicity index developed in this

paper. These findings are significant to public health because they could help to estimate the risk of estrogenic exposure posed to those who consume channel catfish from the Three Rivers Area of Pittsburgh. The findings could also help describe the impact of estrogenic exposure in wildlife.

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1.0 INTRODUCTION

1.1 PITTSBURGH FISH CONTAMINATION AND CONSUMPTION PROJECT

The Pittsburgh Fish Contamination and Consumption Project, initiated in September 2005, consists of three major components:

1. Understanding the attitudes, behaviors, values, perceptions, and beliefs of local anglers regarding the contamination in the Three Rivers Area and other area streams and water bodies, fish eating behavior, and cleaning and cooking techniques.
2. The screening analysis of white bass and channel catfish for a panel of metals including mercury, arsenic and the metalloestrogens.
3. The screening assessment of extracts of fish for the ability of their flesh and fat to bind to the estrogen receptor or proliferate the MCF-7 breast cancer cell line.

The descriptive study initially intended to describe the levels of metals in area fish, to help develop a new method for determining an estrogenicity index for fish, and to assess the relative safety of fish as food for subsistence fishers and anglers. This thesis focuses on the third component of the study involving the ability of fish extracts to bind to the estrogen receptor and

proliferate the MCF-7 human breast cancer cell line. The MCF-7 human breast cancer cell line is exquisitely sensitive to the presence of estrogens or estrogen-mimicking chemicals.

1.2 RESEARCH QUESTIONS

The objective of this analysis is to compare the estrogenicity of channel catfish fillet tissue, based on the ability to proliferate MCF7 human breast cancer cells for fish obtained from areas with different densities of combined and sanitary sewers outfalls. The fish are sampled from locations on the Allegheny, Monongahela and Ohio Rivers near Pittsburgh, PA, at the Kittanning Dam, approximately 40 miles upstream from Pittsburgh on the Allegheny River (a more undisturbed site environmentally), and store bought.

A combined sewer overflow (CSO) occurs when storm water and sewage, carried in a single pipe, overload the sewer system and flow untreated into rivers and streams (Weather 2007). A sanitary sewer overflow (SSO) occurs when a line designed to carry only sewage becomes overloaded with storm water (Weather 2007). This causes untreated sewage to overflow from manholes or back up into basements and rivers.

In the area served by the Allegheny County Sanitary Authority (ALCOSAN), 83 communities and approximately 124 major industries discharge aqueous waste to the sewer system. Pittsburgh has 317 outfall structures, more than any other city in the U.S (Weather 2002). The Environmental Protection Agency has identified over 50 of these outfalls as SSOs, the remaining outfalls are classified as CSOs. The total number of SSOs and CSOs within the satellite sewer systems owned by the municipalities has not yet been determined (USDOJ 2007).

All CSO and sanitary sewer overflows SSOs in the Three Rivers Area were identified by latitude and longitude by Three Rivers Wet Weather (Weather 2007). Within a two-mile radius of the Point, there are approximately 74 CSO outfalls. Within five miles of the Point, there are approximately 184 CSO/SSO outfalls. Within a 10 mile radius of the point, there are 268 CSO outfalls. The remaining CSO/SSO outfalls are all at least 10 miles away from the Point. The locations of these outfalls are shown in Figure 1.

Between the Point and the Highland Park Dam, a distance of approximately 7 miles, there are approximately 72 CSO/SSO outfalls (Figure 1). The density of CSO outfalls decreases near the Highland Park Dam sampling site. Between the Point and Braddock, a distance of approximately 11 miles, there are approximately 62 CSO/SSO outfalls. Additionally, there are approximately 12 SSOs and 15 CSOs on a major feeder stream immediately upstream from the Braddock Dam site. There are only 9 CSOs on a 4-mile stretch of the Allegheny River near the Kittanning catch site.

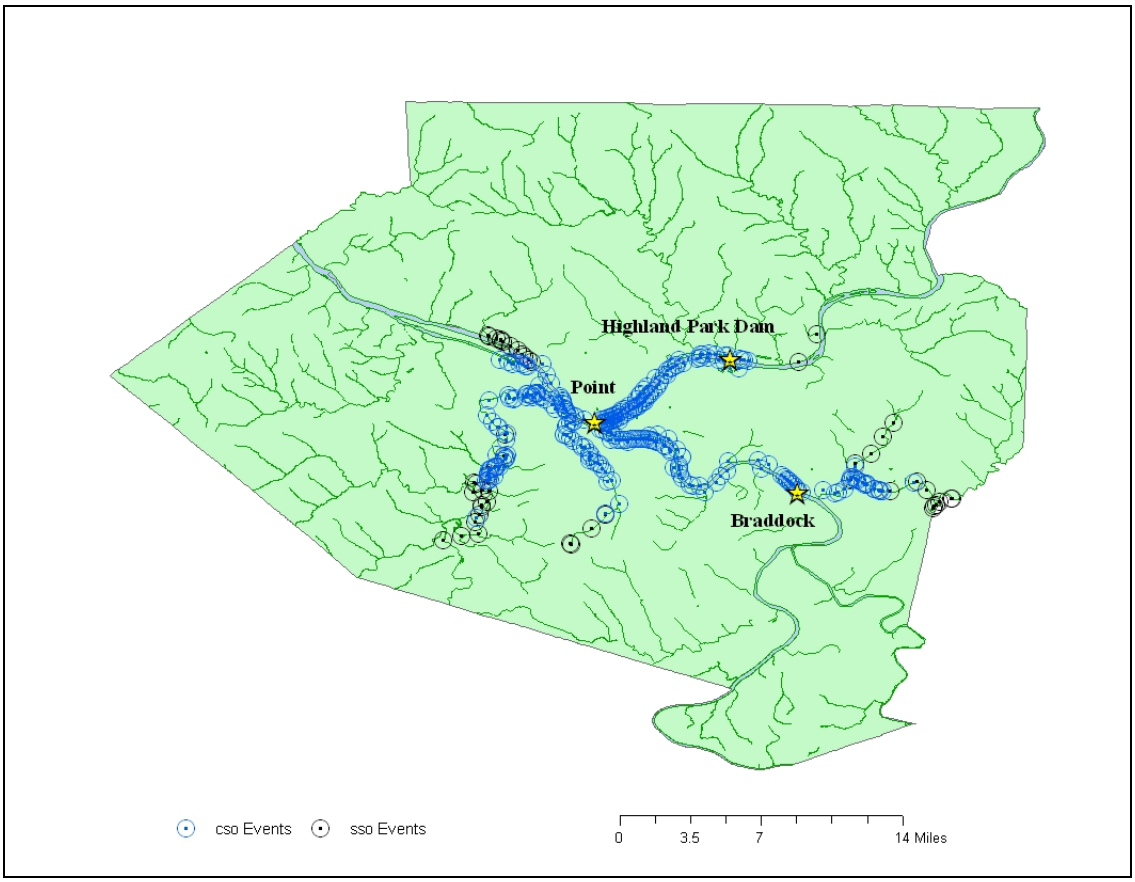


Figure 1: Map of CSO and SSO Locations in Allegheny County

The hypotheses for this study are driven by geography and knowledge of the sewage system in Allegheny County. Because several of the materials present in waste-waters may contain estrogenic or xenoestrogenic materials, we hypothesize that fish from areas with a greater density of CSO/SSO outfalls will proliferate the MCF-7 cell line more than fish that are from Kittanning (where there are very few CSO/SSO locations) and store bought fish that were farm raised in Georgia (and never exposed to CSO/SSO wastewaters).

The first research question examines whether there are differences in estrogenicity of fish across locations:

- Are there differences in estrogenicity between catfish caught at the Point, caught near the Highland Park Dam, caught near Kittanning, caught near the Braddock Dam, and store bought fish (farm raised in Georgia)?

If at least one location differs from the others, we will test all pair-wise comparisons to identify specific differences.

Because store bought fish have never been exposed to waters from CSO/SSO outfalls, we will examine the differences in estrogenicity of fish from all locations, excluding store-bought fish.

- Are there differences in estrogenicity between catfish caught at the Point, caught near the Highland Park Dam, caught near Kittanning, and caught near the Braddock Dam?

We would also like to examine any differences in estrogenicity between the Pittsburgh Pool (Braddock Dam, Highland Park Dam, and Point) and the non-Pittsburgh Pool (Kittanning and store bought). This grouping is of interest because the Pittsburgh Pool can be viewed as a lake with restricted fish movement bounded by the Emsworth Dam on the Ohio River, the Highland Park Dam on the Allegheny River, and the Braddock Dam on the Monongahela River.

- Are there differences between the estrogenicity of catfish caught in the Pittsburgh Pool (Braddock Dam, Highland Park Dam, and the Point) and catfish caught in Kittanning and bought at the store?
- Is there homogeneity in the Pittsburgh Pool?
- Is there homogeneity in the Non-Pittsburgh Pool?

Finally, we will look at estrogenicity of fish caught at Point State Park and near the Braddock Dam on the Monongahela River to see whether they differ in estrogenicity from fish caught on the Allegheny River. We are interested in this grouping because the density of CSO outfalls is greatest at Point State Park and near the Braddock Dam on the Monongahela River. Additionally a feeder stream emptying into the Monongahela River immediately upstream from the Braddock Dam has approximately 27 CSO/SSO outfalls. CSO outfall density decreases as you move upstream on the Allegheny River near the Highland Park Dam, there are only 9 CSO outfalls in a 4-mile length near Kittanning PA, on the Allegheny River. In general, the Allegheny River has better water quality than the Monongahela River due to the relatively undisturbed environmental condition of its source watersheds and waters.

- Is there a difference in estrogenicity between fish caught at Point State Park and near the Braddock Dam on the Monongahela River and fish caught near Kittanning and the Highland Park Dam on the Allegheny River?

2.0 REVIEW OF RELEVANT LITERATURE

The endocrine system is one of the body's main communication networks. It is a network of glands and hormones that regulates many of the body's functions including growth, development and maturation, as well as the way various organs operate (Council 2007). The endocrine gland, (the pituitary, thyroid, adrenal, thymus, pancreas, ovaries, and testes) release regulated amounts of hormones into the bloodstream that act as messengers, traveling to parts of the body in order to control many life functions (Council 2007).

Chemicals known as endocrine disruptors may interfere with the endocrine system and produce adverse effects in wildlife and humans. Endocrine disruptors may interfere with a body's own hormone signals by blocking or mimicking naturally occurring hormones such as estrogens, androgens, and thyroid hormones (NIEHS 2007). The blocking or mimicking of naturally occurring hormones can alter normal hormone levels by halting or stimulating the production of hormones or changing the way hormones travel through the body, thereby affecting the functions that those hormones control (Council 2007).

Chemicals that affect the endocrine system include synthetic and natural estrogens. There is also a group of endocrine disruptors known as phytoestrogens. Together the synthetic estrogens and phytoestrogens are termed xenoestrogens (Shaw 2002). All xenoestrogens are endocrine disruptors, but not all endocrine disruptors are xenoestrogens. For example, tamoxifen

is an endocrine disruptor, but is not a xenoestrogen. Recent public concern has focused on the possible hormonal effects of estrogen disruptors in wildlife and humans.

Several studies demonstrate the potential effects of endocrine disrupting chemicals in humans. For example, populations that consume fish from areas high in xenoestrogens are at higher risk for developing cancer, neurological problems, and reproductive problems (Colborn 1993). Testicular cancer in males is increasing and may be attributed to endocrine disruptors in the environment (Adami 1994). Other studies show that over the past 50 years there has been a steady decline in sperm count in humans, which could be attributed to estrogenic effects (Carlsen 1992; Auger 1995; VanWaeleghem 1996). Additional studies have shown that endocrine disrupting chemicals are linked to developmental deficiencies and learning disabilities in children (EPA 2001).

Humans may be exposed to xenoestrogens by direct contact with pesticides and other chemicals or through intake of contaminated water, food, or air. It is unlikely that adverse human health effects are caused by any specific xenoestrogen in the water, due to their minute concentrations compared to estrogenic compounds found in food sources (Snyder 2003). However, researchers are concerned that a combination of estrogens may have an additive effect that could result in adverse health effects (Shaw 2002).

Contamination of surface and subsurface waters can be caused by agricultural runoff, industrial effluents, and municipal wastewaters (Kale 1995). Agricultural runoff is responsible for the presence of most pesticides found in surface waters (EPA 2001). Examples of pesticides include DDT (an insecticide banned in the U.S. in 1988), endosulfan (primarily used on food crops including tea, coffee, fruits and vegetables, rice, cereals, maize, sorghum, and other

grains), and methoxychlor (an insecticide similar to DDT but with a relatively low toxicity and low persistence in biological systems) (EPA 2001).

Chemicals released into the environment from industrial uses include polychlorinated biphenyls (PCBs) and dioxin. All manufacturing of PCBs has been banned since 1974 and previous use in electrical capacitors and transformers has been greatly reduced (EPA 2001). Despite the manufacturing ban, large quantities remain in the environment because of their chemical-resistant properties. Dioxin is formed during incineration of chlorinated industrial compounds such as plastic and medical waste. Dioxins bioaccumulate throughout the food web because of their lipophylic properties and slow metabolic destruction (EPA 2001).

Municipal wastewaters include materials released from sewage treatment plants. Contained in this water are alkylphenols and alklylphenol ethoxylates such as nonylphenol and octylphenol. Nonylphenol and octylphenol are used to make surfactants, which are the primary active ingredient used to make cleaning and sanitizing agents (EPA 2001). The surfactants do not break down completely in sewage treatment plants or the environment. Also contained in municipal waste waters are natural steroids (17*B*-estradiol and estrone), and the synthetic estrogen used in contraceptives; all of these are excreted by women in domestic sewage (Christiansen 2002). Now, hormone replacement products being excreted in domestic sewage have a greater presence in wastewaters than contraceptives.

Humans and wildlife may also be exposed to estrogen disrupting chemicals by interacting with plastic additives such as bisphenol A (an industrial chemical commonly used for food and drink packaging materials) and diethyl phthalate (DEP; a synthetic substance that increases the flexibility of plastics used to make toothbrushes, automobile parts, tools, toys, and food packaging). DEP is also commonly found in cosmetics, insecticides, and aspirin (EPA 2001).

Exposure to xenoestrogens through persistent environmental contamination can affect wildlife species. In the 1980's it was reported that gulls living in areas with DDT exhibited skewed sex ratios and deformed organs (Fry et al., 1987). In certain amphibian populations, supernumary limbs and missing limbs have been attributed to certain pesticides (Sparling, 2000), and the inability of captive cheetahs to reproduce at the Cincinnati Zoo was linked to a diet high in phytoestrogens (Setchell et al., 1987).

Often, fish are used as sentinels of ecological health because they play a number of roles in the tropic web, they bioaccumulate toxic substances, and they respond to low concentrations of mutagens (Stegeman 2000). Channel catfish are used in the Pittsburgh Fish Consumption and Contamination Project because they are numerous, widespread, and a popular fish for both sport fishing groups and subsistence anglers in the waters of the Three Rivers Area. Catfish are territorial and typically stay within a 10-mile range.

Measuring the amount of contaminants in Channel catfish is important because of the risk that such contaminants pose to higher-level carnivores such as larger fish, predatory birds, and humans (Burger 2006). Channel catfish can also be directly indicative of the health and well-being of the organisms they ingest, of their own populations, of the populations with which they interact, and of the populations that consume them (Burger 2006).

Numerous studies that have shown that wastewaters containing estrogenic chemicals affect the reproductive health of wild fish. For example, male fish downstream of some waste-water outfalls have early stage eggs in their testes and produce vitellogenin, a protein normally synthesized by females during oocyte maturation (Kidd 2007). In another study, fish exposed for 300 days to untreated wastewater showed induced feminization in male fish, altered kidney development, modulated immune function, and genotoxic damage (Liney 2006).

3.0 HUMAN BREAST CANCER CELL PROLIFERATION ASSAY

The screening assessment of the extracts of fish flesh and fat for their ability to bind to the estrogen receptor or proliferate the MCF-7 breast cancer cell line is a proxy technique to determine whether estrogen mimicking chemicals may have been bio-concentrated in fish tissues from water and food sources (Volz 2006). MCF-7 cells are a well characterized estrogen receptor (ER) positive control cell line (i.e. cells are positive for cytoplasmic estrogen receptors) and therefore are a useful in vitro model to study the role of estrogen in breast cancer (Abcam 2007).

For the cell proliferation assay, the CellTiter 96 Aqueous One Solution Cell Proliferation Assay, a colorimetric method for determining the number of viable cells in proliferation, was used. This is an indirect cell counting method.

To begin, an extract of fish flesh and fat was taken for each fish by removing a one gram section of the fish (from backbone to stomach) and then combining it with organic solvents (chloroform methanol 80:20 - the ratio is 9:1 by volume) to solubilize hormonally active substances. The chloroform methanol was then blown off with nitrogen, leaving a residue. This residue is stored at 20^{o}C until needed. One ml of ethanol glycerine (3:1) was then added to the residue.

A stock solution of 1/100 was then formed by combining 13.720 ml of a steroid free medium (95% RPI without phenol red and 5% dextran-charcoal treated serum) and 2.8 ml of 1/1

fish extract, resulting in a total volume of 14.0 ml. To arrive at the final dilution concentrations of 1/100, 1/200, 1/500, 1/1000, 1/1500, 1/2000, 1/3000, 1/4000, various amounts of the steroid-free medium were added to varying volumes of fish extract (Table 1).

Table 1: Preparation of Dilutions from Fish Extracts

Final Concentration	Volume of Fish Extract	Volume of Medium	Total Volume
1/100	2.8 mL of 1/1 Fish Extract	13.720 mLs of charcoal-stripped medium	14.0 mLs
1/200	5 mLs of 1/100 Fish Extract	5 mls of charcoal-stripped medium	10 mLs
1/500	4 mLs of 1/200 Fish Extract	6 mls of charcoal-stripped medium	10 mLs
1/1000	5 mLs of 1/500 Fish Extract	5 mLs of charcoal-stripped medium	10 mLs
1/1500	1.340 mLs of 1/100 Fish Extract	8.660 mLs of charcoal-stripped medium	10 mLs
0.0005	5 mLs of 1/1000 Fish Extract	5 mLs of charcoal-stripped medium	10 mLs
0.000333333	5 mLs of 1/1500 Fish Extract	5 mLs of charcoal-stripped medium	10 mLs
0.0005	5 mLs of 1/2000 Fish Extract	5 mLs of charcoal-stripped medium	10 mLs

Five thousand MCF-7 cells were seeded into every well, except for 3 background control wells, returned to the CO_2 incubator at 37^{o}C and 5% CO_2 and allowed to adhere overnight. The next morning, the 96-well plates were taken out of the incubator, treated with 100ml of the various concentrations of fish extract and returned to the humidified CO_2 incubator at 37^{o}C and 5% CO_2 and allowed to incubate.

After 72 hours, the plates were removed from the incubator, the treatment medium was removed, and 100ml of fresh medium was added in addition to 20ml of the CellTiter96 Aqueous One Solution Reagent. The absorbance of each well was then measured at 490 nm with a Bio-Tek Synergy HT well plate reader. The quantity of fish extract as measure by the amount of

490nm absorbance is directly proportional to the number of living cells in culture. This indirect method is used because it would be impractical to use direct cell counting methods given the amount of time required.

A slight amount of spontaneous 490nm absorbance occurs in culture medium incubated with the reagent used in this assay. Several variables may contribute to the background 490nm absorbance. To correct for the background absorbance, a triplicate set of control wells (without any fish cells) was prepared using the same volumes of culture medium and CellTiter96 Aqueous One Solution Reagent. The average 490nm absorbance from the "no cell" control wells for each dilution was subtracted from all other absorbance values to yield background corrected absorbance values. This is a recommended method by the Promega Corporation who developed the assay (Promega 2004).

Background corrected absorbance value for sample *i* at dilution *j* =

$$(absorption.value) - (avg.value.of.background.ctls.for.dilution.j)$$

Figure 2: Calculation of Background Corrected Absorbance Value

Estradiol controls have the same initial cell dilution (5000 cells/well) and the same dilution of estradiol (1nM) applied to each well. The estradiol controls establish the cells' response to the physiological dose of estradiol.

To account for the variation in cell cultures, a proliferation index was calculated. The proliferation index provides an absolute index of proliferation relative to the control wells. The proliferation index is the background corrected absorbance value divided by the mean background corrected absorbance value for all the control wells (on that plate).

$$\text{proliferation index} = \frac{background.corrected.absorbance.value}{mean.background.corrected.absorbance.value.of.all.control.wells.on.plate}$$

Figure 3: Calculation of Proliferation Index

Estradiol controls and blank controls were included in each run because cell cultures evolve and change. Even though the same amount of estradiol is used each time, the control wells will have varying proliferation values because the numbers of estrogen receptors differ.

Similarly a number of factors, such as differences in the number of estrogen receptors, result in variable responses in the estradiol control wells. Therefore, the proliferation index for the estradiol control wells varies across wells and across fish. To normalize the proliferation index across all fish, an estrogenicity index was calculated. It provides a relative index that is more useful than the proliferation index for comparisons between fish and across locations. The relative index is more useful than an absolute index because it allows for the proliferation values at each location to be on the same scale so that proper comparisons can be made

$$\text{estrogenicity index} = \frac{proliferation.index - mean.PI.of.all.control.wells.on.plate}{mean.PI.of.all.estradiol.wells.on.plate - mean.PI.of.all.control.wells.on.plate}$$

Figure 4: Calculation of Estrogenicity Index

The estrogenicity index is the difference between the proliferation index and the average of all control wells on the plate divided by the difference between the average of all estradiol wells on the plate and the average of all control wells on the plate. The value of the estrogenicity

index tells how much the cells proliferate with varying dilutions of fish extracts relative to how much the cells proliferate with pure estradiol added, adjusted for control response.

A value of one would tell us that the cells in that well proliferated as much as the average of the cells in the estradiol well and a value near 0 tells us that the cells proliferated as much as the average of the cells in the blank control well. Negative values inform us that the cells in that well proliferated less than the cells in the blank control well, which likely represents measurement error.

3.1 EXAMPLE CALCULATIONS

Example calculation for background corrected absorbance value

- To calculate the background corrected absorbance value for one cell in a well plate, you begin with absorbance values. The absorbance value for sample 1 at dilution 1/4000 is 0.81.

Absorbance Values

	1/4000	1/3000	control	1/2000	1/1500	estradiol	1/1000	1/500	control	1/200	1/100	estradiol
background ctl	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.14
background ctl	0.13	0.13	0.12	0.12	0.12	0.12	0.13	0.13	0.13	0.13	0.13	0.13
background ctl	0.13	0.13	0.12	0.12	0.12	0.12	0.13	0.12	0.12	0.13	0.13	0.13
sample 1	0.81	0.79	0.71	0.68	0.67	1.00	0.61	0.64	0.66	0.77	0.88	1.11
sample 2	0.83	0.75	0.72	0.69	0.66	1.03	0.61	0.64	0.64	0.79	0.86	1.11
sample 3	0.78	0.78	0.72	0.71	0.72	1.02	0.66	0.66	0.69	0.77	0.88	1.12
sample 4	0.78	0.79	0.76	0.74	0.75	1.07	0.71	0.74	0.72	0.81	0.89	1.16
sample 5	0.82	0.80	0.79	0.76	0.78	1.12	0.77	0.77	0.77	0.85	0.92	1.16

Then a correction on the absorbance values is made to correct for background 490nm absorbance.

Background corrected absorbance value for sample i at dilution j =

$$(absorption.value) - (avg.value.of\ .background.ctls.\ for.dilution.\ j)$$

For example, for sample 1 at dilution =1/4000, the background corrected absorbance value is equal to $0.81 - \left(\frac{0.14 + 0.13 + 0.13}{3}\right) = 0.68.$

The background corrected absorbance values for all cells are below.

Background Corrected Absorbance Values for Each Dilution

	1/4000	1/3000	control	1/2000	1/1500	estradiol	1/1000	1/500	control	1/200	1/100	estradiol
sample 1	0.68	0.67	0.58	0.56	0.54	0.88	0.48	0.52	0.54	0.64	0.75	0.98
sample 2	0.69	0.62	0.59	0.57	0.54	0.90	0.48	0.51	0.51	0.66	0.73	0.97
sample 3	0.65	0.65	0.59	0.58	0.59	0.89	0.53	0.54	0.56	0.64	0.75	0.99
sample 4	0.65	0.66	0.63	0.61	0.62	0.94	0.58	0.61	0.60	0.68	0.76	1.02
sample 5	0.68	0.67	0.66	0.63	0.66	0.99	0.64	0.64	0.64	0.72	0.79	1.02

Figure 5: Example Calculation for Background Corrected Absorbance Value

Example calculation for Proliferation Index

- Using the background corrected absorbance values in the previous table, a proliferation index is calculated.

$$proliferation\ index = \frac{background.corrected.absorbance.value}{mean.background.corrected.absorbance.value.of\ .all.control.wells.on.plate}$$

The proliferation index for sample 1 at dilution=1/4000 is calculated as follows.
Note that the mean of the background corrected absorbance values of all the control wells on the well plate is 0.59

$$Proliferation\ Index = \frac{0.68}{0.59} = 1.15$$

The proliferation index values for all cells are in the following table. The proliferation index is a scaled absorbance value, scaled by the mean control response on the plate.

Proliferation Index Values for Each Dilution:

	1/4000	1/3000	control	1/2000	1/1500	estradiol	1/1000	1/500	control	1/200	1/100	estradiol
sample 1	1.15	1.13	0.99	0.94	0.92	1.48	0.82	0.88	0.91	1.08	1.27	1.66
sample 2	1.18	1.05	1.00	0.96	0.91	1.53	0.81	0.87	0.86	1.13	1.23	1.65
sample 3	1.10	1.10	1.00	0.98	1.00	1.51	0.90	0.91	0.95	1.09	1.27	1.67
sample 4	1.10	1.12	1.07	1.04	1.06	1.59	0.98	1.03	1.01	1.15	1.28	1.74
sample 5	1.16	1.14	1.12	1.07	1.11	1.68	1.09	1.09	1.08	1.22	1.35	1.74

Figure 6: Example Calculation for Proliferation Index

Example calculation for Estrogenicity Index

- Using the proliferation index values in the previous table, an estrogenicity index is created.

$$estrogenicity\ index = \frac{proliferation.index - mean.PI.of\ .all.control.wells.on.plate}{mean.PI.of\ .all.estradiol.wells.on.plate - mean.PI.of\ .all.control.wells.on.plate}$$

The estrogenicity index for sample 1 at dilution=1/4000 is calculated as follows:
Note that the mean proliferation index for all 10 estradiol wells on the plate is 1.63 and the mean proliferation index for all 10 control wells in the plate is 1.

$$Estrogenicity\ Index = \frac{1.15 - 1}{1.63 - 1} = 0.23$$

The figure below illustrates resulting estrogenicity index values for our example. The estrogenicity index is a relative measure of the proliferation index in a given well relative to the mean estradiol response on that plate, both adjusted for the mean control response on that plate.

Estrogenicity Index Values:

	1/4000	1/3000	control	1/2000	1/1500	estradiol	1/1000	1/500	control	1/200	1/100	estradiol
sample 1	0.23	0.20	-0.02	-0.09	-0.13	0.77	-0.29	-0.19	-0.14	0.13	0.42	1.04
sample 2	0.28	0.08	0.01	-0.07	-0.14	0.85	-0.29	-0.20	-0.22	0.20	0.37	1.03
sample 3	0.16	0.16	0.01	-0.03	0.00	0.82	-0.16	-0.14	-0.08	0.15	0.43	1.06
sample 4	0.16	0.18	0.11	0.07	0.09	0.94	-0.03	0.05	0.02	0.23	0.45	1.17
sample 5	0.25	0.22	0.19	0.11	0.18	1.08	0.14	0.14	0.13	0.34	0.55	1.17

Figure 7: Example Calculation for Estrogenicity Index

4.0 STATISTICAL METHODS AND RESULTS

4.1 SAMPLING SCHEME

The Pittsburgh Fish Contamination and Consumption Project is a Community Based Participatory Research Project, so anglers were enlisted to catch the fish. An advantage of this approach is that, on average, anglers catch larger fish than scientists (Burger et al., 2006). The larger fish, typically, are older, have accumulated more estrogenic compounds, and are representative of what actual anglers would catch.

A total of 127 Channel catfish, White Bass, and Gizzard Shad were caught in this project. Nineteen farm-raised fish were purchased from a local fish market. The distribution of these fish across locations is shown in Table 2.

Table 2: Description of Total Fish Caught for Project

Location		Channel Catfish	White Bass	Gizzard Shad	
Monongahela	(Braddock Dam)	8	11	6	
Allegheny1	(Highland Park Dam)	12	37	0	
Allegheny2	(Kittaning)	13	0	0	
Point		20	1	0	
Store-bought		10	9	0	
TOTAL		63	58	6	**127**

For cell proliferation analysis, 21 of the 127 fish were used. For this analysis we focus on Channel catfish. They are of interest because of their role in the tropic web. The 21 Channel catfish analyzed for cell proliferation were chosen at random from the original sample of channel

catfish caught. Not all fish were analyzed for cell proliferation due to budget constraints. Six Channel catfish were used from the Braddock Dam on the Monongahela River, three from the Highland Park Bridge on the Allegheny River, four from Point State Park, and six from up-stream on the Allegheny River at Kittanning. In addition, two catfish that were farm-raised in Georgia were selected for analysis.

4.2 DATA DESCRIPTION

Table 3: Description of Fish Used for Cell Proliferation Analysis

Location		Channel catfish
Braddock Dam	(Monongahela River)	6
Highland Park Dam	(Allegheny River)	3
Kittanning	(Allegheny River)	6
Point		4
Store-bought	(farm raised in Georgia)	2
		n=21

Figure 8 illustrates the five proliferation indices and the five estrogenicity indices calculated at each dilution for one fish.

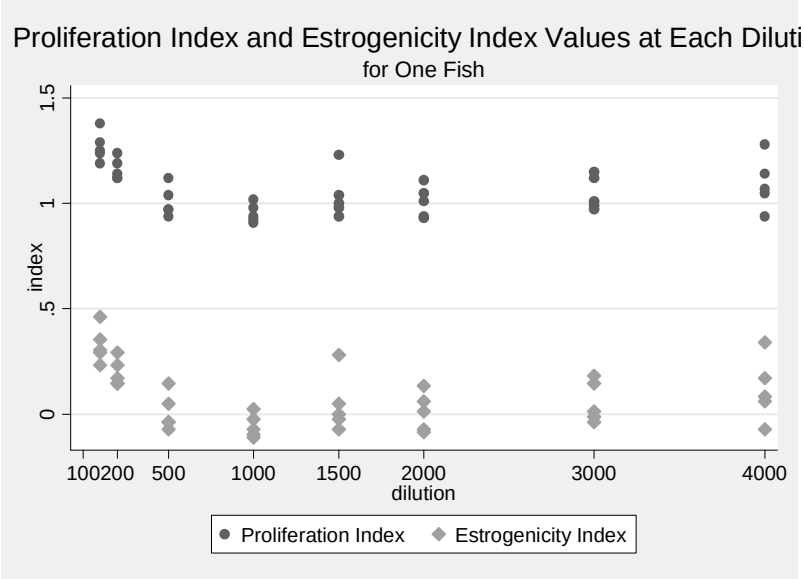


Figure 8: Proliferation Index and Estrogenicity Index Values for the Same Fish at All Dilutions

Figure 9 shows boxplots for proliferation indices and estrogenicity indices for each fish across dilutions. The patterns are similar for both indices, although proliferation index is consistently higher.

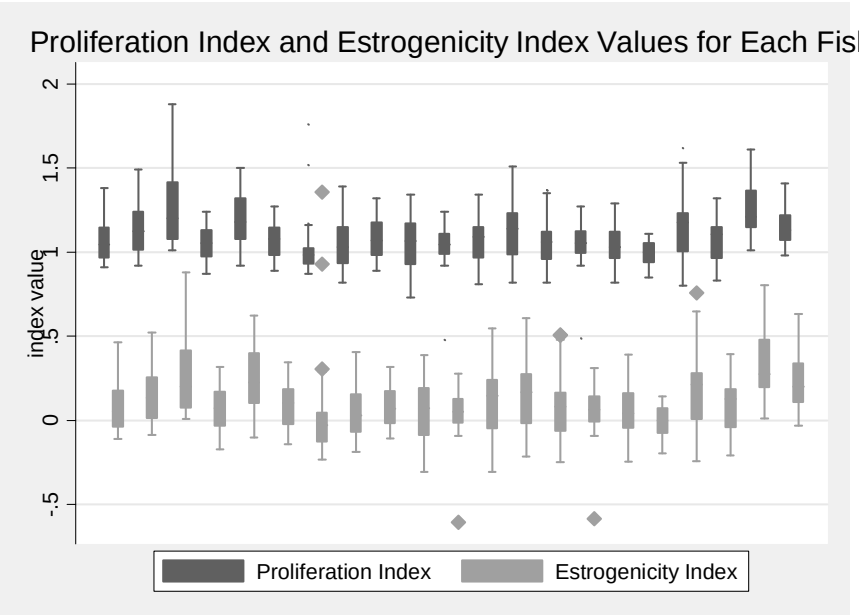


Figure 9: Box-plot of Proliferation Index and Estrogenicity Index Values for Each Fish

Figure 10 shows the average estrogenicity index at each location by dilution.

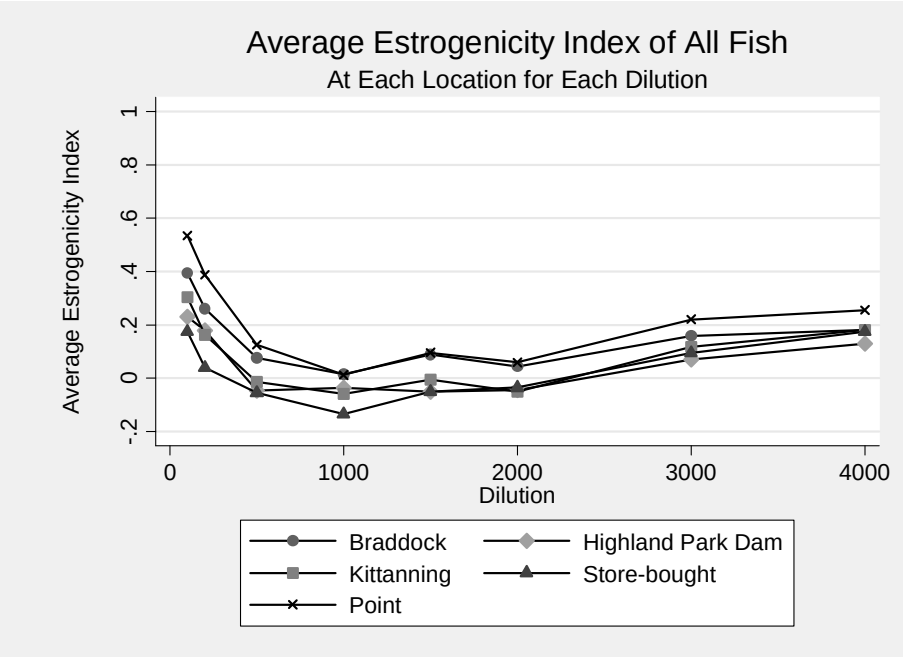


Figure 10: Average Estrogenicity Index of All Fish at Each Location for Each Dilution

The curves in the graphs show how human cells, and not fish cells, respond to various levels of fish extract, from fish that have bioaccumulated estrogenic compounds in the water. Based on Figure 10, it appears that fish from the Point generally have the highest estrogenicity indices across all dilutions, followed by Braddock, then Kittanning, Highland Park Dam, and store bought. The estrogenicity indices for the Point and Braddock tend to be very similar to each other. The estrogenicity indices for fish at Kittanning, Highland Park Dam, and store bought also seem similar to each other.

The pattern of having a biphasic response, where low concentrations of fish extract have a moderate proliferation response that decreases with increasing, but still low, dilution concentrations followed by increasing proliferative responses at higher concentrations is repeated

throughout literature pertaining to cell response when estrogenic compounds are added. The pharmacokinetic explanation for this pattern is not clear.

Figures 11-15 describe the estrogenicity indices for each fish at each location. The dilution response curves show the mean estrogenicity index for each dilution.

Braddock

Dilution	mean	min	max	std.dev.
1/100	0.40	0.17	0.67	0.19
1/200	0.26	0.07	0.05	0.17
1/500	0.08	-0.06	0.23	0.11
1/1000	0.02	-0.06	0.12	0.06
1/1500	0.09	-0.01	0.22	0.08
1/2000	0.04	0.01	0.12	0.05
1/3000	0.16	0.06	0.29	0.08
1/4000	0.18	0.07	0.26	0.07

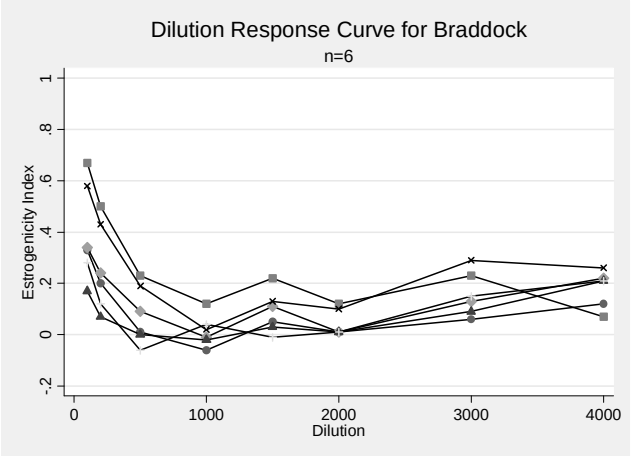


Figure 11: Fish Specific Estrogenicity Indices and Dilution Response Curve for Braddock

Highland Park Dam

Dilution	mean	min	max	std.dev.
1/100	0.23	0.13	0.34	0.11
1/200	0.18	0.07	0.33	0.13
1/500	-0.05	-0.10	0.00	0.05
1/1000	-0.04	-0.50	-0.01	0.02
1/1500	-0.05	-0.14	0.02	0.08
1/2000	-0.43	-0.13	0.00	0.08
1/3000	0.07	-0.03	0.14	0.09
1/4000	0.13	-0.01	0.20	0.12

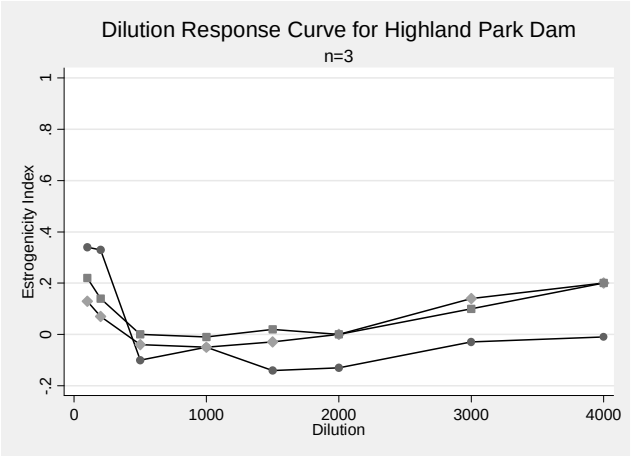


Figure 12: Fish Specific Estrogenicity Indices and Dilution Response Curve for Highland Park Dam

Kittanning

Dilution	mean	min	max	std.dev.
1/100	0.31	0.20	0.44	0.11
1/200	0.16	0.20	0.23	0.06
1/500	-0.01	-0.07	0.03	0.05
1/1000	-0.06	-0.13	0.02	0.06
1/1500	-0.01	-0.03	0.01	0.02
1/2000	-0.05	-0.14	0.04	0.08
1/3000	0.12	0.05	0.20	0.06
1/4000	0.18	0.09	0.24	0.07

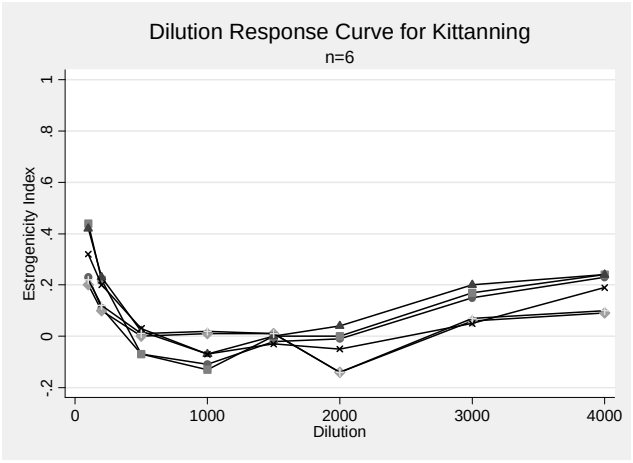


Figure 13: Fish Specific Estrogenicity Indices and Dilution Response Curve for Kittanning

Point

Dilution	mean	min	max	std.dev.
1/100	0.54	0.31	0.67	0.15
1/200	0.39	0.17	0.52	0.15
1/500	0.13	-0.05	0.29	0.14
1/1000	0.01	-0.08	0.12	0.09
1/1500	0.10	-0.04	0.23	0.12
1/2000	0.06	0.00	0.15	0.06
1/3000	0.22	0.14	0.34	0.09
1/4000	0.26	2.00	0.38	0.08

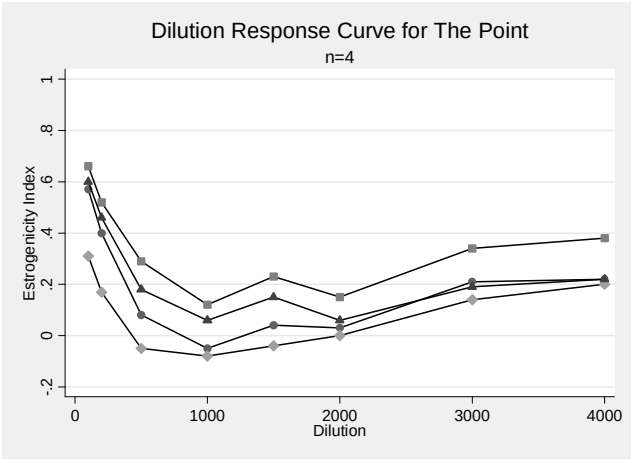


Figure 14: Fish Specific Estrogenicity Indices and Dilution Response Curve for the Point

Store Bought

Dilution	mean	min	max	std.dev.
1/100	0.18	0.06	0.29	0.16
1/200	0.04	0.03	0.05	0.01
1/500	-0.55	-0.11	0.00	0.07
1/1000	-0.14	-0.15	-0.12	0.02
1/1500	-0.05	-0.05	-0.05	0.00
1/2000	-0.04	-0.04	-0.03	0.01
1/3000	0.10	0.07	0.12	0.04
1/4000	0.18	0.10	0.25	0.11

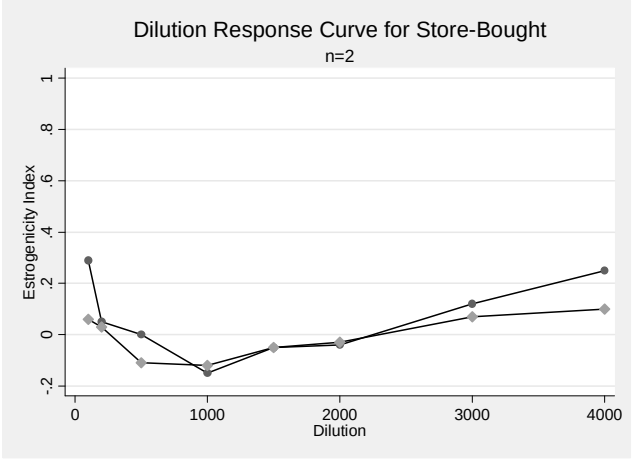


Figure 15: Fish Specific Summary Statistics and Dilution Response Curve for Store-bought

4.3 RESEARCH QUESTIONS

The hypotheses for this study are driven by geography and knowledge of the sewage system in Allegheny County. Because several of the materials present in waste-waters may contain estrogenic or xenoestrogenic materials, we hypothesize that fish from areas with a greater density of CSO/SSO outfalls will have higher estrogenicity index values than fish from Kittanning, where there are very few CSO/SSO locations, and store bought fish that were farm raised in Georgia and were never exposed to CSO/SSO wastewaters.

To answer the questions posed in this study, the data were modeled using both proliferation index and estrogenicity index as a function of fish, dilution, and location. Because we are mostly interested in comparing fish across locations, the estrogenicity index is the focus of this paper. The estrogenicity index allows for the proliferation values at each location to be on the same scale so that proper comparisons can be made.

A measurement was taken 5 times for each of 8 dilutions for each fish, giving a total of 40 measurements per fish. The observations within each fish are more likely to be like each other than are observations from different fish. We take this correlation into account by using random intercept linear regression, which includes variance terms to account for repeated measures (Figure 16). The estrogenicity index for extract of the i^{th} fish, j^{th} dilution, and k^{th} replicate is modeled as a function of an overall mean, μ, a random fish effect, γ_i, and fixed effects for fish and dilution. One variance term (ε_{ijk}) is for the within-fish effects and one (γ_i) is for the between-fish effects. This allows us to capture both within-fish and between-fish differences.

Estrogenicity $\text{Index}_{ijk} = \mu + \gamma_i + \text{dilution}_j + \text{location}_{j(l)} + \varepsilon_{ijk}$

Assume that $\gamma_i \sim N(0, \sigma^2)$, $\varepsilon_{ijk} \sim N(0, \sigma^2)$, and γ_i and ε_{ijk} are independent.

Figure 16: Regression Model Using Estrogenicity Index Data

The random intercept linear regression model was fitted using the xtreg command in Stata 9. Indicator variables were used to model the fixed effects of dilutions and locations. The baseline scenario is a fish caught near Kittanning at dilution 1/100. The fixed effects in this model are interpreted as the estimated index for an average fish at dilution *j* from a particular location.

The same conclusions were reached for both the proliferation index (Table 4) and the estrogenicity index (Table 5). Both models have greater between-fish variation than within-fish variation. In the model for the proliferation index, the between fish variation is 0.10 while the

between-fish variation is 0.13 in the model for the estrogenicity index. Both models have approximately the same amount of within fish variation (sigma_u(proliferation)=0.05, sigma_u(estrogenicity)=0.06).

Table 4: Regression Results Using Proliferation Index

	Coef.	Std.Err	z	p>|z|
1/200	-0.104	0.014	-7.380	0.000
1/500	-0.256	0.014	-18.210	0.000
1/1000	-0.303	0.014	-21.580	0.000
1/1500	-0.255	0.014	-18.190	0.000
1/2000	-0.281	0.014	-20.050	0.000
1/3000	-0.167	0.014	-11.910	0.000
1/4000	-0.131	0.014	-9.320	0.000
Braddock	0.071	0.032	2.240	0.025
HPD	-0.012	0.039	-0.320	0.750
Store	-0.043	0.045	-0.960	0.338
Point	0.095	0.035	2.690	0.007
cons	1.250	0.024	51.560	0.000
sigma_u	0.052	0.009		
sigma_e	0.102	0.003		
rho	0.210	0.057		

Likelihood-ratio test of sigma_u=0: chibar2(01)= 146.46
Prob>=chibar2 = 0.000

Observations: 840
Number of fish: 21
Observations per fish: 40

LR chi2(11) = 561.79
Prob > chi2 = 0.000

Table 5: Regression Results Using Estrogenicity Index

	Coef.	Std.Err	z	p>|z|
1/200	-0.129	0.018	-7.25	0.000
1/500	-0.322	0.018	-18.17	0.000
1/1000	-0.381	0.018	-21.45	0.000
1/1500	-0.323	0.018	-18.21	0.000
1/2000	-0.353	0.018	-19.92	0.000
1/3000	-0.212	0.018	-11.96	0.000
1/4000	-0.166	0.018	-9.33	0.000
Braddock	0.072	0.036	1.99	0.047
HPD	-0.025	0.045	-0.57	0.569
Store	-0.053	0.051	-1.04	0.301
Point	0.132	0.041	3.23	0.001
cons	0.316	0.028	11.18	0.000
sigma_u	0.060	0.010		
sigma_e	0.129	0.003		
rho	0.177	0.051		

Likelihood-ratio test of sigma_u=0: chibar2(01)= 116.23
Prob>=chibar2 = 0.000

Observations: 840 LR chi2(11) = 561.11
Number of fish: 21 Prob > chi2 = 0.000
Observations per fish: 40

The intra-class correlation coefficient, rho, for the model for the proliferation index is 0.210; rho for the model for the estrogenicity index is 0.177. This means that 21% of the variation in the model for the proliferation index is attributable to fish traits whereas 18% of the variation in the model for this estrogenicity index data is attributable to fish traits.

Because we are interested in comparing across fish sites, the estimates in Table 5 will be interpreted in more detail. In Table 5, the coef. column provides the coefficients for the regression model. The coefficients indicate how much the estrogenicity index for each attribute differs from the baseline (Kittanning, 1/100). A coefficient of zero indicates that there is no change from the baseline. The standard error reflects the variability of the regression coefficient.

In Table 5, the estimated estrogenicity index for an average fish from Kittanning at dilution 1/100 is 0.316. A fish from Kittanning at dilution 1/100 one that is 1.96 standard deviations away from 0.316 would have an estimated estrogenicity index of

0.316±1.96*0.028=(0.261,0.371). A small interval such as this one shows that the estrogenicity index at this dilution is relatively precisely measured.

For an average fish from Kittanning, the estimated estrogenicity index for at dilution 1/200 is 0.187. This means that the proliferation index at this dilution is almost 20% of the estradiol response, controlling for background. .

Table 6 shows that an average fish from Kittanning has the highest estimated estrogenicity index at dilution 1/100. The estrogenicity index decreases until dilution 1/1000 and then increases again. The negative estimated estrogenicity indices represent responses below background.

Table 6: Estrogenicity Index for an Average Fish from Kittanning at Each Dilution

River	Dilution	Estrogenicity Index
Kittanning	1/100	0.316
	1/200	0.187
	1/500	-0.006
	1/1000	-0.065
	1/1500	-0.007
	1/2000	-0.037
	1/3000	0.104
	1/4000	0.150

For an average fish from Braddock at dilution 1/100, the expected estrogenicity index is 0.39 (Table 5). This is 0.072 units more than the expected estrogenicity index of an average fish from Kittanning at dilution1/100.

Table 7 shows that fish from the Point have the highest estimated estrogenicity index followed by Braddock, Highland Park Dam, and then the store. Fish from Kittanning, Highland Park Dam, and the store all have very similar estimated estrogenicity indices ranging from 0.26 to 0.32.

Table 7: Estrogenicity Index for an Average Fish from Each Location at Dilution 1/100

River	Dilution	Estrogenicity Index
Kittanning	1/100	0.32
Braddock		0.39
HPD		0.29
Store		0.26
Point		0.45

The z-statistic is used to calculate the p-value. It is the ratio of the coefficient to the standard error of the respective predictor. This p-value is the two-tailed p-value for the null hypothesis that a given regression coefficient is zero given the rest of the predictors in the model.

Based on Table 5, all dilution coefficients are significantly different from 1/100 ($p<0.001$ for each). All location coefficients are significantly different from 0 except for Highland Park Dam and Store. The p-values ($p=0.569$, $p=0.301$) tell us that their coefficients are not significantly different from that for Kittanning.

Sigma_u tells how much between fish variation is present and sigma_e tells how much within-fish variation is present. In our model, sigma_u=0.060 and sigma_e=0.29. The likelihood ratio test of sigma_u in Table 5 shows that there is significant variation among fish ($p<0.001$).

Rho is the intraclass correlation coefficient. In this case, it tells about how much the observations within each fish are correlated with one another. To calculate rho, the between subject variation is divided by the between subject variation plus within subject variation. $rho = \left(\frac{sigma_u^2}{sigma_u^2 + sigma_e^2} \right)$. The higher rho is, the less unique information each observation within a fish provides. For this model, rho=0.177. This means that 18% of the total variance is attributable to fish traits.

The results from the regression model can be used to compare estrogenicity indices for average fish from different locations and at various dilutions. The results can also be used to test whether estrogenicity indices for fish from different locations and/or at different dilutions differ from one another.

To test whether the estrogenicity indices are the same at two different locations, we test that the difference in coefficients equals 0 using Wald's test. Because linear combinations of normally distributed variables are also normally distributed, Wald's Test statistics can be defined in terms of linear contrasts of the estimated parameters in Table 5.

4.3.1 Are there differences in estrogenicity index between catfish caught at each location?

H_0: $\mu_{Braddock} = \mu_{HPD} = \mu_{Kit\,tan\,ning} = \mu_{Po\,int} = \mu_{store}$
H_A: The mean estrogenicity index for at least one location differs from the other locations.

We hypothesize that at least one sampling location will differ significantly from the other locations. Because the store-bought catfish were farm-raised in Georgia and never exposed to CSO/SSO wastewaters they will probably differ from the fish that are exposed to high densities of CSO/SSO outfall waters. Also, the fish from the Highland Park Dam may differ from the fish caught near the Point since there are fewer SSO outfalls near the Highland Park Dam than at the Point.

To test this null hypothesis using the estrogenicity index, Wald's test showed that there is significant variation among locations. (X^2_{4df} =20.00, p=0.005). The null hypothesis is rejected and we conclude that the estrogenicity index for at least one location differs significantly from the others.

```
STATA CMD:          test Braddock=HPD=Kittanning=Store=Point

STATA OUTPUT:       (1)  Braddock= 0
                    (2)  HPD     = 0
                    (3)  Store   = 0
                    (4)  Point   = 0

                   chi2(4)       =   20.00
                    Prob > chi2  =   0.0005
```

4.3.2 Pair-wise contrasts

Because there is a significant variation among locations, we performed pair-wise contrasts using Wald tests to identify which locations differ from each other. When testing pair-wise contrasts, the same final conclusions were achieved with both the proliferation index and estrogenicity index (Table 8). We will interpret the results for the estrogenicity index in detail.

Table 8: Comparison of Results for Pair-wise Contrasts

Proliferation Index

	store	HPD	Kittanning	Braddock
HPD	p=0.54			
Kittannning	p=0.34	p=0.75		
Braddock	p=0.01	p=0.03	p=0.03	
Point	p<0.01	p=0.01	p=0.01	p=0.49

Estrogenicity Index

	store	HPD	Kittanning	Braddock
HPD	p=0.63			
Kittannning	p=0.30	p=0.57		
Braddock	p=0.01	p=0.03	p=0.05	
Point	p<0.01	p<0.01	p<0.01	p=0.15

First, we compared fish that were bought at the store to each location. The catfish from the store-bought location are hypothesized to have lower estrogenicity indices than all other locations since those catfish have never been exposed to any CSO/SSO outfalls. As shown in the

lower section of Table 8, the estrogenicity indices for fish from the Highland Park Dam and store-bought fish are not significantly different from one another (p=0.63), nor were those for Kittanning and store bought (p=0.30) or Kittanning and Highland Park Dam (p=0.57). Estrogenicity indices at both Braddock and the Point were significantly different from the store bought, Highland Park Dam, and Kittanning at α =0.05.

These results are consistent with the graph of average estrogenicity index for all fish by dilution. We hypothesized that the estrogenicity index for store bought fish, fish from Kittanning, and fish from the Highland Park Dam do not differ significantly because of the small amount of SSOs to which they are exposed. We hypothesize that the estrogenicity index for Point and Braddock do not significantly differ from one another because of the high density of SSOs to which they are exposed.

Below are the Stata commands and output used for these pair-wise comparisons.

H_0: $\mu_{store} \equiv \mu_{Braddock}$

H_A: $\mu_{store} \neq \mu_{Braddock}$

```
STATA CMD:          test store=Braddock

STATA OUTPUT:       (1) store-Braddock = 0

                    chi2(1)      =    5.95
                    Prob>chi2    =    0.0147
```

Using Wald's Test, we reject the null hypothesis and conclude that the mean estrogenicity index for Braddock and Store-bought fish are significantly different (X^2_{1df} =5.95, p=0.01).

$H_0: \mu_{store} \equiv \mu_{HPD}$

$H_A: \mu_{store} \neq \mu_{HPD}$

```
STATA CMD:          test store= HPD

STATA OUTPUT:       (1) store-HPD = 0

                    chi2(1)      =    0.23
                    Prob>chi2    =    0.6282
```

Using Wald's Test, we fail to reject the null hypothesis and conclude that the mean estrogenicity index for Highland Park Dam and Store-bought fish are not significantly different (X^2_{1df} =0.23, p=0.6282).

$H_0: \mu_{store} \equiv \mu_{Kittanning}$

$H_A: \mu_{store} \neq \mu_{Kittanning}$

```
STATA CMD:          test store= Kittanning

STATA OUTPUT:       (1)  store = 0

                    chi2(1)      =    1.07
                    Prob>chi2    =    0.3006
```

Using Wald's Test, we fail to reject the null hypothesis and conclude that the mean estrogenicity index for Kittanning and Store-bought fish are not significantly different (X^2_{1df} = 1.07, p=0.3006)

$H_0: \mu_{store} \equiv \mu_{Point}$

$H_A: \mu_{store} \neq \mu_{Point}$

```
STATA CMD:          test store= Point

STATA OUTPUT:       (1) store - point = 0

                    chi2(1)      =    11.47
                    Prob>chi2    =    .0007
```

Using Wald's Test, we reject the null hypothesis and conclude that the mean estrogenicity index for the Point and Store-bought fish are significantly different (X^2_{1df} =11.47, p=0.0007).

Next we compared Highland Park Dam and Kittanning to see if they are different from one another because the estrogenicity index from store bought fish was not significantly different from the estrogenicity index for those two locations.

H_0: $\mu_{Kittanning} \equiv \mu_{HPD}$

H_A: $\mu_{Kittanning} \neq \mu_{HPD}$

```
STATA CMD:          test Kittanning= HPD

STATA OUTPUT:       (1) HPD = 0

                    chi2(1)      =    0.33
                    Prob>chi2    =    0.5685
```

Using Wald's Test, we fail to reject the null hypothesis and conclude that the mean estrogenicity index for the Kittanning and Highland Park fish are not significantly different (X^2_{1df} =0.33, p=0.569).

We continued testing to see if the estrogenicity index for fish from Kittanning differs significantly from fish from other locations.

H_0: $\mu_{Kittanning} \equiv \mu_{Braddock}$

H_A: $\mu_{Kittanning} \neq \mu_{Braddock}$

```
STATA CMD:          test Kittanning = Braddock

STATA OUTPUT:       (1) Braddock = 0

                    chi2(1)      =    3.94
                    Prob>chi2    =    0.0471
```

Using Wald's Test, we reject the null hypothesis and conclude that the mean estrogenicity index for the Kittanning and Braddock fish are significantly different (X^2_{1df} =3.94, p=0.0471).

H_0: $\mu_{Kittanning} \equiv \mu_{Point}$

H_A: $\mu_{Kittanning} \neq \mu_{Point}$

```
STATA CMD:          test Kittanning = Point

STATA OUTPUT:       (1) Point = 0

                    chi2(1)      =    10.46
                    Prob>chi2    =    0.0012
```

Using Wald's Test, we reject the null hypothesis and conclude that the mean estrogenicity index for fish from Kittanning and the Point are significantly different (X^2_{1df} =10.46, p=0.001).

Next the estrogenicity index for the Highland Park Dam is compared with the estrogenicity index for fish from the Point and Braddock using Wald's Test. Then, also using Wald's Test, the estrogenicity index for fish from Braddock is compared with estrogenicity index of fish from the Point.

H_0: $\mu_{HPD} \equiv \mu_{Point}$

H_A: $\mu_{HPD} \neq \mu_{Point}$

```
STATA CMD:          test HPD = Point

STATA OUTPUT:       (1)  HPD - Point = 0

                     chi2(1)      =    10.64
                     Prob>chi2    =    0.0011
```

Using Wald's Test, we reject the null hypothesis and conclude that the mean estrogenicity index for fish from Highland Park Dam and the Point are significantly different (X^2_{1df} =10.64, p=0.001).

H_0: $\mu_{HPD} \equiv \mu_{Braddock}$

H_A: $\mu_{HPD} \neq \mu_{Braddock}$

```
STATA CMD:          test HPD = Braddock

STATA OUTPUT:       (1)  HPD - Braddock = 0

                     chi2(1)      =    4.80
                     Prob>chi2    =    0.0284
```

Using Wald's Test, we reject the null hypothesis and conclude that the mean estrogenicity index for fish from Highland Park Dam and Braddock are significantly different (X^2_{1df} =4.80, p=0.028).

H_0: $\mu_{Point} \equiv \mu_{Braddock}$

H_A: $\mu_{Point} \neq \mu_{Braddock}$

```
STATA CMD:          test Point = Braddock

STATA OUTPUT:       (1)  Point - Braddock = 0

                     chi2(1)      =    2.13
                     Prob>chi2    =    0.1446
```

Using Wald's Test, we fail to reject the null hypothesis and conclude that the mean estrogenicity index for fish from the Point are not significantly different from the mean estrogenicity index for fish from Braddock (X^2_{1df} =2.13, p=0.145).

4.3.3 Additional contrasts

Additional contrasts were made for hypotheses based on knowledge of the sewage system and geography of the rivers (Table 9). The same conclusions were reached for the proliferation index data and the estrogenicity index data, so we will interpret the estrogenicity index in detail.

Table 9: Summary of p-values for Contrasts using Proliferation Index and Estrogenicity Index

Proliferation Index Data

Null Hypothesis	p-value
$\mu_{Braddock} = \mu_{HPD} = \mu_{Kittanning} = \mu_{Point} = \mu_{store}$	p=0.003
$\mu_{Braddock} = \mu_{HPD} = \mu_{Kittanning} = \mu_{Point}$	p=0.008
$\mu_{Braddock} = \mu_{HPD} = \mu_{Point}$	p=0.028
$(\mu_{point} + \mu_{Braddock}) = (\mu_{Kittanig} + \mu_{HPD})$	p=0.001

Estrogenicity Index Data

Null Hypothesis	p-value
$\mu_{Braddock} = \mu_{HPD} = \mu_{Kittanning} = \mu_{Point} = \mu_{store}$	p=0.0005
$\mu_{Braddock} = \mu_{HPD} = \mu_{Kittanning} = \mu_{Point}$	p=0.0015
$\mu_{Braddock} = \mu_{HPD} = \mu_{Point}$	p=0.0048
$(\mu_{point} + \mu_{Braddock}) = (\mu_{Kittanig} + \mu_{HPD})$	P<0.0001

Based on the p-values in Table 9, estrogenicity index for at least one location differs significantly from the others at $\alpha = 0.05$. This is still true when store bought fish are excluded (p=0.0015). The Pittsburgh Pool (Braddock, Highland Park Dam and the Point) is not homogeneous (p=0.0048), and estrogenicity index for fish from Braddock and the Point differs significantly from the estrogenicity index for fish from Kittanning and Highland Park Dam (p<0.0001).

4.3.3.1 Are there differences in estrogenicity index between catfish caught at every location excluding store bought?

H_0: $\mu_{Braddock} = \mu_{HPD} = \mu_{Kittanning} = \mu_{Point}$
H_A: The mean estrogenicity index for at least one location differs from the other locations.

```
STATA CMD:          test Braddock=HPD=Kittanning =Point

STATA OUTPUT:       (1)  Braddock = 0
                    (2)  HPD      = 0
                    (3)  Point    = 0

                    chi2(3)       =   15.46
                    Prob > chi2   =   0.0015
```

This research question is almost the same as the first research question, however store bought fish are excluded from the analysis. Since the store-bought fish have never been exposed to waste-waters from CSO/SSO outfalls, we would like to exclude them to see if at least one sampling location still differs significantly from the other locations. We hypothesize that there will still be a difference among all locations even when store bought fish are excluded.

To test the null hypothesis, Wald's test was used and showed that there is significant variation among locations. (X^2_{3df} =15.46, p=0.0015). The null hypothesis is rejected and we conclude the estrogenicity index for at least one location differs significantly from the others even when store bought fish is excluded from the comparison.

4.3.3.2 Is there homogeneity in the Pittsburgh Pool?

One of the questions of interest was if there was a difference between the Pittsburgh Pool (Point, Highland Park Dam, and Braddock) and the non-Pittsburgh Pool (store bought and Kittanning). Before testing null hypothesis that the mean estrogenicity index of the Pittsburgh Pool is equal to

the mean estrogenicity index of the non-Pittsburgh Pool, we first ask whether there is homogeneity in the Pittsburgh and non-Pittsburgh Pools.

The homogeneity of the non-Pittsburgh Pool was verified above when all possible pair-wise contrasts were made. Using Wald's Test, we concluded that the mean estrogenicity index for Kittanning and Store-bought fish are not significantly different (X^2_{1df} =1.07, p=0.30). There is no significant evidence of heterogeneity in the non-Pittsburgh Pool.

Next, we examine whether there is homogeneity in the Pittsburgh-Pool.

$H_0: \mu_{Braddock} = \mu_{HPD} = \mu_{Point}$
H_A: The mean estrogenicity index for at least one location differs from the other locations.

```
STATA CMD:          test Braddock=HPD=Point

STATA OUTPUT:       (1)   Braddock-HPD=0
                    (2)   Braddock-Point=0

                    chi2(2)      = 10.69
                    Prob<chi2    = 0.0048
```

We rejected the null hypothesis and concluded that there is heterogeneity in the Pittsburgh Pool (X^2_2=10.69, p=0.005). Because there is not homogeneity in the Pittsburgh Pool, we do not test whether the estrogenicity index in the Pittsburgh Pool is the same as that in the non-Pittsburgh Pool.

4.3.3.3 Are there differences in estrogenicity index of catfish caught at the Point and near the Braddock Dam and fish caught near Kittanning and the Highland Park Dam?

$H_0: (\mu_{Point} + \mu_{Braddock}) = (\mu_{Kittanning} + \mu_{HPD})$
$H_A: (\mu_{Point} + \mu_{Braddock}) \neq (\mu_{Kittanning} + \mu_{HPD})$

```
STATA CMD:          lincom (Braddock+Point)- HPD

STATA OUTPUT:       (1)  Braddock - HPD + Point = 0

       ------------------------------------------------------------------------------
                pi_ |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
       -------------+----------------------------------------------------------------
                (1) |   .2291688   .0603261     3.80   0.000     .1109318    .3474057
       ------------------------------------------------------------------------------
```

There are only two SSO outfall locations near the Highland Park Dam on the Allegheny River. The remaining SSO outfall locations are on the Ohio River, near the Point, and on the Monongahela River, near Braddock. We hypothesize that there is a difference in estrogenicity index between catfish from the Point and Braddock and fish caught near Kittanning and the Highland Park Dam due to the difference in the amount of SSO locations in the two groups.

A linear combination of coefficients was used to test the null hypothesis. We rejected the null hypothesis and concluded that there is a significant difference in estrogenicity index of catfish caught at the Point and near the Braddock Dam and fish caught near Kittanning and the Highland Park Dam ($z=3.80$, $p<0.001$).

Figure 17 shows that the estimated average estrogenicity indices for the Point and Braddock are higher than those for the Highland Park Dam and Kittanning at all dilutions. This is not surprising with a main effects model. The estimated difference is 22.9%, with a 95% confidence interval of (11.1%,34.7%)

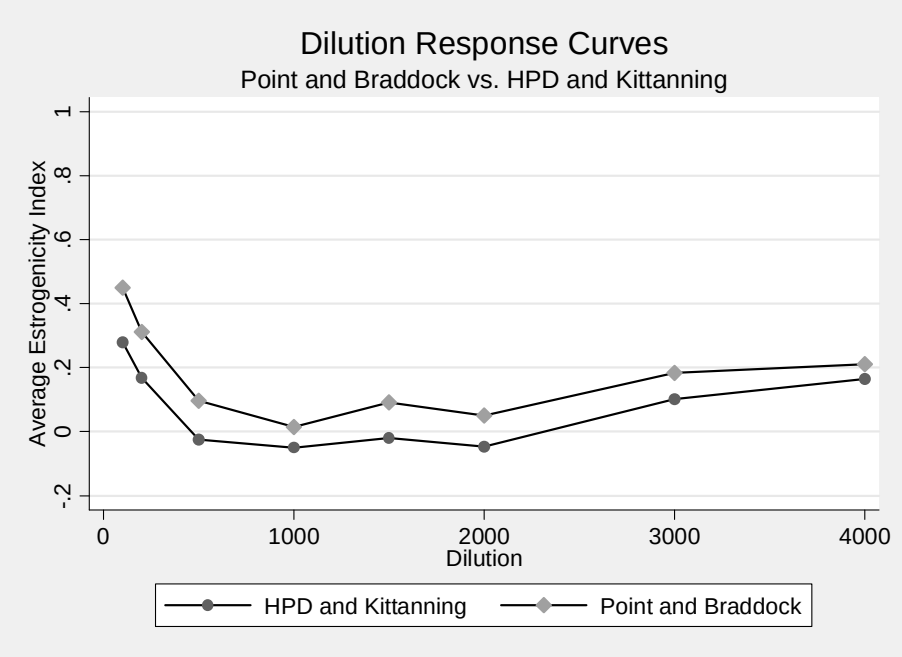


Figure 17: Point and Braddock and Highland Park Dam and Kittanning: Averaged Estrogenicity Indices by Dilution

5.0 DISCUSSION

Our results show that both the proliferation index and the estrogenicity index for fish from Highland Park Dam are not statistically different from store bought fish or fish from Kittanning. Also, the estrogenicity index and proliferation index for fish from Kittanning and the Highland Park Dam are not significantly different from one another.

We hypothesize that estrogenicity index for fish from the Highland Park Dam, Kittanning, and the Store are not significantly different from one another because they are exposed to fewer outfalls than fish at other locations. Store bought fish should not be exposed to any CSO outfalls, Kittanning fish are exposed to few or no CSO outfalls and there are approximately 17 CSO outfalls within a one mile radius of the Highland Park Dam sampling site. Fish from Braddock and the Point are likely not statistically different from one another due to the high amount of SSO outfalls to which they are exposed.

Further analysis shows that there is heterogeneity in the Pittsburgh Pool (Braddock, Highland Park Dam, and Kittanning). This is most likely due to the difference in the number of CSO and/or SSOs fish are exposed to at the Highland Park Dam versus Braddock or the Point. There are only 2 SSO outfalls near Highland Park Dam. Within a ten mile radius of Braddock there are 13 SSO outfalls and within a ten mile radius of the point there are 35 SSO outfalls.

Also, we have found that estrogenicity index and proliferation index for fish from Point and Braddock are significantly different than fish from Kittanning and Highland Park Dam. This

is probably due to the greater amount of CSO/SSO outfall locations in the Point and Braddock than at Kittanning and Highland Park Dam. The Point and Braddock have a total of 53 CSO outfalls within a 1 mile radius of each site. Kittanning and the Highland Park Dam have a total of 17 CSO outfalls within a 1 mile radius of each site. This is why we hypothesize that estrogenicity index is higher for fish from the Point and Braddock than for fish from Highland Park Dam or Kittanning.

While results show that estrogenicity index of fish from areas with higher densities of CSO/SSO outfalls is higher than estrogenicity index of fish from areas with fewer CSO/SSO outfalls, it also shows that the outfalls may not diffuse since there is heterogeneity in the Pittsburgh Pool.

Adjustments for multiple comparisons were not made due to the exploratory nature of the study. All hypotheses were made a priori. However, similar conclusions would not change greatly with a Bonferroni multiple comparison correction had been used, except for differences between Braddock and both the Highland Park Dam and Kittanning.

We acknowledge several limitations to this study. First, while our results are consistent with a causal effect of CSO/SSO outfalls, alternative explanations are possible in these observed data. We cannot rule out factors associated with the prevalence of CSO/SSO outfalls or population density.

The second limitation in this paper is the small sample size. Because the Pittsburgh Fish Contamination and Consumption Project was originally intended to be a descriptive study, a large sample size of fish from each location was not obtained. The analyses of this paper were hypothesis driven based on knowledge of the geography in the Pittsburgh area and were meant to

be exploratory. This study should be replicated with a larger number of fish in each location for more power and to validate the results.

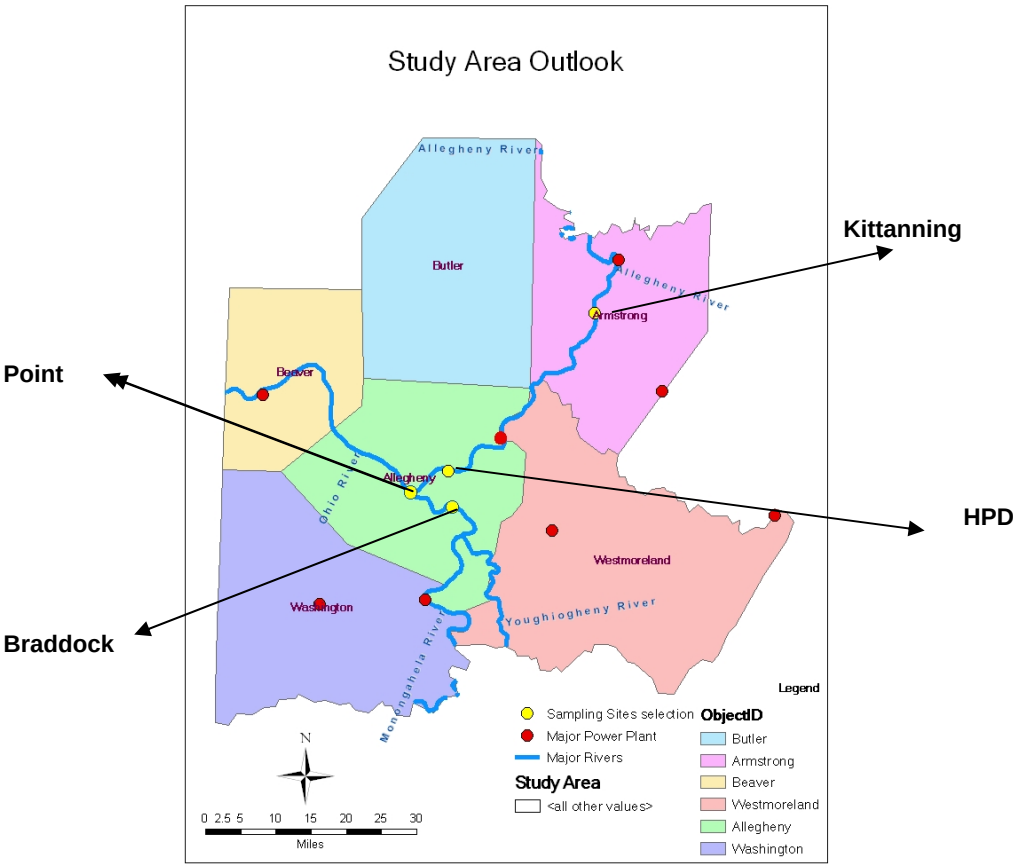
A third limitation is that only one species of fish was used in this analysis. It would have been interesting to include other species of fish, such as white bass and gizzard shad, in our analysis to see if species makes a difference or not in regression analysis. Due to the small sample sizes, this was not possible in the present study.

Another limitation is that a general assay was used for cell proliferation. The assay is not specific to any particular compounds and is not able to identify the individual estrogenic compounds in the fish extract. It would be useful to analyze the fish for specific substances causing estrogenic effects, to conduct an environmental assessment to pinpoint the source(s) of the harmful substances, and to perform a risk assessment to determine the fish consumption safety.

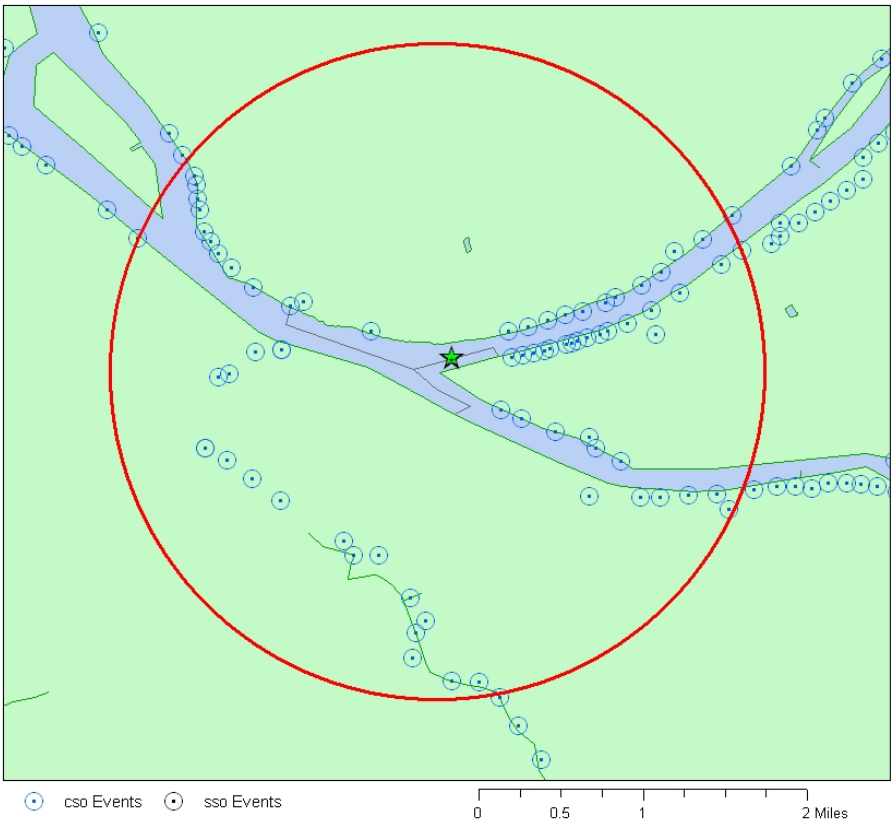
In summary, our results showed that fish tissue from areas with high densities of CSO/SSO outfalls proliferate the MCF-7 human breast cancer cell line more than fish tissue from areas with lower densities of CSO/SSO outfalls. This research is relevant to public health because the information concerning cumulative estrogenicity in channel catfish may provide a linkage between the ecological compounds contained in wastewaters and human health.

APPENDIX A Maps

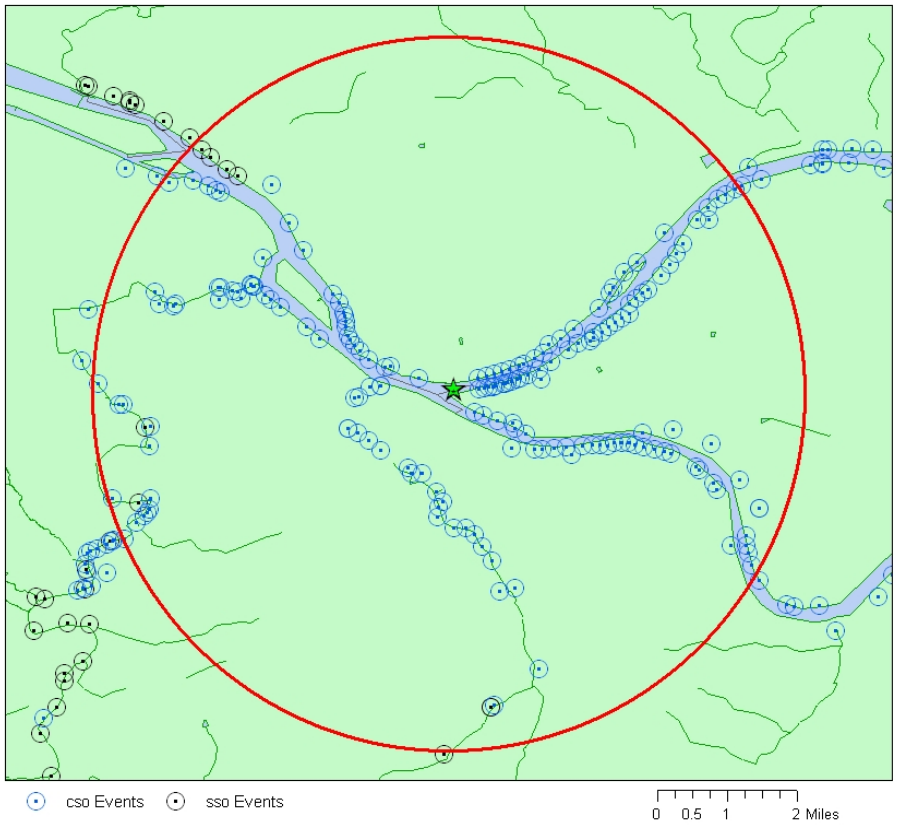
A.1.1 Study area



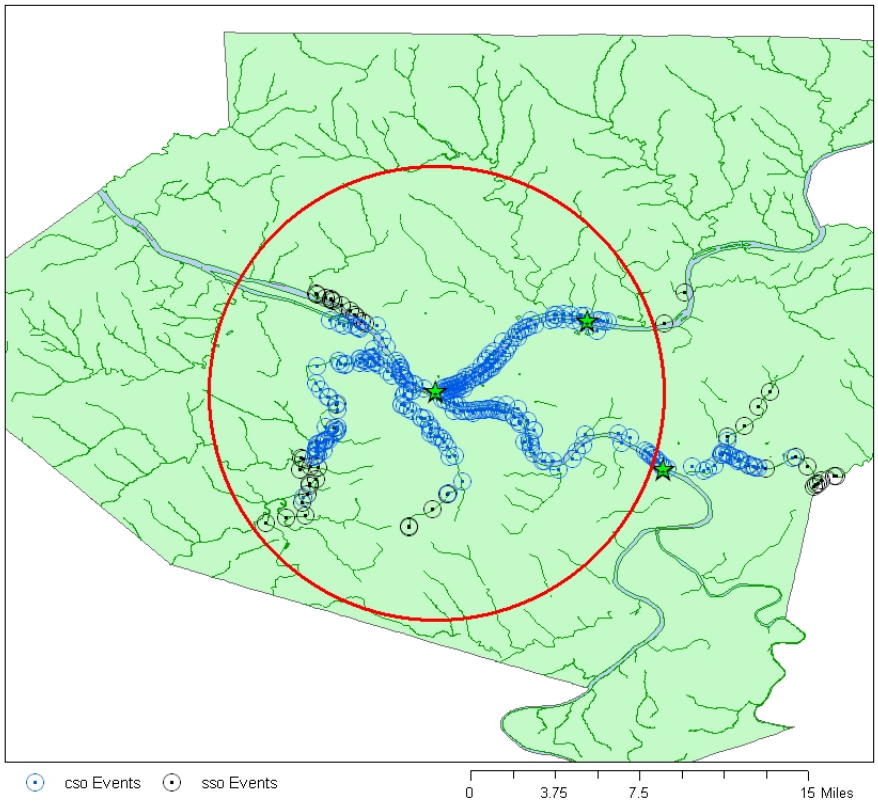
A.1.2 CSO/SSO Outfall Locations within 2 Miles of Point State Park



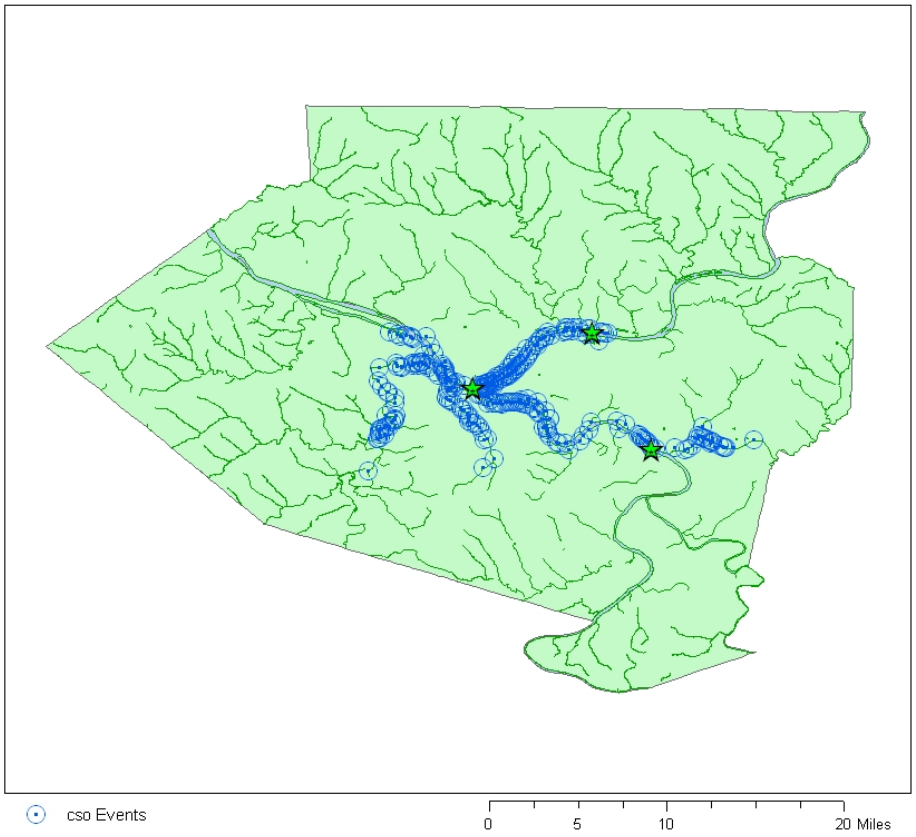
A.1.3 CSO/SSO Locations within 5 miles of Point State Park



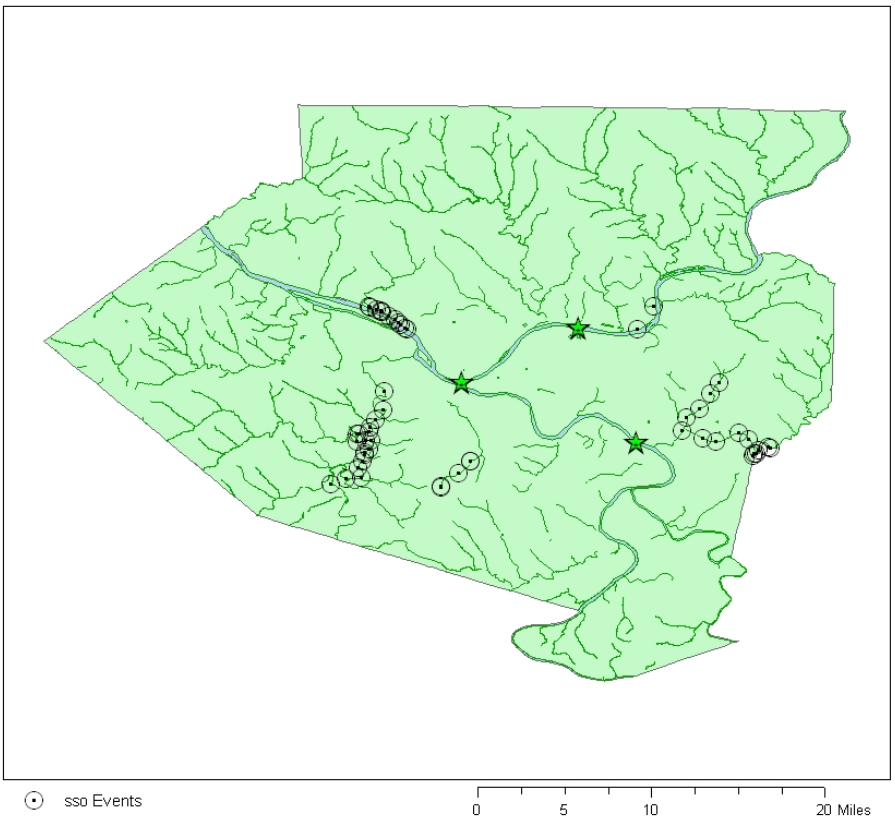
A.1.4 CSO/SSO Locations within 10 miles of points state park



A.1.5 Map of all CSO Outfall Locations in Allegheny County



A.1.6 Map of all SSO Outfall Locations in Allegheny County



APPENDIX B DATA

B.1.1 Proliferation Index data

Proliferation Index Data

fish	run	river	control1	control2	estradial1	estradial2	3E-04	3E-04	5E-04	1/1500	1/1000	1/500	1/200	1/100
1	1	1	1.05	0.96	1.7	1.9	0.94	0.97	0.94	1	0.92	0.97	1.12	1.38
	2	1	0.95	0.95	1.68	1.96	1.05	1.01	0.93	0.94	0.91	0.97	1.14	1.24
	3	1	1	0.92	1.7	1.86	1.07	0.99	1.01	0.98	0.94	0.94	1.19	1.19
	4	1	1.01	1	1.73	1.94	1.14	1.12	1.05	1.04	0.98	1.04	1.12	1.25
	5	1	1.09	1.07	1.82	1.91	1.28	1.15	1.11	1.23	1.02	1.12	1.24	1.29
2	1	1	0.99	0.86	1.82	2.08	1.08	1.04	0.95	1.02	0.92	0.97	1.19	1.28
	2	1	1.02	0.87	1.74	1.91	1.15	1.13	0.97	1.01	0.93	0.99	1.11	1.33
	3	1	0.97	1	1.8	1.99	1.18	1.07	0.96	0.98	0.95	1.06	1.24	1.28
	4	1	1.06	0.95	1.81	2.11	1.26	1.15	1.01	1.11	1.03	1.14	1.2	1.24
	5	1	1.2	1.08	2.05	2.04	1.36	1.24	1.17	1.4	1.12	1.27	1.37	1.49
3	1	1	0.96	0.92	1.71	1.99	1.07	1.1	1.01	1.15	1.01	1.16	1.47	1.49
	2	1	0.93	0.89	1.91	2.01	1.07	1.21	1.02	1.08	1.03	1.11	1.41	1.6
	3	1	0.94	0.91	1.81	2.08	1.07	1.13	1.08	1.16	1.05	1.19	1.39	1.64
	4	1	1.05	1.08	1.94	2.1	1.07	1.29	1.18	1.29	1.22	1.34	1.58	1.76
	5	1	1.13	1.19	2.14	2.29	1.07	1.42	1.31	1.44	1.27	1.37	1.64	1.88
4	1	1	0.98	0.88	1.66	1.77	1.14	0.96	0.95	0.97	0.87	0.93	0.98	1.05
	2	1	1.06	0.93	1.64	1.84	1.14	1.04	0.99	0.97	0.9	0.99	1	1.17
	3	1	0.97	0.93	1.68	1.87	1.16	1.08	0.94	0.97	0.97	0.98	1.09	1.06
	4	1	1.1	0.99	1.73	1.81	1.13	1.15	1.07	1.02	1.05	1.02	1.07	1.14
	5	1	1.14	1.02	1.8	1.72	1.21	1.11	1.08	1.18	1.13	1.08	1.12	1.24
5	1	1	1.02	0.99	1.62	2	1.17	1.2	1	1.01	0.95	1.17	1.36	1.5
	2	1	0.99	0.91	1.62	1.98	1.18	1.23	1.03	1.03	0.92	1.08	1.32	1.44
	3	1	0.97	0.92	1.61	1.95	1.21	1.16	1.04	1.07	0.99	1.16	1.39	1.43
	4	1	1.08	0.97	1.65	1.9	1.18	1.24	1.1	1.13	1.08	1.17	1.32	1.48
	5	1	1.16	0.98	1.87	1.84	1.29	1.35	1.24	1.27	1.13	1.2	1.34	1.49
6	1	1	1.07	0.92	1.64	1.88	1.1	1.09	0.97	0.99	1	0.91	1.11	1.24
	2	1	1.11	0.94	1.55	1.88	1.17	1.12	0.97	0.91	1.21	0.89	1.07	1.27
	3	1	0.99	0.93	1.62	1.88	1.13	1.09	0.97	0.96	0.91	0.91	1.11	1.16
	4	1	1.03	0.93	1.7	1.92	1.19	1.1	1	1.03	0.96	1.02	1.07	1.17
	5	1	1.08	1	1.85	1.86	1.24	1.2	1.12	1.07	1.06	1.03	1.12	1.24
7	1	2	1.03	0.93	1.61	1.47	1.01	0.96	0.93	0.89	0.94	0.88	1.12	1.06
	2	2	1.17	1.05	1.55	1.55	1.01	1.02	0.93	0.89	1.17	0.95	1.76	1.52
	3	2	0.97	0.91	1.56	1.53	1.03	0.98	0.9	0.89	0.9	0.97	1.03	1.07
	4	2	0.97	0.95	1.54	1.54	1	0.99	0.9	0.93	0.87	0.97	1	1.13
	5	2	1.02	1.01	1.62	1.6	0.92	0.98	0.99	1.01	0.99	0.96	1.01	1.16
8	1	2	0.99	0.88	1.78	2.01	1.2	1.02	0.93	0.83	0.84	0.87	0.96	1.1
	2	2	0.99	0.79	1.69	2.04	1.14	1.03	0.96	0.92	0.82	0.86	0.95	1.05
	3	2	1.03	0.9	1.81	2.05	1.1	1.03	0.94	0.9	0.92	0.91	0.98	1.09
	4	2	1.1	1.05	1.96	2.14	1.15	1.23	1.02	1.06	1.02	1.03	1.13	1.15
	5	2	1.17	1.09	2.05	2.06	1.39	1.36	1.15	1.17	1.16	1.15	1.32	1.23
9	1	2	0.99	0.94	1.84	2.13	1.32	1.11	1	0.93	0.89	0.97	1.05	1.19
	2	2	0.93	0.91	1.84	2.08	1.2	1.09	0.96	0.96	0.92	0.92	1.05	1.16
	3	2	1.03	0.94	1.88	2.08	1.16	1.03	0.95	0.97	0.96	0.99	1.1	1.24
	4	2	1	1.07	1.91	2.11	1.15	1.06	1.02	1.07	1.03	1.03	1.19	1.25
	5	2	1.06	1.11	2.06	2.15	1.2	1.19	1.07	1.15	1.15	1.08	1.31	1.29
10	1	3	0.93	0.84	1.57	1.97	1.17	1.06	0.9	0.81	0.73	0.79	1	1.07
	2	3	0.98	0.84	1.58	2.04	1.16	1.09	0.88	0.88	0.75	0.78	1.03	1.17
	3	3	1.01	0.9	1.65	1.98	1.17	1.06	0.97	0.92	0.83	0.93	1.07	1.19
	4	3	1.1	1.03	1.88	2.05	1.22	1.19	1.03	1.06	1.05	1.05	1.16	1.26
	5	3	1.21	1.15	1.99	2.09	1.31	1.25	1.16	1.23	1.17	1.14	1.24	1.34
11	1	3	1	1.02	1.8	2.01	1.01	1.05	0.96	0.93	0.99	0.95	1.04	1.17
	2	3	0.96	1	1.8	1.91	1.06	0.99	0.93	1	0.92	0.99	1.08	1.12
	3	3	0.93	0.89	1.7	2.01	1.07	1.02	1.02	0.94	0.99	0.99	1.06	1.14
	4	3	0.98	0.93	1.61	1.86	1.11	1.09	0.99	1.03	1.05	1.06	1.07	1.17
	5	3	1.07	1.21	1.99	1.94	1.15	1.11	0.48	1.13	1.11	1.01	1.16	1.24
12	1	3	0.99	0.91	1.48	1.65	1.15	1.13	0.94	0.92	0.82	0.88	1.08	1.26
	2	3	1	0.86	1.53	1.65	1.15	1.05	0.96	0.91	0.81	0.87	1.13	1.23
	3	3	1	0.95	1.51	1.67	1.15	1.1	0.98	1	0.9	0.91	1.09	1.27
	4	3	1.07	1.01	1.59	1.74	1.15	1.12	1.04	1.06	0.98	1.03	1.15	1.28
	5	3	1.12	1.08	1.68	1.74	1.15	1.14	1.07	1.11	1.09	1.09	1.22	1.34
13	1	3	0.94	0.86	1.57	1.96	1.16	1.06	0.94	0.91	0.83	0.88	1.08	1.29
	2	3	0.97	0.88	1.66	1.83	1.16	1.12	0.92	0.9	0.82	0.9	1.16	1.28
	3	3	0.95	0.92	1.72	1.98	1.15	1.15	0.99	0.98	0.88	1.01	1.17	1.33
	4	3	1.15	1.02	1.78	1.87	1.24	1.22	1.07	1.08	1.03	1.11	1.21	1.37
	5	3	1.18	1.12	2.04	2.03	1.29	1.31	1.25	1.15	1.13	1.18	1.35	1.51
14	1	3	0.94	0.88	1.64	1.67	1.08	0.98	0.92	0.89	0.83	0.93	0.99	1.15
	2	3	0.97	0.89	1.48	1.88	1.18	1.01	0.88	0.88	0.82	0.95	1.06	1.11
	3	3	0.96	1.01	2.04	1.7	1.27	1.01	1	0.94	0.92	1.01	1.08	1.18
	4	3	1.04	1.02	1.78	1.65	1.05	1.06	0.96	1.06	1.07	1.08	1.24	1.37
	5	3	1.16	1.13	1.75	1.69	1.11	1.12	1.06	1.12	1.11	1.15	1.35	1.37
15	1	3	1	1.02	1.58	2.08	1.01	1.05	0.95	0.92	0.99	0.95	1.06	1.19
	2	3	0.98	0.87	1.57	1.97	1.07	1	0.94	1	0.92	0.99	1.09	1.14
	3	3	0.95	0.91	1.77	2.08	1.07	1.02	1.01	0.94	0.99	1	1.08	1.17
	4	3	1.01	0.95	1.67	1.91	1.12	1.1	1	1.04	1.06	1.07	1.09	1.19
	5	3	1.09	1.22	2.06	1.99	1.17	1.13	0.49	1.16	1.13	1.03	1.19	1.27
16	1	4	1.13	0.98	1.71	1.84	1.28	1.06	0.98	0.93	0.82	0.96	1.05	1.22
	2	4	1.01	0.94	1.71	1.83	1.2	1.08	0.9	0.89	0.83	1.07	1.02	1.26
	3	4	0.98	0.92	1.63	1.74	1.11	1.06	0.97	0.96	0.85	0.92	1.03	1.17
	4	4	1.06	0.93	1.65	1.79	1.17	1.09	0.99	1.01	0.99	1.01	1.06	1.13
	5	4	1.08	0.97	1.65	1.81	1.16	1.17	1.03	1.02	0.96	1.03	1.04	1.29
17	1	4	1.03	0.98	1.73	1.79	1.08	1.04	0.96	1	0.85	0.89	1.02	1.06
	2	4	1.03	0.93	1.94	1.81	1.02	1.04	0.97	0.92	0.86	0.85	1	1.04
	3	4	0.98	0.96	1.73	1.8	1.11	0.99	0.91	0.91	0.92	0.87	1.06	1.1
	4	4	1.1	0.94	1.7	1.79	1.1	1.11	0.99	0.98	1	0.97	1.01	0.99
	5	4	1.08	0.97	1.66	1.78	1.07	1.1	1.05	1	0.91	0.99	1.02	1.06
18	1	6	0.95	0.83	1.54	1.9	1.12	1.19	0.91	0.89	0.8	0.92	1.21	1.38
	2	6	0.96	0.92	1.63	2.01	1.17	1.08	0.94	0.9	0.83	0.96	1.22	1.4
	3	6	1.05	0.89	1.65	1.96	1.16	1.17	0.99	1.03	0.9	1.02	1.31	1.44
	4	6	1.11	1.03	1.73	1.92	1.21	1.22	1.1	1.1	1.04	1.18	1.39	1.51
	5	6	1.19	1.08	1.91	1.97	1.23	1.22	1.18	1.23	1.22	1.25	1.53	1.62
19	1	6	0.99	0.85	1.61	1.93	1.13	1.11	0.97	0.87	0.83	0.85	1.05	1.27
	2	6	1.02	0.9	1.63	1.86	1.15	1.08	0.93	0.87	0.83	0.85	1.1	1.2
	3	6	0.99	0.98	1.64	1.92	1.2	1.09	0.97	0.96	0.87	0.94	1.13	1.2
	4	6	1.08	1.01	1.83	1.94	1.14	1.14	0.99	1.02	1.02	1.03	1.16	1.27
	5	6	1.08	1.09	1.86	1.92	1.18	1.15	1.13	1.12	1.12	1.11	1.26	1.32
20	1	6	0.96	0.94	1.58	1.79	1.26	1.29	1.03	1.14	1.04	1.24	1.42	1.52
	2	6	0.99	0.94	1.63	1.78	1.61	1.26	1.11	1.14	1.01	1.17	1.35	1.5
	3	6	0.97	1.01	1.67	1.84	1.19	1.2	1.12	1.14	1.09	1.23	1.41	1.48
	4	6	1.08	1.02	1.78	1.8	1.2	1.2	1.09	1.18	1.16	1.19	1.38	1.51
	5	6	1.06	1.02	1.9	1.82	1.18	1.33	1.22	1.28	1.17	1.29	1.43	1.51
21	1	6	1.01	0.92	1.57	1.71	1.15	1.13	1.03	1.08	1.02	1.14	1.28	1.38
	2	6	1.04	0.92	1.51	1.69	1.1	1.09	1.04	1.07	0.98	1.08	1.32	1.35
	3	6	1	0.95	1.44	1.77	1.15	1.1	1.01	1.07	1.02	1.06	1.29	1.4
	4	6	1.06	0.99	1.54	1.74	1.15	1.13	1.02	1.07	1.09	1.19	1.25	1.41
	5	6	1.1	1	1.74	1.78	1.16	1.18	1.08	1.19	1.1	1.13	1.37	1.4

B.1.2 Data with proliferation indices averaged

The table below summarizes averaged proliferation index values for each fish at each dilution. Values are obtained by averaging all five values from the original data at each dilution for each fish.

Averaged Proliferation Index Data

fish	river	control	estradial	1/4000	1/3000	1/2000	1/1500	1/1000	1/500	1/200	1/100
1	1	1.00	1.82	1.10	1.05	1.01	1.04	0.95	1.01	1.16	1.27
2	1	1.00	1.94	1.21	1.13	1.01	1.10	0.99	1.09	1.22	1.32
3	1	1.00	2.00	1.27	1.23	1.12	1.22	1.12	1.23	1.50	1.67
4	1	1.00	1.75	1.16	1.07	1.01	1.02	0.98	1.00	1.05	1.13
5	1	1.00	1.80	1.21	1.24	1.08	1.10	1.01	1.16	1.35	1.47
6	1	1.00	1.78	1.17	1.12	1.01	0.99	1.03	0.95	1.10	1.22
7	2	1.00	1.56	0.99	0.99	0.93	0.92	0.97	0.95	1.18	1.19
8	2	1.00	1.96	1.20	1.13	1.00	0.98	0.95	0.96	1.07	1.12
9	2	1.00	2.01	1.21	1.10	1.00	1.02	0.99	1.00	1.14	1.23
10	3	1.00	1.88	1.21	1.13	0.99	0.98	0.91	0.94	1.10	1.21
11	3	1.00	1.86	1.08	1.05	0.88	1.01	1.01	1.00	1.08	1.17
12	3	1.00	1.62	1.14	1.11	1.00	1.00	0.92	0.96	1.13	1.28
13	3	1.00	1.84	1.20	1.17	1.03	1.00	0.94	1.02	1.19	1.36
14	3	1.00	1.73	1.14	1.04	0.96	0.98	0.95	1.02	1.14	1.24
15	3	1.00	1.87	1.09	1.06	0.88	1.01	1.02	1.01	1.10	1.19
16	4	1.00	1.74	1.18	1.09	0.97	0.96	0.89	1.00	1.04	1.21
17	4	1.00	1.77	1.08	1.06	0.98	0.96	0.91	0.91	1.02	1.05
18	6	1.00	1.82	1.18	1.18	1.02	1.03	0.96	1.07	1.33	1.47
19	6	1.00	1.81	1.16	1.11	1.00	0.97	0.93	0.96	1.14	1.25
20	6	1.00	1.76	1.29	1.26	1.11	1.18	1.09	1.22	1.40	1.50
21	6	1.00	1.65	1.14	1.13	1.04	1.10	1.04	1.12	1.30	1.39

B.1.3 Estrogenicity index data

Estrogenicity Index Data

fish	run	river	estradiol	control	1/4000	1/3000	1/2000	1/1500	1/1000	1/500	1/200	1/100
1	1	1	1.00	0.00	-0.073	-0.037	-0.073	0.000	-0.098	-0.037	0.146	0.463
	2	1	1.00	0.00	0.061	0.012	-0.085	-0.073	-0.110	-0.037	0.171	0.293
	3	1	1.00	0.00	0.085	-0.012	0.012	-0.024	-0.073	-0.073	0.232	0.232
	4	1	1.00	0.00	0.171	0.146	0.061	0.049	-0.024	0.049	0.146	0.305
	5	1	1.00	0.00	0.341	0.183	0.134	0.280	0.024	0.146	0.293	0.354
2	1	1	1.00	0.00	0.085	0.043	-0.053	0.021	-0.085	-0.032	0.202	0.298
	2	1	1.00	0.00	0.160	0.138	-0.032	0.011	-0.074	-0.011	0.117	0.351
	3	1	1.00	0.00	0.191	0.074	-0.043	-0.021	-0.053	0.064	0.255	0.298
	4	1	1.00	0.00	0.277	0.160	0.011	0.117	0.032	0.149	0.213	0.255
	5	1	1.00	0.00	0.383	0.255	0.181	0.426	0.128	0.287	0.394	0.521
3	1	1	1.00	0.00	0.070	0.100	0.010	0.150	0.010	0.160	0.470	0.490
	2	1	1.00	0.00	0.070	0.210	0.020	0.080	0.030	0.110	0.410	0.600
	3	1	1.00	0.00	0.070	0.130	0.080	0.160	0.050	0.190	0.390	0.640
	4	1	1.00	0.00	0.070	0.290	0.180	0.290	0.220	0.340	0.580	0.760
	5	1	1.00	0.00	0.070	0.420	0.310	0.440	0.270	0.370	0.640	0.880
4	1	1	1.00	0.00	0.184	-0.053	-0.066	-0.039	-0.171	-0.092	-0.026	0.066
	2	1	1.00	0.00	0.184	0.053	-0.013	-0.039	-0.132	-0.013	0.000	0.224
	3	1	1.00	0.00	0.211	0.105	-0.079	-0.039	-0.039	-0.026	0.118	0.079
	4	1	1.00	0.00	0.171	0.197	0.092	0.026	0.066	0.026	0.092	0.184
	5	1	1.00	0.00	0.276	0.145	0.105	0.237	0.171	0.105	0.158	0.316
5	1	1	1.00	0.00	0.211	0.249	0.000	0.012	-0.062	0.211	0.448	0.622
	2	1	1.00	0.00	0.225	0.288	0.038	0.038	-0.100	0.100	0.400	0.550
	3	1	1.00	0.00	0.263	0.200	0.050	0.088	-0.013	0.200	0.488	0.538
	4	1	1.00	0.00	0.225	0.300	0.125	0.163	0.100	0.213	0.400	0.600
	5	1	1.00	0.00	0.363	0.438	0.300	0.338	0.163	0.250	0.425	0.613
6	1	1	1.00	0.00	0.129	0.116	-0.039	-0.013	0.000	-0.116	0.141	0.308
	2	1	1.00	0.00	0.218	0.154	-0.038	-0.115	0.269	-0.141	0.090	0.346
	3	1	1.00	0.00	0.167	0.115	-0.038	-0.051	-0.115	-0.115	0.141	0.205
	4	1	1.00	0.00	0.244	0.128	0.000	0.038	-0.051	0.026	0.090	0.218
	5	1	1.00	0.00	0.308	0.256	0.154	0.090	0.077	0.038	0.154	0.308
7	1	2	1.00	0.00	0.018	-0.072	-0.126	-0.197	-0.108	-0.215	0.215	0.108
	2	2	1.00	0.00	0.018	0.036	-0.125	-0.196	0.304	-0.089	1.357	0.929
	3	2	1.00	0.00	0.054	-0.036	-0.179	-0.196	-0.179	-0.054	0.054	0.125
	4	2	1.00	0.00	0.000	-0.018	-0.179	-0.125	-0.232	-0.054	0.000	0.232
	5	2	1.00	0.00	-0.143	-0.036	-0.018	0.018	-0.018	-0.071	0.018	0.286
8	1	2	1.00	0.00	0.209	0.021	-0.073	-0.177	-0.167	-0.136	-0.042	0.104
	2	2	1.00	0.00	0.146	0.031	-0.042	-0.083	-0.188	-0.146	-0.052	0.052
	3	2	1.00	0.00	0.104	0.031	-0.063	-0.104	-0.083	-0.094	-0.021	0.094
	4	2	1.00	0.00	0.156	0.240	0.021	0.063	0.021	0.031	0.135	0.156
	5	2	1.00	0.00	0.406	0.375	0.156	0.177	0.167	0.156	0.333	0.240
9	1	2	1.00	0.00	0.317	0.109	0.000	-0.069	-0.109	-0.030	0.050	0.188
	2	2	1.00	0.00	0.198	0.089	-0.040	-0.040	-0.079	-0.079	0.050	0.158
	3	2	1.00	0.00	0.158	0.030	-0.050	-0.030	-0.040	-0.010	0.099	0.238
	4	2	1.00	0.00	0.149	0.059	0.020	0.069	0.030	0.030	0.188	0.248
	5	2	1.00	0.00	0.198	0.188	0.069	0.149	0.149	0.079	0.307	0.287
10	1	3	1.00	0.00	0.193	0.068	-0.114	-0.216	-0.307	-0.239	0.000	0.080
	2	3	1.00	0.00	0.182	0.102	-0.136	-0.136	-0.284	-0.250	0.034	0.193
	3	3	1.00	0.00	0.193	0.068	-0.034	-0.091	-0.193	-0.080	0.080	0.216
	4	3	1.00	0.00	0.250	0.216	0.034	0.068	0.057	0.057	0.182	0.295
	5	3	1.00	0.00	0.353	0.285	0.182	0.262	0.194	0.159	0.273	0.387
11	1	3	1.00	0.00	0.012	0.058	-0.046	-0.081	-0.012	-0.058	0.046	0.197
	2	3	1.00	0.00	0.070	-0.012	-0.081	0.000	-0.093	-0.012	0.093	0.140
	3	3	1.00	0.00	0.081	0.023	0.023	-0.070	-0.012	-0.012	0.070	0.163
	4	3	1.00	0.00	0.128	0.105	-0.012	0.035	0.058	0.070	0.081	0.198
	5	3	1.00	0.00	0.174	0.128	-0.605	0.151	0.128	0.012	0.186	0.279
12	1	3	1.00	0.00	0.240	0.208	-0.096	-0.128	-0.288	-0.192	0.128	0.417
	2	3	1.00	0.00	0.242	0.081	-0.065	-0.145	-0.306	-0.210	0.210	0.371
	3	3	1.00	0.00	0.242	0.161	-0.032	0.000	-0.161	-0.145	0.145	0.435
	4	3	1.00	0.00	0.242	0.194	0.065	0.097	-0.032	0.048	0.242	0.452
	5	3	1.00	0.00	0.242	0.226	0.113	0.177	0.145	0.145	0.355	0.548
13	1	3	1.00	0.00	0.190	0.071	-0.071	-0.107	-0.201	-0.142	0.095	0.344
	2	3	1.00	0.00	0.190	0.143	-0.095	-0.119	-0.214	-0.119	0.190	0.333
	3	3	1.00	0.00	0.179	0.179	-0.012	-0.024	-0.143	0.012	0.202	0.393
	4	3	1.00	0.00	0.286	0.262	0.083	0.095	0.036	0.131	0.250	0.440
	5	3	1.00	0.00	0.345	0.369	0.298	0.179	0.155	0.214	0.417	0.607
14	1	3	1.00	0.00	0.110	-0.027	-0.110	-0.151	-0.234	-0.096	-0.014	0.206
	2	3	1.00	0.00	0.247	0.014	-0.164	-0.164	-0.247	-0.068	0.082	0.151
	3	3	1.00	0.00	0.370	0.014	0.000	-0.082	-0.110	0.014	0.110	0.247
	4	3	1.00	0.00	0.068	0.082	-0.055	0.082	0.096	0.110	0.329	0.507
	5	3	1.00	0.00	0.151	0.164	0.082	0.164	0.151	0.205	0.479	0.507
15	1	3	1.00	0.00	0.012	0.058	-0.058	-0.092	-0.012	-0.058	0.069	0.219
	2	3	1.00	0.00	0.080	0.000	-0.069	0.000	-0.092	-0.011	0.103	0.161
	3	3	1.00	0.00	0.080	0.023	0.011	-0.069	-0.011	0.000	0.092	0.195
	4	3	1.00	0.00	0.138	0.115	0.000	0.046	0.069	0.080	0.103	0.218
	5	3	1.00	0.00	0.195	0.149	-0.586	0.184	0.149	0.034	0.218	0.310
16	1	4	1.00	0.00	0.380	0.082	-0.027	-0.095	-0.245	-0.054	0.068	0.299
	2	4	1.00	0.00	0.270	0.108	-0.135	-0.149	-0.230	0.095	0.027	0.351
	3	4	1.00	0.00	0.149	0.081	-0.041	-0.054	-0.203	-0.108	0.041	0.230
	4	4	1.00	0.00	0.230	0.122	-0.014	0.014	-0.014	0.014	0.081	0.176
	5	4	1.00	0.00	0.216	0.230	0.041	0.027	-0.054	0.041	0.054	0.392
17	1	4	1.00	0.00	0.103	0.052	-0.052	0.000	-0.194	-0.142	0.026	0.078
	2	4	1.00	0.00	0.026	0.052	-0.039	-0.104	-0.182	-0.195	0.000	0.052
	3	4	1.00	0.00	0.143	-0.013	-0.117	-0.117	-0.104	-0.169	0.078	0.130
	4	4	1.00	0.00	0.130	0.143	-0.013	-0.026	0.000	-0.039	0.013	-0.013
	5	4	1.00	0.00	0.091	0.130	0.065	0.000	-0.117	-0.013	0.026	0.078
18	1	6	1.00	0.00	0.146	0.231	-0.109	-0.134	-0.243	-0.097	0.255	0.462
	2	6	1.00	0.00	0.207	0.098	-0.073	-0.122	-0.207	-0.049	0.268	0.488
	3	6	1.00	0.00	0.195	0.207	-0.012	0.037	-0.122	0.024	0.378	0.537
	4	6	1.00	0.00	0.256	0.268	0.122	0.122	0.049	0.220	0.476	0.622
	5	6	1.00	0.00	0.280	0.268	0.220	0.280	0.268	0.305	0.646	0.756
19	1	6	1.00	0.00	0.160	0.135	-0.037	-0.160	-0.209	-0.184	0.061	0.332
	2	6	1.00	0.00	0.185	0.099	-0.086	-0.160	-0.210	-0.185	0.123	0.247
	3	6	1.00	0.00	0.247	0.111	-0.037	-0.049	-0.160	-0.074	0.160	0.247
	4	6	1.00	0.00	0.173	0.173	-0.012	0.025	0.025	0.037	0.198	0.333
	5	6	1.00	0.00	0.222	0.185	0.160	0.148	0.148	0.136	0.321	0.395
20	1	6	1.00	0.00	0.343	0.382	0.040	0.184	0.053	0.316	0.553	0.685
	2	6	1.00	0.00	0.803	0.342	0.145	0.184	0.013	0.224	0.461	0.658
	3	6	1.00	0.00	0.250	0.263	0.158	0.184	0.118	0.303	0.539	0.632
	4	6	1.00	0.00	0.263	0.263	0.118	0.237	0.211	0.250	0.500	0.671
	5	6	1.00	0.00	0.237	0.434	0.289	0.368	0.224	0.382	0.566	0.671
21	1	6	1.00	0.00	0.231	0.200	0.046	0.123	0.031	0.216	0.431	0.586
	2	6	1.00	0.00	0.154	0.138	0.062	0.108	-0.031	0.123	0.492	0.538
	3	6	1.00	0.00	0.231	0.154	0.015	0.108	0.031	0.092	0.446	0.615
	4	6	1.00	0.00	0.231	0.200	0.031	0.108	0.138	0.292	0.385	0.631
	5	6	1.00	0.00	0.246	0.277	0.123	0.292	0.154	0.200	0.569	0.615

B.1.4 Data with Estrogenicity index averaged

Averaged Estrogenicity Averaged Data

fish	run	river	avg_ei_ctl	avg_ei_E2	avg_ei_4000	avg_ei_3000	avg_ei_2000	avg_ei_1500	avg_ei_1000	avg_ei_500	avg_ei_200	avg_ei_100
1	1	1	0.00	1.00	0.12	0.06	0.01	0.05	-0.06	0.01	0.20	0.33
2	1	1	0.00	1.00	0.22	0.13	0.01	0.11	-0.01	0.09	0.24	0.34
3	1	1	0.00	1.00	0.07	0.23	0.12	0.22	0.12	0.23	0.50	0.67
4	1	1	0.00	1.00	0.21	0.09	0.01	0.03	-0.02	0.00	0.07	0.17
5	1	1	0.00	1.00	0.26	0.29	0.10	0.13	0.02	0.19	0.43	0.58
6	1	1	0.00	1.00	0.21	0.15	0.01	-0.01	0.04	-0.06	0.12	0.28
7	1	2	0.00	1.00	-0.01	-0.03	-0.13	-0.14	-0.05	-0.10	0.33	0.34
8	1	2	0.00	1.00	0.20	0.14	0.00	-0.03	-0.05	-0.04	0.07	0.13
9	1	2	0.00	1.00	0.20	0.10	0.00	0.02	-0.01	0.00	0.14	0.22
10	1	3	0.00	1.00	0.23	0.15	-0.01	-0.02	-0.11	-0.07	0.11	0.23
11	1	3	0.00	1.00	0.09	0.06	-0.14	0.01	0.01	0.00	0.10	0.20
12	1	3	0.00	1.00	0.24	0.17	0.00	0.00	-0.13	-0.07	0.22	0.44
13	1	3	0.00	1.00	0.24	0.20	0.04	0.00	-0.07	0.02	0.23	0.42
14	1	3	0.00	1.00	0.19	0.05	-0.05	-0.03	-0.07	0.03	0.20	0.32
15	1	3	0.00	1.00	0.10	0.07	-0.14	0.01	0.02	0.01	0.12	0.22
16	1	4	0.00	1.00	0.25	0.12	-0.04	-0.05	-0.15	0.00	0.05	0.29
17	1	4	0.00	1.00	0.10	0.07	-0.03	-0.05	-0.12	-0.11	0.03	0.06
18	1	6	0.00	1.00	0.22	0.21	0.03	0.04	-0.05	0.08	0.40	0.57
19	1	6	0.00	1.00	0.20	0.14	0.00	-0.04	-0.08	-0.05	0.17	0.31
20	1	6	0.00	1.00	0.38	0.34	0.15	0.23	0.12	0.29	0.52	0.66
21	1	6	0.00	1.00	0.22	0.19	0.06	0.15	0.06	0.18	0.46	0.60

APPENDIX C Selected Figures in Color

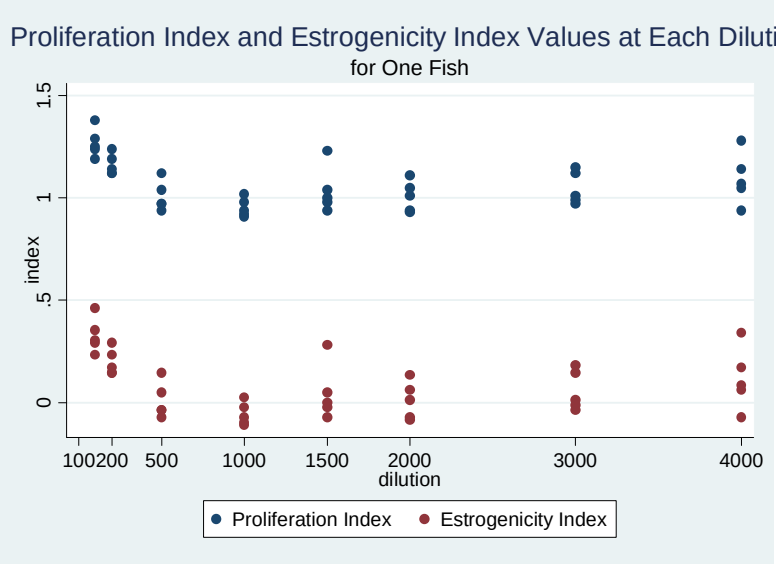


Figure 8: Proliferation Index and Estrogenicity Index Values for the Same Fish at All Dilutions

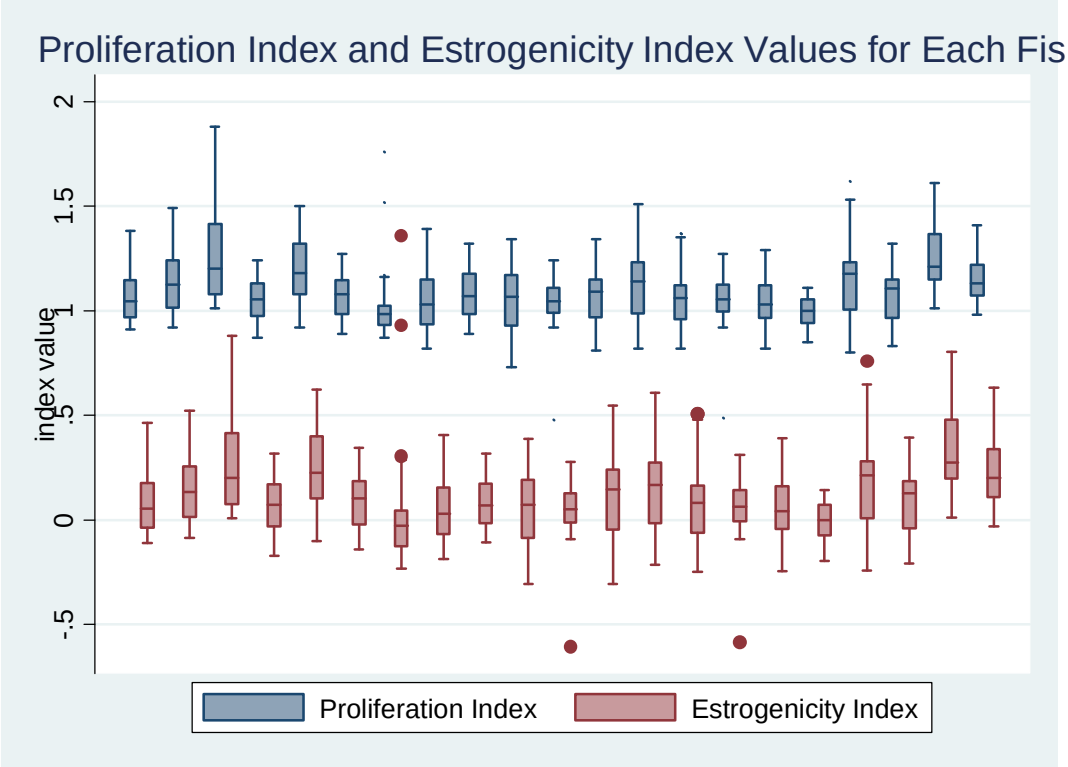


Figure 9: Box-plot of Proliferation Index and Estrogenicity Index Values for Each Fish

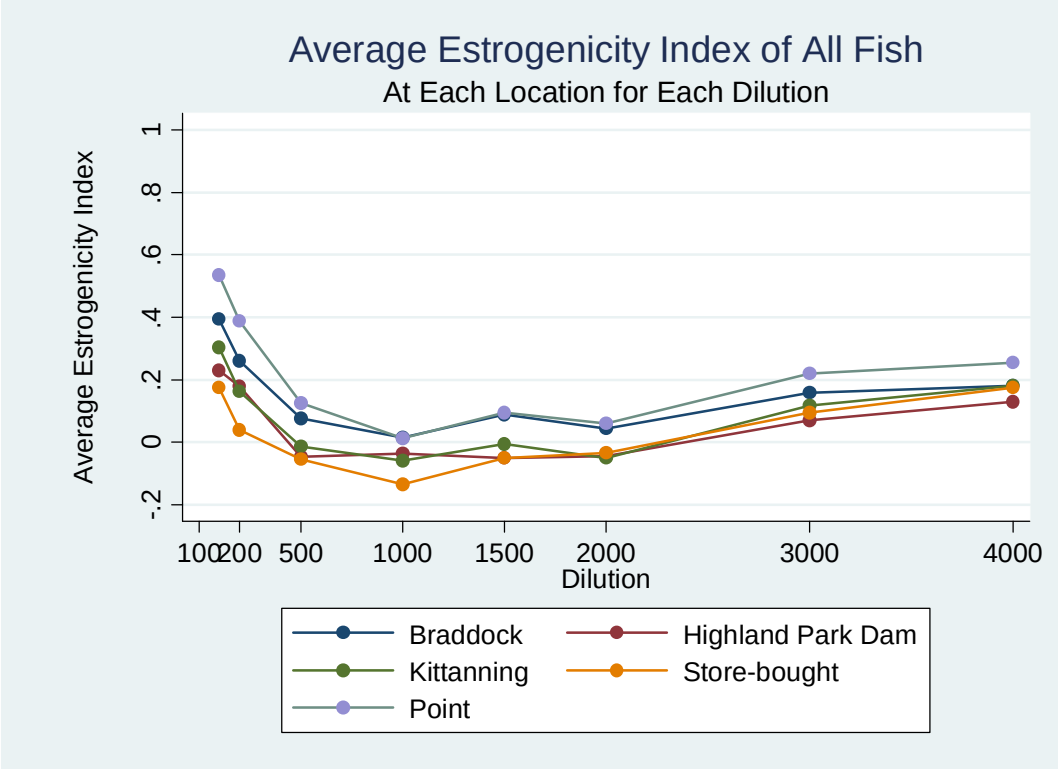


Figure 10: Average Estrogenicity Index of All Fish at Each Location for Each Dilution

Braddock

Dilution	mean	min	max	std.dev.
1/100	0.40	0.17	0.67	0.19
1/200	0.26	0.07	0.05	0.17
1/500	0.08	-0.06	0.23	0.11
1/1000	0.02	-0.06	0.12	0.06
1/1500	0.09	-0.01	0.22	0.08
1/2000	0.04	0.01	0.12	0.05
1/3000	0.16	0.06	0.29	0.08
1/4000	0.18	0.07	0.26	0.07

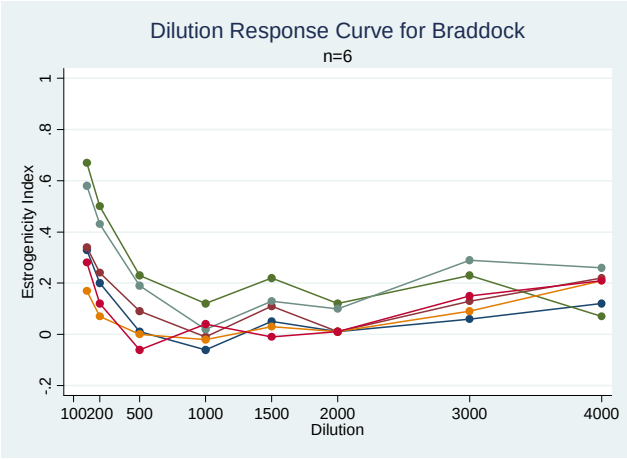


Figure 11: Fish Specific Estrogenicity Indices and Dilution Response Curve for Braddock

Highland Park Dam

Dilution	mean	min	max	std.dev.
1/100	0.23	0.13	0.34	0.11
1/200	0.18	0.07	0.33	0.13
1/500	-0.05	-0.10	0.00	0.05
1/1000	-0.04	-0.50	-0.01	0.02
1/1500	-0.05	-0.14	0.02	0.08
1/2000	-0.43	-0.13	0.00	0.08
1/3000	0.07	-0.03	0.14	0.09
1/4000	0.13	-0.01	0.20	0.12

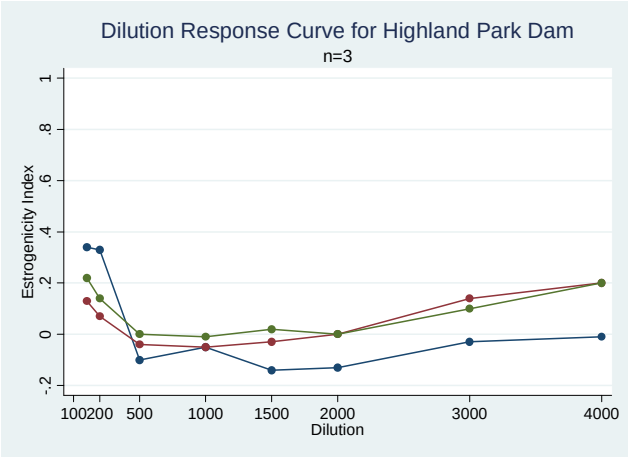


Figure 12: Fish Specific Estrogenicity Indices and Dilution Response Curve for Highland Park Dam

Kittanning

Dilution	mean	min	max	std.dev.
1/100	0.31	0.20	0.44	0.11
1/200	0.16	0.20	0.23	0.06
1/500	-0.01	-0.07	0.03	0.05
1/1000	-0.06	-0.13	0.02	0.06
1/1500	-0.01	-0.03	0.01	0.02
1/2000	-0.05	-0.14	0.04	0.08
1/3000	0.12	0.05	0.20	0.06
1/4000	0.18	0.09	0.24	0.07

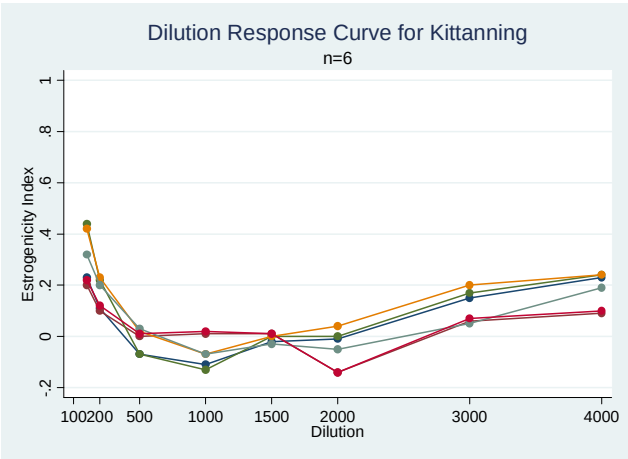


Figure 13: Fish Specific Estrogenicity Indices and Dilution Response Curve for Kittanning

Point

Dilution	mean	min	max	std.dev.
1/100	0.54	0.31	0.67	0.15
1/200	0.39	0.17	0.52	0.15
1/500	0.13	-0.05	0.29	0.14
1/1000	0.01	-0.08	0.12	0.09
1/1500	0.10	-0.04	0.23	0.12
1/2000	0.06	0.00	0.15	0.06
1/3000	0.22	0.14	0.34	0.09
1/4000	0.26	2.00	0.38	0.08

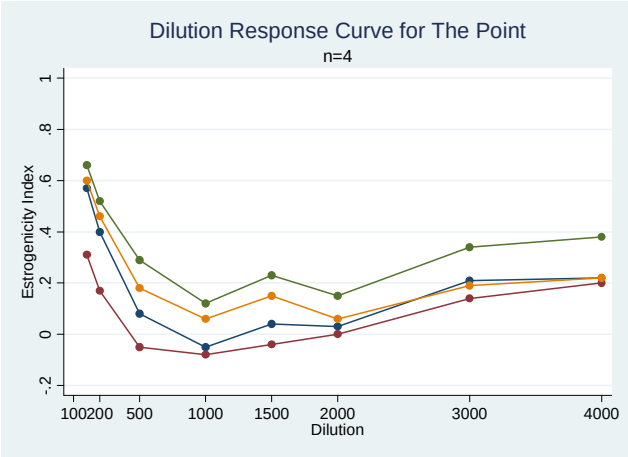


Figure 14: Fish Specific Estrogenicity Indices and Dilution Response Curve for the Point

Store Bought

Dilution	mean	min	max	std.dev.
1/100	0.18	0.06	0.29	0.16
1/200	0.04	0.03	0.05	0.01
1/500	-0.55	-0.11	0.00	0.07
1/1000	-0.14	-0.15	-0.12	0.02
1/1500	-0.05	-0.05	-0.05	0.00
1/2000	-0.04	-0.04	-0.03	0.01
1/3000	0.10	0.07	0.12	0.04
1/4000	0.18	0.10	0.25	0.11

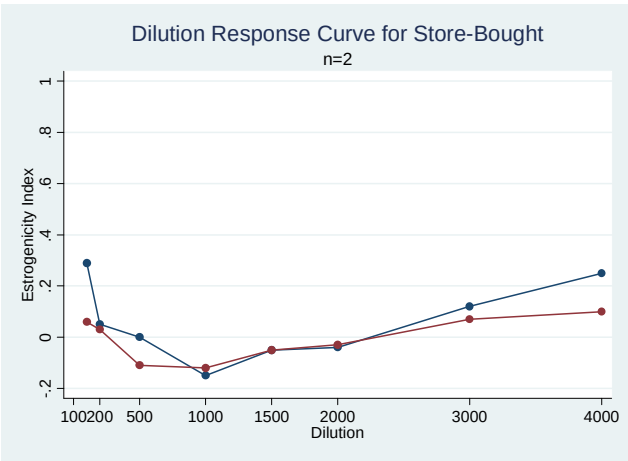


Figure 15: Fish Specific Estrogenicity Indices and Dilution Response Curve for Store-bought

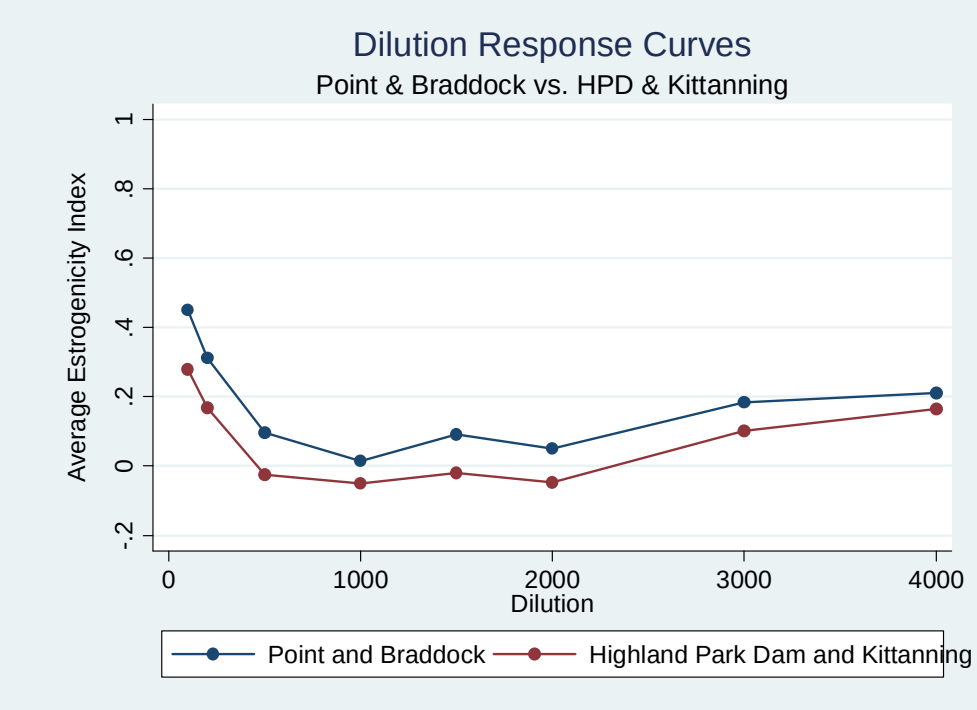


Figure 17: Point & Braddock and HPD & Kittanning: Averaged Estrogenicity Indices by Dilution

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