Determination of Endocrine Disrupting Chemicals in the Pike River

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Abstract

Endocrine disrupting chemicals (EDCs) are synthetic and natural compounds that, when absorbed by an organism, can change the function of the endocrine system (Stuart, 2006). Researchers all over the world are studying the potentially harmful effects of these chemicals, particularly those effects on fish and amphibians. These effects include, reproductive, neurological, and immunologic dysfunction, among others (Harding et al. 2006).Research in our lab has focused on the reproductive effects of EDCs in aquatic habitats. Fish and amphibians are exposed to EDCs through their environment. To our knowledge, the concentrations of three specific EDCs-- atrazine, 17 α ethynylestradiol, and 17 β estradiol--have not been determined for the Pike River in Kenosha, WI. We hypothesize that surface waters in Lake Michigan will have higher levels of 17α ethynylestradiol and 17β estradiol. In agricultural areas, we hypothesize that atrazine will be detected in higher concentrations. Finally, we hypothesize that there should not be any 17α ethynylestradiol in the Pike River as it should be sewage free; any 17 β estradiol found in the Pike River will be attributed to natural production by organisms living in or near the river. Through filtration, solid phase extraction (SPE), derivatization, and the use of gas chromatography/mass spectrometry (GC/MS) the concentrations of these three EDCs will be determined. After analyzing our GC/MS results, we have seen that a new or improved protocol will be needed to better derivatize the samples and that a new internal standard needs to be used to prevent overlapping peaks. Also, calibration curves for all three EDCs have been made, each having an R^2 value above 0.98. Through the progress made this summer, our research team hopes improve our protocols to continue our research during the 2010-2011 school year in hopes of determining the concentrations of atrazine, 17 α ethynylestradiol, and 17 β estradiol in the Pike River. After the concentrations of these three EDCs are found, future research can focus on the reproductive effects on aquatic organisms at the determined concentrations.

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Introduction

Endocrine disrupting chemicals (EDCs) are natural and man-made compounds that may alter the function of the endocrine system when an organism absorbs them (Stuart, 2006). The tissues and receptors that participate in many hormone pathways like biosynthesis, transport, and metabolism are particularly sensitive to EDCs (Maruya, 2010). Endocrine disrupting chemicals alter the endocrine system by many different mechanisms. A few of these mechanisms include, acting as an antiandrogen, androgen, inhibitor or activator of steroid hormone synthesis, antithyroid substance, or retinoid agonist (Hotchkiss et al. 2008).

Two decades ago, little research had been done to understand the effects of endocrine disrupting chemicals (EDCs). However, in 1991 at the first World Wildlife Federation Wingspread Conference a group of scientists said, "Many compounds introduced into the environment by human activity are capable of disrupting the endocrine system of animals, including fish, wildlife, and humans" (Hotchkiss et al. 2008).

There are many classes of EDCs including, pharmaceuticals, natural and synthetic estrogens, pesticides, plasticizers, and personal care products (Comerton et al. 2010). Organisms are exposed to EDCs through a variety of ways; some common routes of exposure are through industry and manufacturing. These manufactured products are then used in homes, hospitals, work places, are put on crops, fed to livestock, and are directly or indirectly put into the ground water and air (Colborn et al, 1996). Another way organisms can be exposed to EDCs is through human and non-human waste excretion into water systems.

Researchers are finding that small concentrations of EDCs are causing a decline in many animal populations due to reproductive failure. There have also been reports of sex reversal and infertility in several species of fish and amphibians (WHO, 2002) due to the increase of EDCs in aquatic habitats. For example, Tyrone Hayes (2010) reported that, at atrazine concentrations as low as 2.5 ppb, *Xenopus laevis* (African clawed frog) males are becoming demasculinized to the point of complete feminization as adults and 10% of exposed genetic males developed into functioning females that can copulate and produce viable eggs.

Our experiment zeroes in on three specific endocrine disrupting chemicals; atrazine, 17α ethynylestradiol, and 17β-estradiol. Atrazine is a common herbicide used by many farmers to rid their fields of weeds. In fact, Tyrone Hayes states that, "approximately 80 million pounds of atrazine are applied annually in the US, primarily in corn growing states (Hayes, 2010)," even though it has found to be an EDC (Atrazine Updates, 2010).

Atrazine is a pertinent EDC in Wisconsin because a large portion of the state is primarily agricultural and utilizes this herbicide (Hayes, 2010). In agricultural counties of Wisconsin, it is estimated that, 9.321 - 34.596 lbs/sq.mile of atrazine is used each year (Hayes, 2010). Atrazine can persist in soils, leaving it susceptible to get into ground water through run off for sixty days (Ma et al. 2003). In addition, atrazine can remain in surface and ground water significantly longer than 60 days (Ma et al. 2003). In 2002, Hayes and coworkers exposed *Xenopus laevis* larvae to 0.01-200 ppm of atrazine and allowed the frogs to develop. Post gonadal developmental analysis revealed that, atrazine exposure produced pseudohermaphroditism and

demasculinization in males. In 2010, Hayes and coworkers also presented results that atrazine induced females (genetic males) produced only male offspring. This could potentially wipe out the community because no female frogs would be born. However, other studies of atrazine have shown that frog larval exposure of varying concentrations (0.1 ppb to 25 ppb) has no internal or external reproductive organ effect (Coady et al, 2004). This controversy has researchers pondering reasons for varying results; some of which could be due to differences in exposure time and intensity used in different experiments. In order to resolve this controversy, more research needs to be done using a unified protocol to show whether or not atrazine causes intersex.

17 α ethynylestradiol and 17 β estradiol are estrogenic compounds; these lipid derived steroid hormones are estrogens that aid in the feminization of animals. 17 α ethynylestradiol is a synthetic estrogen that is used in hormone replacement therapy and birth control; while 17 β estradiol is a naturally occurring hormone. These compounds are causing male reproductive dysfunction at levels between 5 ppt and 25 ppt (Stuart 2006). One example of this is the induction of vitellogenin in male fish. Vitellogenin is an estrogen-responsive egg yolk protein precursor not normally expressed in male fish. For example, Kidd and colleagues found that in fathead minnows 5-6 ppt of 17 α ethynylestradiol was sufficient to induce the production of vitellogenin mRNA. The presence of estrogen later caused impacts on gonadal development (intersex) in males and altered oogenesis in females (Kidd et al. 2007). This marker, when present in male fish, suggests an increase in estrogen levels that can lead to reproductive failure (Sumpter, 1995). Also, in 2000, Miyata and Kubo exposed male *Xenopus laevis* to a range of concentrations of 17 β-estradiol. They found that at a concentration as low as 1 ppb 44% of exposed males became feminized. At a concentration of 100 ppb over 90% of the exposed males were feminized. It is therefore, imperative that we determine the concentrations of these estrogens because even low levels are more than sufficient to threaten a community's survival.

With increasing evidence of the effects of atrazine, 17 α ethynylestradiol, 17 β estradiol, and other EDCs, it is no wonder why research in this area has become so prevalent. Scientists across the globe are determining the concentrations of EDCs in rivers, lakes, and other watersheds. To our knowledge, no one has determined the concentrations of atrazine, 17 α ethynylestradiol, and 17 β estradiol in the Pike River in Kenosha Wisconsin. In this experiment we would like to design a method based on previous research for the analysis of these three EDCs. In doing so, we hope determine the concentrations at which atrazine, 17 α ethynylestradiol, and 17 β estradiol are present in the Pike River. We hypothesize that surface waters in Lake Michigan will have higher levels of 17α ethynylestradiol and 17β estradiol due to the accumulation of human and non human waste from urban areas. In rural areas, atrazine will be detected in higher concentrations in surface waters due to agricultural use. There should not be any 17α ethynylestradiol in the Pike River because it should be sewage free. Any 17 β estradiol found in the Pike River will be attributed to natural production by organisms living in or near the river.

Methods

        Sample Collection

        Samples will be collected in three locations along the Pike River. These locations are upstream at the origin of the Pike River, on the Carthage College campus, and at the mouth of the river where it meets Lake Michigan. All samples will be collected in 2.5 liter Fisher Brand amber glass jugs and stored at 4°C until filtration.

        Filtration

        Several different filtration methods will be used to determine which has the best recovery of our analytes. These methods include filter paper filtration, glass wool filtration, filter paper then glass wool filtration, and glass wool then paper filtration. Each of these methods will be compared to an unfiltered spiked sample to determine the best possible percent recovery. This will be done by creating a spiked water sample of 35ng/µL. Once the most efficient filtration method is determined, this will be the method used to filter water samples.

        Solid Phase Extraction (SPE)

        After consulting the Supelco *Guide to Solid Phase Extraction*, we determined that Reversed Phase SPE was the best method to use because reversed phase SPE utilizes a nonpolar stationary phase, a polar liquid phase, and mid to nonpolar analytes. We used a 60mL ENVI-18 Sorbent tube from Supelco to separate our analytes. The tube was conditioned with 30mL of ethyl acetate and 30mL of methanol, followed by 60mL of deionized water. After conditioning, we added the sample to the sorbent tube and vacuumed it through at a rate of 5mL/min. Next, we dried the tube under a vacuum to remove any excess water before elution. The compounds of interest were eluted with 2 portions of 30mL of methanol at a flow rate of 5mL/min. We collected the elution in a 250mL side arm flask. Then, we dried down the eluate compound using a rotovacuum to about 5mL and then it was transferred into a 5mL conical vial wrapped in foil. Drying was continued using a clean nitrogen stream until only 2mL remained in the conical vial. Next, we added 10µL of 10mg/L cholesteryl acetate to the vial as an internal standard and drying was continued to complete dryness.

        Derivatization

        Derivatization methods were obtained from Zhang et al. 2006. After the sample was dried, we derivatized it by adding 50µL of bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA (1% TMCS)) and 50µL of pyridine. Then, mixture was heated for 30 minutes at 60°C and then allowed to cool to room temperature. Next, 100µL of hexane was added to the sample to act as the final solvent and improve the sensitivity of the method.

        Gas Chromatography Mass Spectrometry (GC/MS)

        Methods for the GC/MS were obtained from Ma et al. 2003. A Finnigan Mat GCQ with a 15m X .25mm column was used. This column had a 0.25 µm film thickness and 5% equivalent polysilphenylene siloxane material within the column. The Xcalibur software program was then used to analyze different samples. We used the splitless injection mode on the GCQ and the temperature at the injector port was set to 280°C. The initial oven temperature was set at 90°C with a hold time of 0.5 minutes. Then, the oven was heated to 160°C at a rate of 15°C/min with

no hold time before increasing to the final temperature of 280°C at a rate of 25°C/min with a hold time of 5.0 minutes. Then, the pressure was set at a velocity of 60-cm/sec. The transfer line was set to have a temperature of 300°C. A 4 minute delay was set and the scanning parameters were set between 50 and 500 m/z. We used a 1μL injection for all injections into the GC/MS.

Preliminary data concerning the calibration curves were tested using an HP GC/MS from Lawrence University in Appleton, WI and a Griffin Analytical 400 GC/MS from Beloit College in Beloit, WI.

Calibration Curve

A calibration curve was created for each sample using several different concentrations well above and below the expected concentrations. The concentrations used were 0.5ng/μL, 1.0ng/μL, 5.0ng/μL, 10.0ng/μL, 25.0ng/μL, 50.0ng/μL, 75.0ng/μL, 100.0ng/μL and 150.0ng/μL. Each of these samples were run in triplicate to create precise calibration curves which will be used to determine percent recovery. Later, a fresh calibration curve will be used for comparison of collected samples.

Results

Derivatization

Upon derivatizing our estrogenic compounds, we expected the polar OH groups to be removed and replaced with nonpolar silyl groups. We expected that either one or two OH groups would be replaced from 17 α ethynylestradiol, creating a mono or di substitution. 17 β estradiol however, only has a single OH therefore; we expect this OH group to be replaced. According to analysis of our m/z ratio graphs, 17 α ethynylestradiol and 17 β estradiol did not derivatize. We followed the derivatization steps from Zhang and colleagues (2006) and used Figure 1 below from their paper to compare with our samples because Figure 1 shows two different m/z (mass to charge) ratio graphs representing the mono and di substituted forms of 17 α ethynylestradiol derivatization. The major m/z ratios can be seen at 73, 285, 368, and 425.

When we attempted to derivatize our sample following Zhang's methods, our results did not show derivatization of 17 α ethynylestradiol as can be seen in Figure 2 which shows a m/z ratio graph of 100ng/μL of 17 α ethynylestradiol that was obtained during out time at Beloit College. The underivatized 17 α ethynylestradiol has m/z ratios of 296, 228, 213, 172, 160, and 159. These ratios were confirmed as underivatized using the Spectral Database for Organic Compounds.

When we compared the two figures, we saw that Figure 2 shows underivatized 17 α ethynylestradiol.

Figure 1: Derivatized 17 α ethynylestradiol
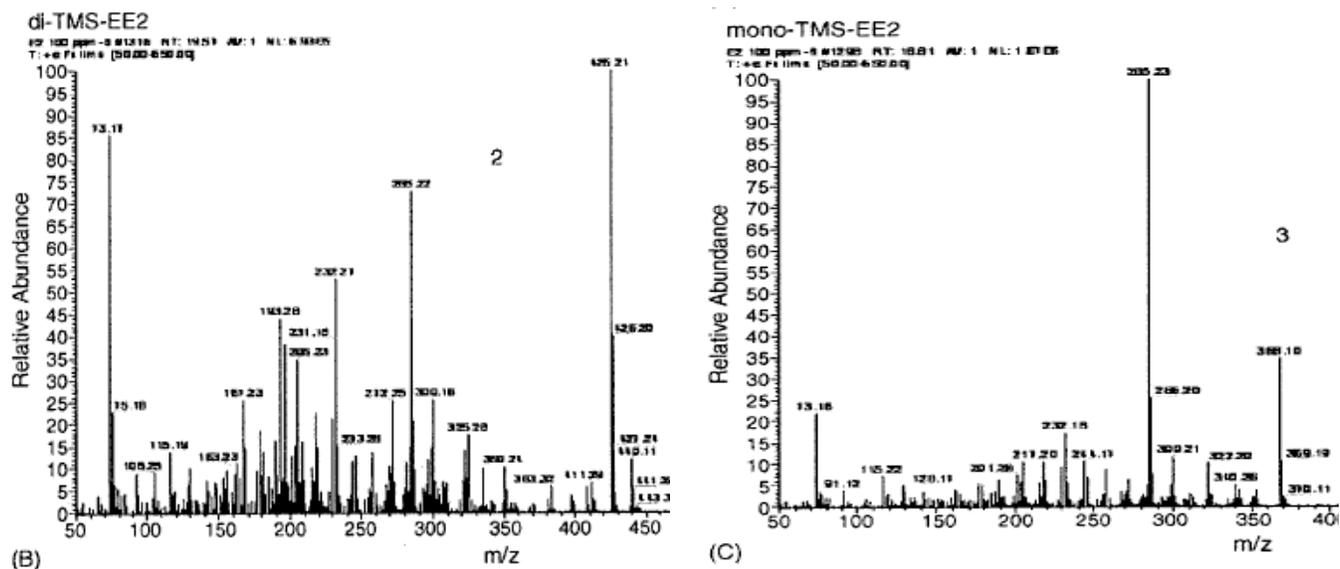
Zhang et al. (2006). Optimization of derivatisation for the analysis of estrogenic compounds in water by solid-phase extraction gas chromatography—mass spectrometry.Analytica Chimica Acta, 577, 57.

Figure 2: Underivatized 100ng/µL 17 α ethynylestradiol
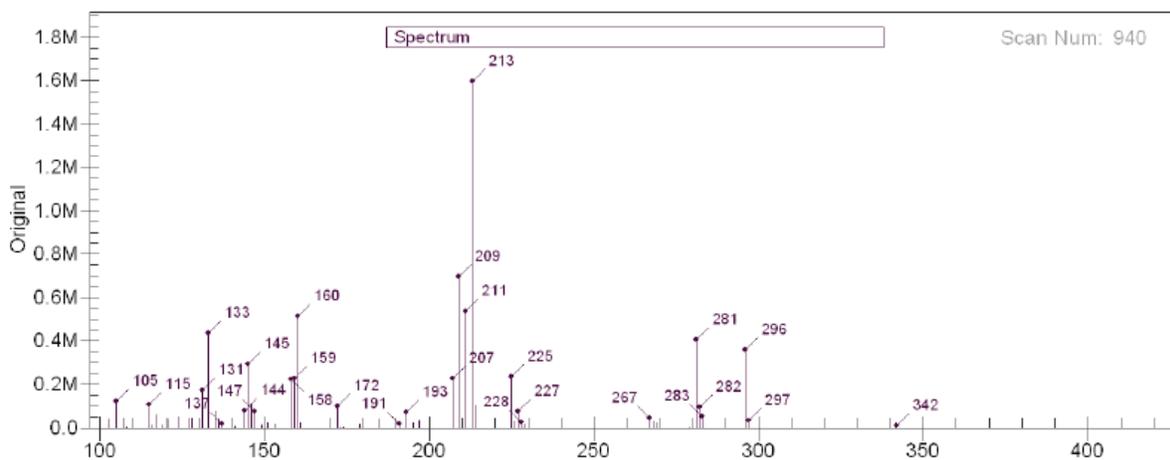
We then decided to adjust our methods and use bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylcholorsoilane (BSTFA (1% TMCS)) only this time, leaving out the pyridine. These results showed, that both 17 α ethynylestradiol and 17 β estradiol partially derivatized. Figure 3 below shows the partially derivatized 17 α ethynylestradiol and when we compared it to Figure 1 from Zhang and the underivatized Figure 2, we saw that some of the 17 α ethynylestradiol derivatized.

Figure 3: Derivatized 500ng/µL 17 α ethynylestradiol
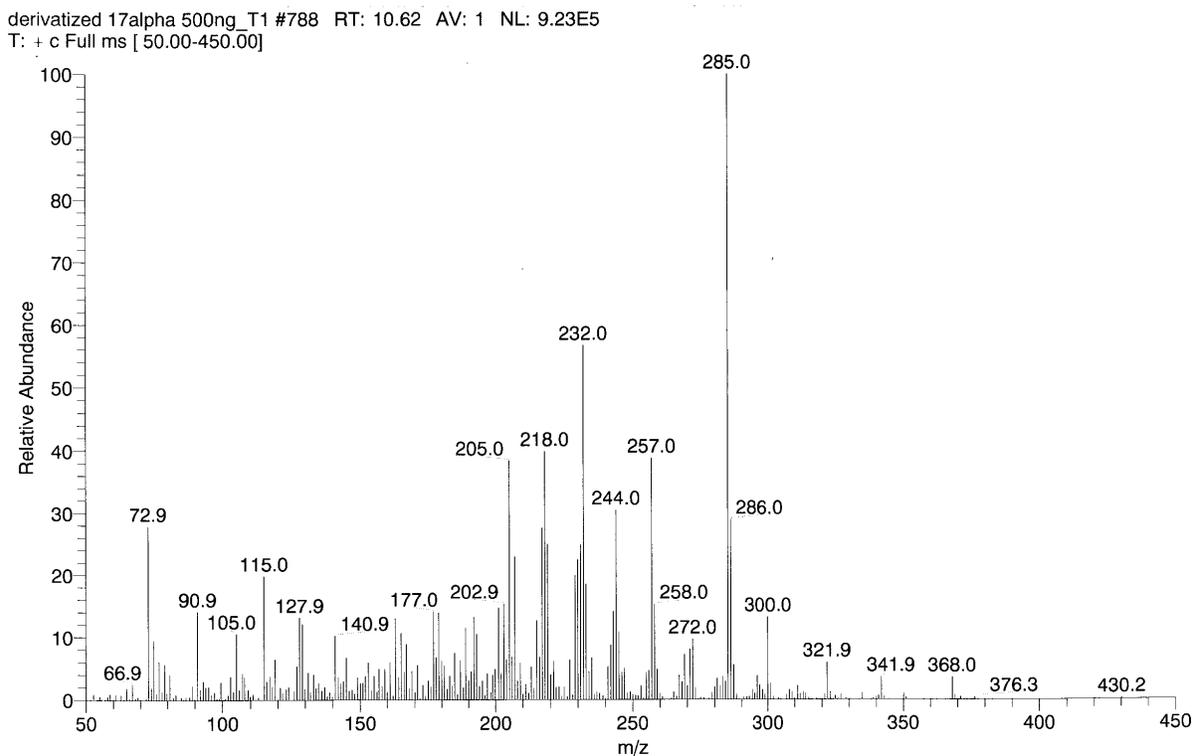
Internal Standard

We expected cholesteryl acetate to be a good internal standard as its structure is similar enough to 17 α ethynylestradiol and 17 β estradiol. 17 α ethynylestradiol and 17 β estradiol have a steroid backbone, which has a benzene ring at carbon 1, cholesteryl acetate however, does not have a benzene ring at carbon 1 but rather a phenyl group. Also, cholesteryl acetate has a carbon carbon double bond between carbons 5 and 6, and 17 α ethynylestradiol and 17 β estradiol do not. Based on these structural differences, we expected the internal standard to have a different retention time than 17 α ethynylestradiol and 17 β estradiol. However, our results showed that, cholesteryl acetate, 17 α ethynylestradiol and 17 β estradiol had similar retention times; therefore, causing peak overlap. Figures 4 and 5 below show two m/z ratio graphs obtained at Lawrence University and Beloit College where the cholesteryl acetate overlapped both the 17 α ethynylestradiol and 17 β estradiol peaks.

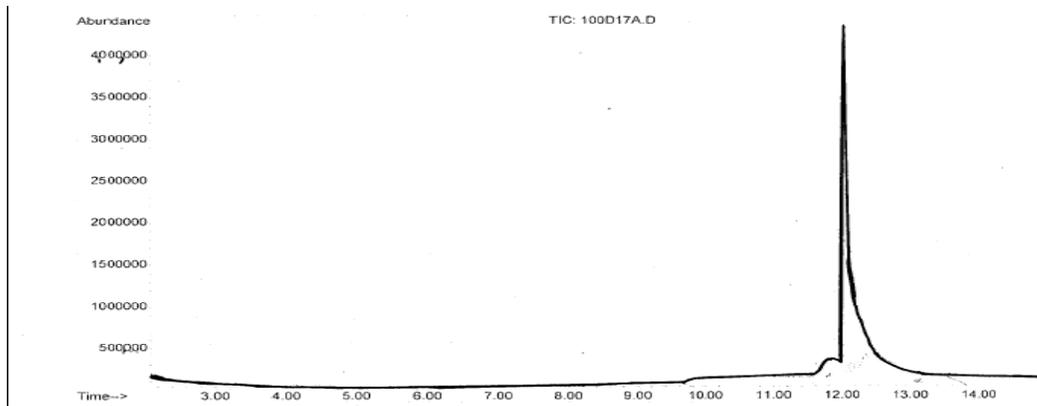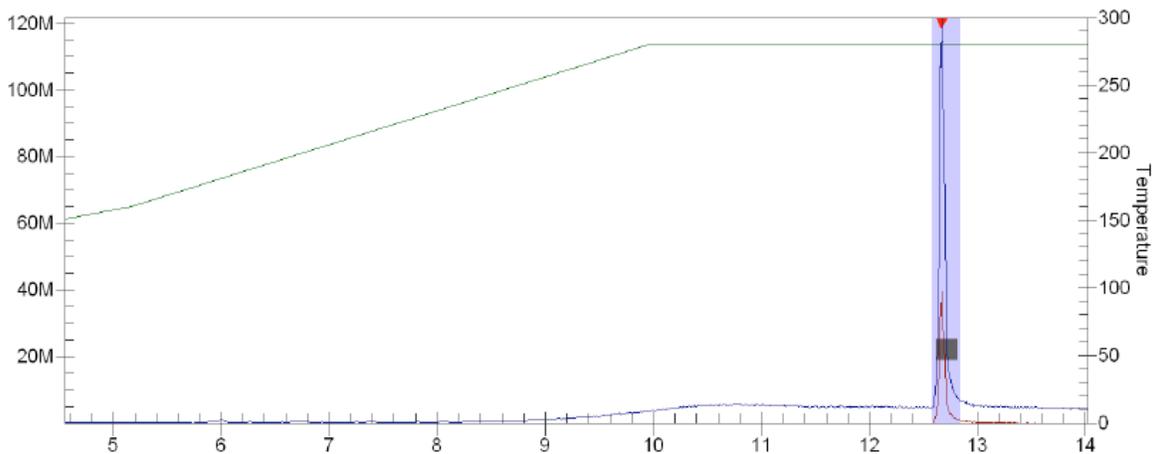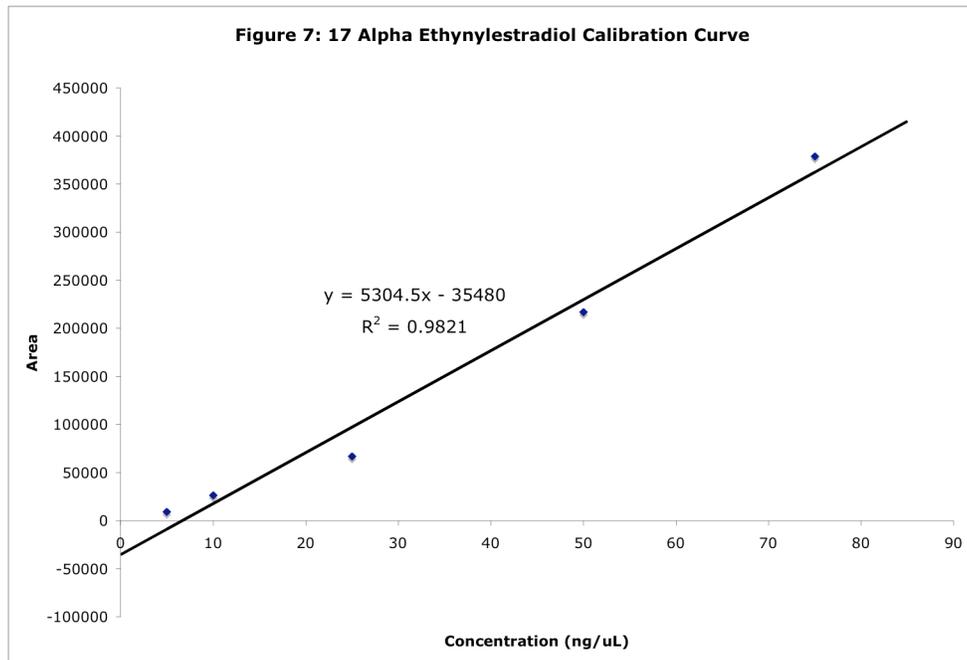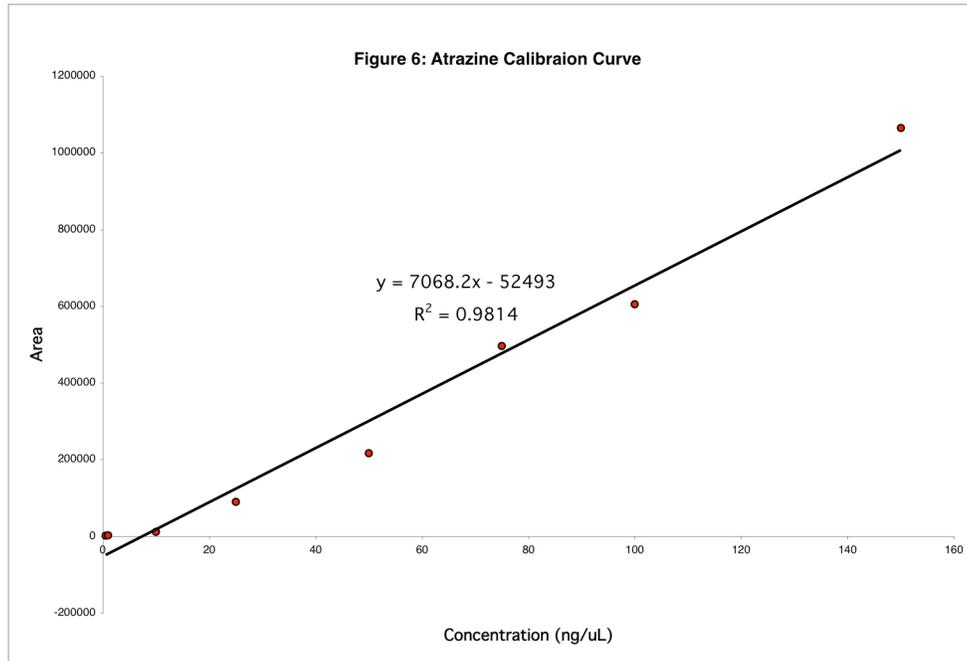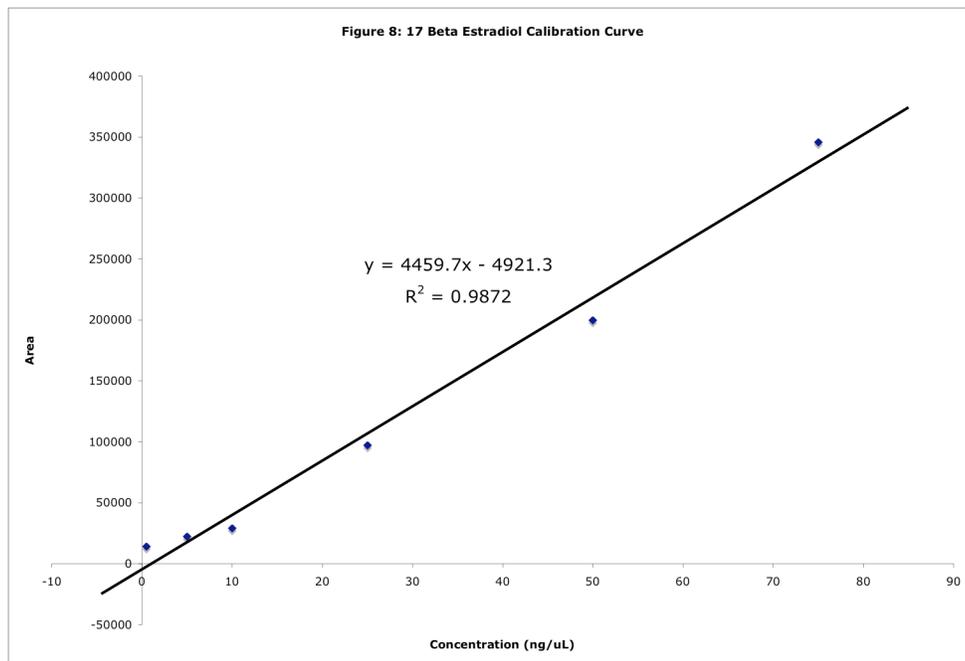Figure 4: 100ng/μL 17 α Ethynylestradiol and Cholesteryl Acetate

Figure 5: 500ng/μL 17 β Estradiol with Cholesteryl Acetate

Calibration Curve

In a calibration curve we expect an R^2 value above 0.98 for each of our EDCs of interest. The calibration curves we obtained for atrazine (Figure 6), 17 α ethynylestradiol (Figure 7) and 17 β estradiol (Figure 8) each portray R^2 values above 0.98. The atrazine curve shown in Figure 6 has a R^2 value of 0.9814, the 17 α ethynylestradiol curve shown in figure 7 has a R^2 value of 0.9821, and the 17 β eastradiol curve shown in figure 8 has R^2 a value of 0.9872.

Figure 6: Atrazine Calibraion Curve

$y = 7068.2x - 52493$
$R^2 = 0.9814$

Area

Concentration (ng/uL)

Figure 7: 17 Alpha Ethynylestradiol Calibration Curve

$y = 5304.5x - 35480$
$R^2 = 0.9821$

Area

Concentration (ng/uL)

Figure 8: 17 Beta Estradiol Calibration Curve

$y = 4459.7x - 4921.3$

$R^2 = 0.9872$

Discussion

This summer we attempted to create an effective method for our experiment and to determine the concentrations of our three EDCs. We found however, that our methods took many hours to develop and refine. So far, many of our initial procedures have proven to be successful. Our preliminary results showed 17 α ethynylestradiol and 17 β estradiol did not derivatize when we followed the methods set forth by Zhang et al. 2006. Upon removing the pyridine from the method we did obtain some derivatization of both estrogens. It's possible that our pyridine was no longer pure because of repeated use and may have accumulated moisture, therefore hindering the derivatization process. However, we are still seeking complete derivatization and further ways to enhance our methods. In the future, we plan to use pure and moisture free pyridine along with fresh BSTFA, which should allow us to completely derivatize both estrogenic compounds.

Also, our results showed that the internal standard peak, cholesteryl acetate, overlapped with 17α ethynylestradiol and the 17 β estradiol peak in the GC spectrum. Their similar retention times made it difficult to determine the peak areas for the internal standard, 17α ethynylestradiol and 17 β estradiol. This overlap could be due to the fact that these compounds are too chemically similar. From this data, we determined that a new internal standard needed to be found. However, due to budget we were unable to use deuterated standards, as most other researchers have done. Some alternate internal standards are cholesteryl-n-butyrate, 17 β estradiol-17-acetate, and 3-*O*-methylestrone. Cholestryl-n-butyrate was used as an internal standard in both Lopez de Alda and Barcelo research in 2001 and Kuch and Ballschmiter's work in 1999. While cholesteryl acetate and cholesteryl-n-butyrate are almost structurally identical they may be different enough to separate at different times, decreasing our chances of overlap with our estrogens. 17 β estradiol-17-acetate is another alternative that is structurally similar to 17 β

estradiol with an additional acetate group. This internal standard has been used both by Ternes and colleagues (1999) and in Lopez de Alda and Barcelo's work in 2001 both of whom were studying various estrogenic compounds for later analytical quantification. 3-*O*-methylestrone was used as the internal standard in Velicu and Suri's (2008) research of steroid hormones in agricultural, suburban, and mixed-use areas. We believe that, 3-*O*-methylestrone would make a good internal standard because of its steroid backbone. However, unlike our estrogens, the oxygen attached to the third carbon has an additional methyl group rather then a hydrogen. While this compound may be similar to our estrogens of interest, we believe that it is distinctive enough to have separate peaks as witnessed by other studies.

As seen from Figures 6, 7, and 8 the calibration curves made for atrazine, 17 α ethynylestradiol, and 17 β estradiol have R^2 values of 0.9814, 0.9821, and 0.9872 respectively. This means that over 98% of the variation in the data can be attributed to the linear relationship between the calibration standard concentrations and the GC/MS quantification. This strong R^2 value will allow us to determine the concentration of atrazine, 17 α ethynylestradiol, and 17 β estradiol in the samples we obtain from the Pike River. Based on the results from the calibration curves, it is also significant to point out that the Finnigan GCQ being used has a sensitivity level that is able to detect concentrations of atrazine, 17 α ethynylestradiol, and 17 β estradiol within ranges detected by previous research. Using the protocol we have designed, we expect to be able to detect concentrations higher then 0.5 ppt. For example, in 2008 Suri and Velicu determined the concentration of 17 β estradiol in streams in Chester County, PA. Concentration averages ranged from 0.09-5.0 ppt. Also, in 1999, Ternes and colleagues tested the concentration of both 17 α ethynylestradiol and 17 β estradiol in Canadian rivers and found concentrations of 9 ppt for 17 α ethynylestradiol and 6 ppt for 17 β estradiol. In 2003 Ma and coworkers determined that the average atrazine concentrations in two Hong Kong rivers were 3.9 and 9.7 ppt. Regardless of the fact that these concentrations vary by area, each value could have been determined by our GC/MS. This gives our team the confidence that we will be able to detect applicable concentrations of atrazine 17 α ethynylestradiol, and 17 ß estradiol in the Pike River.

Reflections

Thus far into the experiment, our research group has discovered that there are many different procedures to analyze endocrine disrupting chemicals. These methods have many steps and thus, many areas to improve upon in the future.

We hope to improve our methods in order to fix the problems that we have found so far. We will contact other researchers who have used similar derivatization methods and ensure our derivatizing reagents are pure and moisture free in order to find a way to fully derivatize the estrogens. Also, we hope to find a new internal standard to use that does not have overlap with the chemicals we are testing. Another goal we have for our research is to determine the best filtration method for water samples by experimentally finding which method gives the highest percent recovery when spiked samples are filtered. Finally, using our revised methods we hope

to collect water samples from our chosen sample sites along the Pike River and to determine the concentrations of atrazine, 17 α ethynylestradiol, and 17 β estradiol.

From this experiment our research group has learned how to understand and analyze research articles which in turn taught us to formulate our own protocols to be used in lab. Another aspect of research we learned about was how to adapt to varying setbacks in lab. Finally, we learned how to troubleshoot through our problems whether by trial and error or by collaborating with other researchers.

While the Summer Undergraduate Research Experience went very quickly, it was an invaluable event. SURE allowed us to experience an aspect of science not often encountered until graduate school and provided us with knowledge and know-how for the future. Over the summer our research has left many unanswered questions and it is for these reasons that our team plans to continue on into the school year to finally determine the concentrations of these three endocrine disrupting compounds in the Pike River watershed.

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