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# EXPLORING THE USE OF PIOX SYSTEM FOR TREATMENT OF ENDOCRINE DISRUPTING COMPOUNDS

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# EXPLORING THE USE OF PIOX SYSTEM FOR TREATMENT OF ENDOCRINE DISRUPTING COMPOUNDS

by

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Abstract

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In this thesis, the Photon Initiated Oxidation (PIOx) system was investigated for its possible use in the treatment of Endocrine Disrupting Compounds (EDCs). It was theorized that the unique design of the system, namely the multiple wavelengths of UV light and the micronfoam would improve removal rates of the test contaminant when compared to other systems. 17β-estradiol was chosen as the test contaminant due to the compounds known sensitivity to ozone exposure. Two methods of testing were used, the first to test an idealized homogenous concentration (method one) and the second to mimic the sudden influx of contaminant that would be common in a water and wastewater treatment environment (method two). Method one showed degradation to below level of detection within 15 minutes. Method two showed a reduction trend but with 60-80% eliminated by the end of 15 minutes. While the PIOx system did not perform above expectations, its simplicity and compact form-factor allows the system to be applicable in a significant number of situations where other AOPs would be too expensive or complex to implement. Continued research is required into the PIOx systems capabilities and its application to low budget and rural environments.

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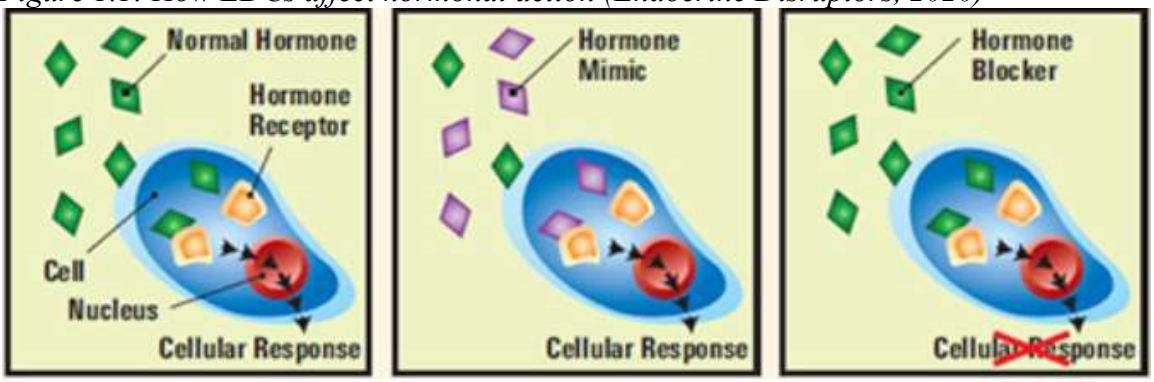
Chapter 1

Introduction and General Information

**Overview**

Over the last several decades, mankind has made use of many natural and synthetic compounds as a part of human industry, agriculture, and general activity. Of the tens of thousands of chemical compounds and products, approximately 1000 are recognized and studied as possible endocrine disrupting chemicals (EDCs). An EDC can be more rigorously defined as "an exogenous chemical, or mixture of chemicals, that can interfere with any aspect of hormone action" (Zoeller et al., 2012, p. 4107).

*Figure 1.1: How EDCs affect hormonal action (Endocrine Disruptors, 2020)*




When absorbed in the body, an endocrine disruptor can decrease or increase normal hormone levels (left), mimic the body's natural hormones (middle), or alter the natural production of hormones (right).

They can interfere with normal hormonal actions in the body in two general ways as shown in Figure 1.1, either by changing the production of hormones either positively or negatively, by mimicking the structure of hormones so it activates the same receptors as the body's natural hormones, or by blocking the receptors and preventing natural

processes. Because of their ability to affect such a key part of the body's chemistry, many of these chemicals have the capacity for doing harm to both humans and wildlife. Even though immediate harm is unlikely at current ecological concentrations, concentrations in the parts per billion (ppb) to parts per trillion (ppt) range, organisms that are exposed at vulnerable time periods such as during or just after pregnancy, or during infancy are at risk of significant illnesses, deformities, and future reproductive problems (Street et al., 2018, p. 1647) .

**History**

EDCs, which are also referred to as hormonally active agents, endocrine active substances, chemicals of emerging concern, or micropollutants, are a growing matter of concern for a wide swath of the scientific field. They have been found to be a consistent part of our environment due to many human activities, which includes agricultural, industrial, and municipal sources. This constant background exposure has been identified within the last several decades due to the effects EDCs were having on the wildlife in heavily impacted ecosystems and from cross disciplinary discoveries.

During the 1960s and 1970s when the public and the scientific community were first starting to realize the possible ramifications of manufactured chemicals on the environment, a number of ecological and environmental studies were being carried out by independent researchers. Several worrying patterns were emerging. In the North American Great Lakes, fish, reptiles, and birds were emerging with reproductive and developmental abnormalities including thinned egg shells, increased number of in-egg

deaths, hermaphroditic characteristics (i.e. displaying both masculine and feminine sexual organs), increasing number of abnormal, malfunctioning, or disable genitalia leading to population declines (Street et al., 2018, p. 1647). Studies in Florida showed similar reproductive deformities in alligators and turtles, leading to similar die-offs. Meanwhile, in England, fish were studied that showed similar reproductive anomalies: testes holding eggs and males displaying an egg protein formed due to presence of estrogen (Schug et al., 2016, p. 844). It was clear that these were not isolated incidents but symptoms of a growing global problem.

In the mid-90s, the United States Environmental Protection Agency (US EPA) conducted 2 international meetings to gather people together for information sharing and to determine research needs. Due to this meeting and several others like it in Europe, many regulations and policies were put in to place to limit EDC exposure. In America, the EPA was directed to assess the hormonal impact of more than 70,000 known compounds which lead to developing a 3-tiered endocrine disruptor screening program testing the estrogen, androgen, and thyroid axis (Schug et al., 2016, p. 844). This program is continuing to grow and be used to this day. In 1968 Japan, a large amount of oil was contaminated with polychlorinated biphenyls (PCBs), a group of industrial compounds used as plasticizers, pigments, and in electrical insulation and heat transfer, leading to thousands of sick people. Later research showed that the exposure had longer term consequences with women that were exposed being more likely to have children with low birth weight and slower neurological development. Due to these events, Japan became

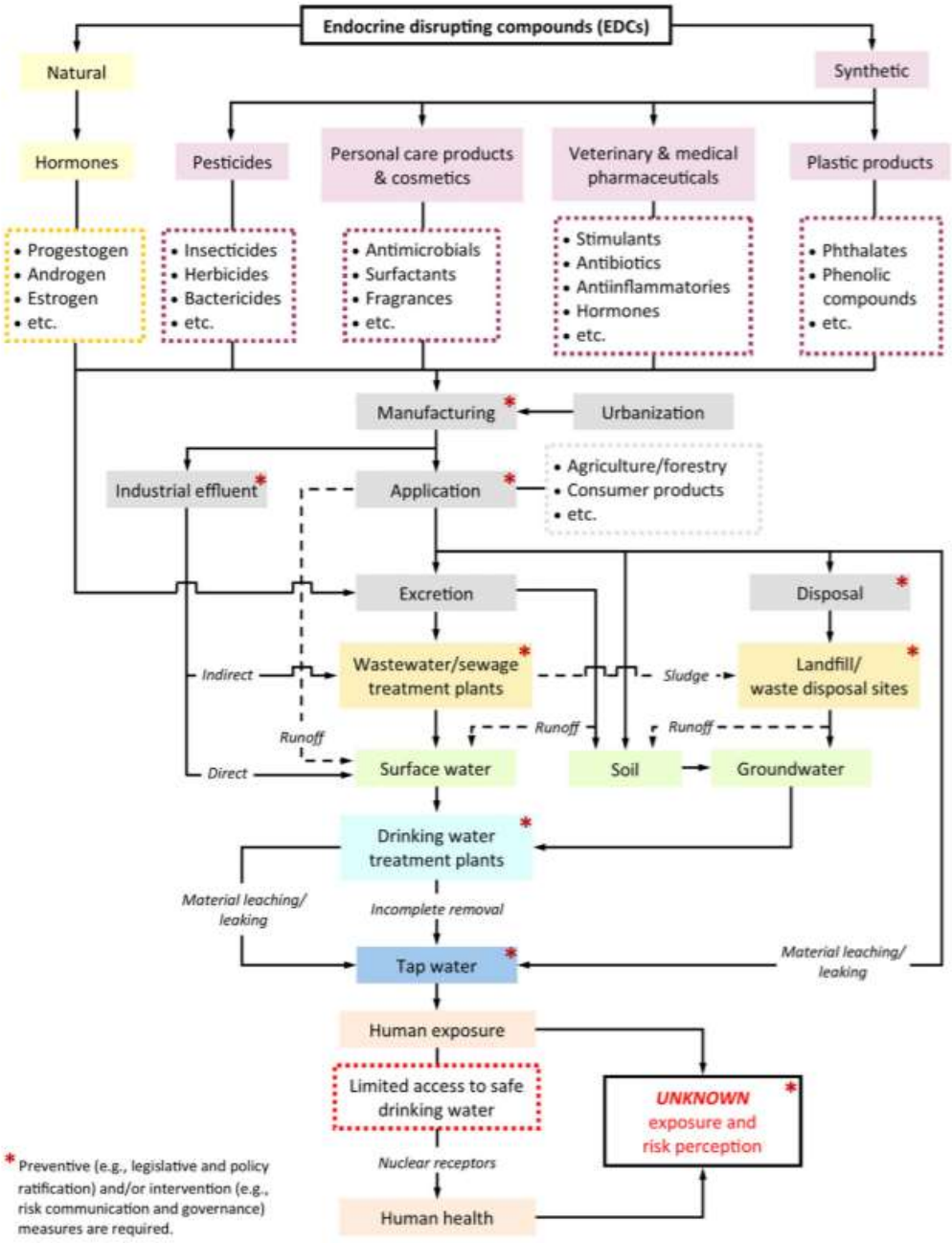
one of the first nations to have a national response to the EDC problem by starting initiatives focused on environmental monitoring and sponsoring several more International meetings discussing EDCs from 1998 to 2007 (Schug et al., 2016, p. 844). In Europe, multiple workshops on the impact of endocrine disruptors on human health and wildlife led to a substantial increase in research funding by the European Union into the effects of EDCs on wildlife and humans as well as their mechanisms of action. These actions culminated in a report in 2012 summarizing the last 15 years of research which reinforces the seriousness of the situation by calling endocrine disruptors "a real phenomenon likely affecting both human and wildlife populations globally."

**Exposure, Standard Treatment, & Risk**

As the international community becomes more cognizant of the effects EDCs have on the environment, monitoring of many EDCs have become more widespread despite how low the environmental concentrations are. While they can be released into the atmosphere via combustion, the primary areas of accumulation are in ground water, rivers, and lakes, all key sources for drinking water (Auriol et al., 2006, p. 538). Drinking water supplies across the world have been observed with EDC concentrations from 0.2 ng/L to 5510 ng/L (Lee et al., 2016, p. 184; Rosa Boleda et al., 2011, p. 1605; Wee & Aris, 2017, p. 224; Yang et al., 2014, p. 57). The mechanisms for contamination are the same for EDCs as they are for any other kind of contaminant, through storm water runoff sweeping chemicals into the water stream, leeching into the ground water, or by just being sent to the wastewater treatment plant. The difference is this contamination can come from a

variety of sources as EDCs are used in a large number of products, like plastics, cosmetics, pharmaceuticals, pesticides, and even just natural hormones.

*Figure 1.2: Means of exposure to EDCs in drinking water (Wee & Aris, 2019, p. 1)*



For mature adult humans the damage in the short term is negligible but that doesn't mean there aren't affects. EDCs have been observed in bodily fluids like breast milk, blood, sweat, and urine (Jönsson et al., 2014, p. 5). Over an entire lifetime, a person will be exposed to a pharmaceutical level of exposure through consumption of drinking water, which equates to <10% of a daily medical dose (Houtman et al., 2014, p. 55). Infants, who are significantly more susceptible to the effects of EDCs, have a significantly higher average level of exposure, 1340 ng/day compared to an adults 148 ng/day, of bisphenol A (BPA), an EDC that leaches out of the plastic baby bottles (Leung et al., 2013, p. 845).

One of the possible reasons for the somewhat sluggish regulatory response to EDCs is the difficulty in accurately assessing the risk involved in their exposure. Risk assessment (RA) is a process for identifying risk factors related to a potentially hazardous action or situation, evaluate the risk associated with the hazard, and finally determine means to either eliminate the hazard or minimize the risk associated with the hazard (*OSH Answers Fact Sheets*, 2020). The previously mentioned data point illustrates two of the problems that have made RA for EDCs difficult. It shows the difficulty in assessing possible exposure, as there is consistent background exposure due to EDCs suffusing the environment and that slight changes to the situation can wildly change the level of exposure. Other issues that have gotten in the way of assessing exposure include the importance of timing, which can drastically change the effect of EDC exposure, and the impact of transgenerational effects from multiple generations of EDC exposure (Futran Fuhrman et al., 2015, p. 607). There is lack of data surrounding chronic exposure in

humans in addition to a steadily growing list of compounds that have been labeled an EDC and need to be researched. In addition, there is little research into the effect of simultaneous exposure to several different EDCs which may have hormonal actions that interfere with each other in unpredictable manners. Finally, the lack of a unified definition as to what an EDC even is makes discussion of the hazards associated with them difficult. While the definition of "exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior," seems to be an effective working answer for what an EDC is, it fails to address the "quality of interference" by omitting reference to any adverse effects. This leads to EDC being a larger more blanket term that covers more edge cases but also makes the situation vague when decisiveness is needed. Overall, the emergence of EDCs has caused RA to have to adapt due to their broad scope and defiance of standard assessment methods.

The problem is that current treatment practices, both for drinking water and wastewater, are not capable of completely removing EDCs from the water stream as shown in Table 1.1. While the removal efficacy is high for all the measure compounds in Table 1.1, the concentrations that remain are still within the range shown to have negative health effects and endocrine disruption in aquatic organisms (Auriol et al., 2006, p. 532). In addition, there really is no consensus on the mechanism of removal for many of the compounds in

the case of standard treatment. This makes improving the existing system to deal with the remaining contaminants difficult if not impossible.

Table 1.1: EDC concentrations and removal efficiency for standard treatment processes

| **Compound** | **Concentration** | | **Removal efficacy (%)** | **Treatment process** | **Matrice type** |
|---|---|---|---|---|---|
| | **Influent** | **Effluent** | | | |
| 17β-Estradiol | 5 ng/L | <1 ng/L | >80 | 1 | Municipal waste landfill |
| | 11 ng/L | 1.6 ng/L | 86 | 2 | Municipal STP |
| | 9.69 ng/L | 4 ng/L | 59 | 2 | Domestic STP |
| | 28.1 ng/L | 1.2 ng/L | 96 | 2 | Domestic STP |
| | – | – | 100 | 2 | Municipal STP |
| Estrone | 44 ng/L | 17 ng/L | 61 | 2 | Municipal STP |
| | 31 ng/L | 24 ng/L | 23 | 2 | Domestic STP |
| | 43.1 ng/L | 12.3 ng/L | 69 | 2 | Domestic STP |
| | – | – | 83 | 2 | Municipal STP |
| Estriol | 72 ng/L | 2.3 ng/L | 97 | 2 | Municipal STP |
| | 57.29 ng/L | 11.71 ng/L | 80 | 2 | Domestic STP |
| | 381.5 ng/L | 5.6 ng/L | 99 | 2 | Domestic STP |
| 17α-Ethinylestradiol | 4.84 ng/L | 1.40 ng/L | 71 | 2 | Domestic STP |
| | – | – | 78 | 2 | Municipal STP |
| Phenol | 6 mg/L | No detected | – | 3 | Municipal + tannery industry STP |
| Nitrophenol | 11 mg/L | No detected | – | 3 | Municipal + tannery industry STP |
| 2,4-Dichlorophenol | 83 mg/L | 16 mg/L | 81 | 3 | Municipal + tannery industry STP |
| NP1EO | 140.03 mg/L | 1.99 mg/L | 99 | 4 | Industrial + domestic STP |
| NP2EO | 140.03 mg/L | 1.99 mg/L | 99 | 4 | Industrial + domestic STP |

| Compound | Concentration | | Removal efficacy (%) | Treatment process | Matrice type |
|---|---|---|---|---|---|
| | Influent | Effluent | | | |
| NP | 2.8 mg/L | <0.05 mg/L | >98 | 1 | Municipal waste landfill |
| | 1.5 mg/L | 6.6 mg/L | – | 3 | Municipal + tannery industry STP |
| | 57.64 mg/L | 0.65 mg/L | 99 | 4 | Industrial + domestic STP |
| | 10 mg/L | 1 mg/L | 90 | 2 | Domestic STP |
| | 73 mg/L | 47.5 mg/L | 35 | 5 | Industrial STP |
| 4-NP | 2.37 mg/L | 0.95 mg/L | 60 | 6 | Municipal STP |
| 4-*t*-OP | 0.88 mg/L | 0.32 mg/L | 64 | 6 | Municipal STP |
| PCBs | 46 ng/L | 1.2 ng/L | 97 | 1 | Municipal waste landfill |
| BPA | 0.13 mg/L | <0.005 mg/L | >96 | 1 | Municipal waste landfill |
| | 7.1 mg/L | No detected | – | 3 | Municipal + tannery industry STP |
| | 2.5 mg/L | No detected | – | 3 | Municipal STP |
| | 1.776 mg/L | 0.210 mg/L | 88 | 6 | Municipal STP |
| | 0.55 mg/L | 0.14 mg/L | 75 | 2 | Domestic STP |
| PCDD | 21 pg/L | 5.2 pg/L | 75 | 1 | Municipal waste landfill |
| PCDF | 8.7 pg/L | 3.3 pg/L | 62 | 1 | Municipal waste landfill |

**Health Effects in Animals**

As technology advanced more EDCs were identified and animal laboratory studies began to be conducted. These studies on EDC exposure in mice and rats showed exposure, even at extremely low concentrations, had noticeable effects, both physical and behavioral (Street et al., 2018, p. 1647). These behavioral changes include increased anxiety like

behaviors, impairment of spatial learning and memory, and reduction of maternal behavior. In addition, there appeared to be an epigenetic aspect to the morphological changes, a change in genome of an individual due to environmental factors that can be inherited. This transgenerational inheritance is a point of worry and concern in the scientific community as it seems to be supported by the studies on exposure in humans.

**Health Effects in Humans**

Studies into the effect general low-level EDC exposure has on human health is less certain due to the constant exposure from the environment, however there are still connections that can be drawn. EDCs have been connected to reproductive illnesses in humans, including testicular, breast, ovarian, cervical, and uterine cancers, and infertility (Mallozzi et al., 2017, p. 334; Prins, 2008, p. 653; Rachoń, 2015, p. 360). In addition, there is evidence that exposure to EDCs may lead to obesity, insulin resistance, attention deficit hyperactivity disorder (ADHD), autism and other neurodevelopmental diseases.

Although there is a sizable body of work connecting EDCs to illnesses, disorders, abnormalities, and other negative health effects, there is not a solid consensus on what health risk EDCs pose to humans. Most EDCs are not recognized as a treatment goal for water and wastewater treatments, though that is changing. Experimental solutions for removing or degrading the EDCs as a tertiary treatment option are being explored for water and wastewater treatment plants. There are a few general categories that these solutions fit into. They are removal by physical means, such as using carbon filters or membranes, by biodegradation, similar to an extension of current wastewater practices,

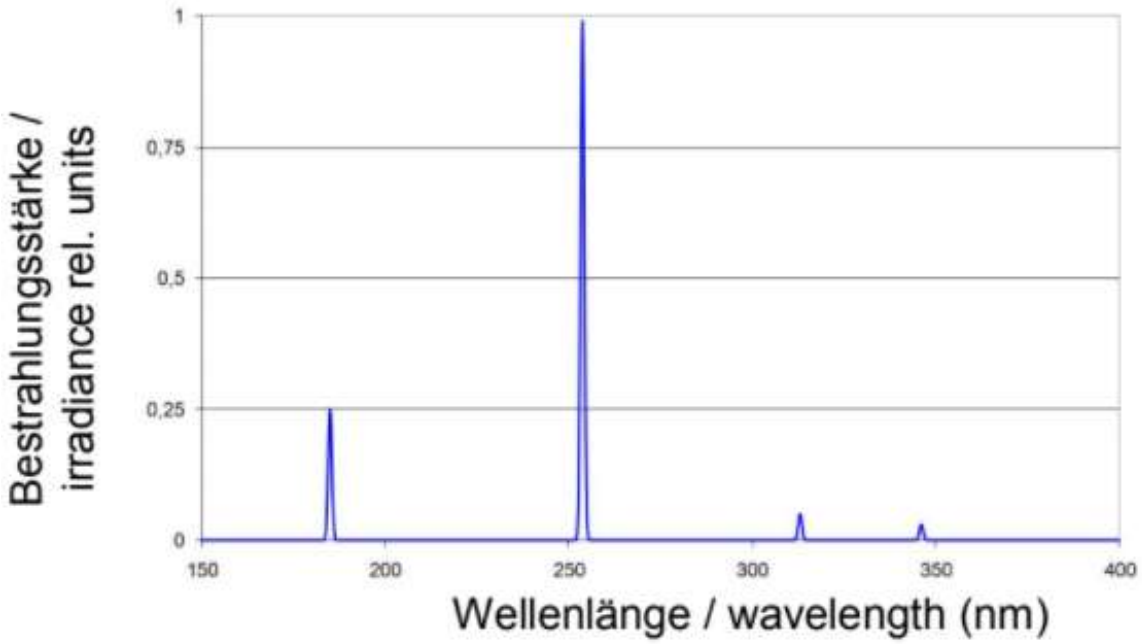
and by chemical advanced oxidation, which typically involves using an oxidizer and sometimes UV light to eliminate contaminants. This final category includes the Photon Initiated Oxidation (PIOx) system which is the focus of this paper.

**The PIOx System**

The PIOx system was originally put together as a means of sanitizing wash water for reuse in dairy plants and similar businesses. The system has several key features that it uses to accomplish this. It contains high intensity UV bulbs that primarily output at several wavelengths as shown in Figure 1.x to serve two functions, a wavelength of 185 nanometers to create ozone from the air and a wavelength of 254 nanometers to sterilize.

As it will be shown in detail in chapter 2, the addition of UV radiation to the ozone oxidation reaction increases the efficiency significantly. This introduces a dilemma of some consequence; where do you source the ozone from. UV light can form ozone by reacting with oxygen and water. However, UV bulbs that are most efficient for synthesizing ozone, which includes the bulbs used in the PIOx system, are the least capable of directly oxidizing organic compounds. They output at too narrow of a wavelength range to be widely effective against most organic compounds. Conversely, radiation sources of a sufficiently broad spectrum to be effective at oxidizing organic compounds makes ozone generation difficult.

*Figure 1.3: Wavelength distribution for PIOx UV lamps*

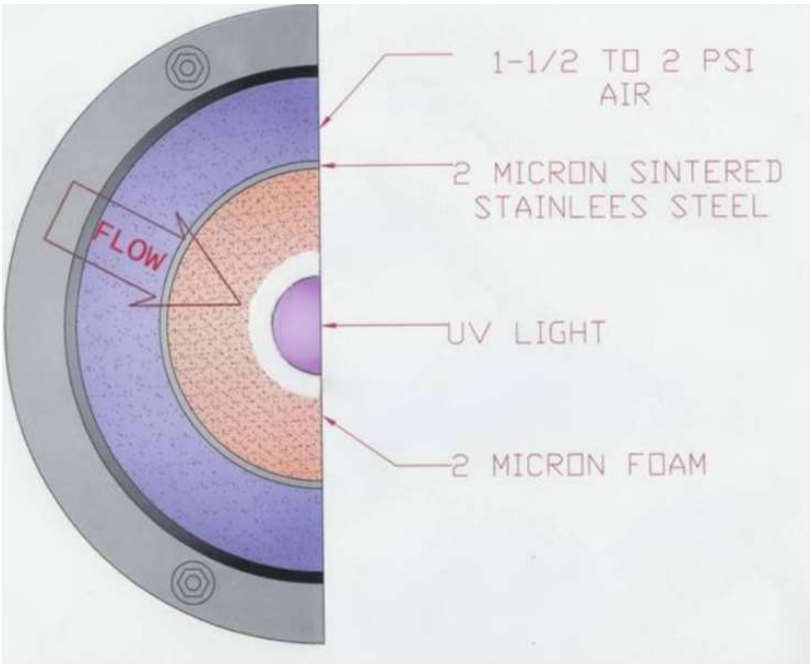



The bulbs used in this system are low pressure vacuum UV lamps with a lamp input of 100 W, with 9 W of radiation at 185 nm. Around the UV lamps is a metal sleeve drilled with 2 micrometer wide holes. The water is pumped past the lamps inside the metal sleeve while an air pump creates a pressure buildup of around 1-2 pounds per square inch (psi). This positive pressure creates a foam that increases surface area which would aid in increasing ozone contact in the water. The similarity of this system to current attempted solutions and the addition of a micron as a new variable is what lead to this exploration of the PIOx system.

The PIOx system used for this project was assembled by myself and an undergraduate who was performing experiments for a research project that ran parallel to my own. The frame and the tank had already been delivered to the university along with the bulbs and

the housing for the bulbs. In order to complete the structure, we had to obtain a water pump, an air pump, nonreactive tubing for the ozone, tubing for the water pump, an electrical panel box, conduit, and a high voltage electrical connection. Assembling the system took place over several months due to waiting for parts, troubleshooting electrical components and issues, and the unfortunate need to reorder the mercury bulbs after the apparatus was inadvertently moved, breaking the bulbs.

*Figure 1.4: Diagram of PIOx use of air pressure*

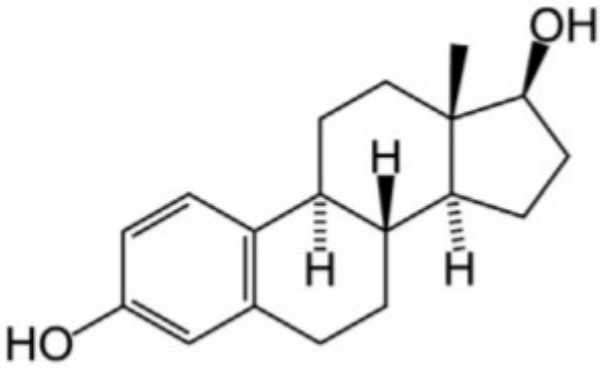



**17β-Estradiol**

The EDC that was chosen to be the target contaminant in this thesis is 17β-Estradiol, which is typically abbreviated as E2. 17β-Estradiol is a steroidal estrogen hormone that is produced mainly in the ovaries, in the placenta during pregnancy, and in very low concentrations in the testis. It is a key hormone in the regulation of the menstrual cycle and in the expression of secondary sex characteristics (Rexroad, 1977, p. 86). It also

impacts bone growth, brain development and maturation, and concentrations of calcium and some messenger molecules between cells. It is a part of the estrogen group of compounds, which includes estradiol, estrone, and estriol of which estradiol is the most concentrated and the most active (*The Serotonin Molecule*, 2006).

*Figure 1.5: Estradiol molecular structure*




Estradiol is also used for medical purposes including birth control and hormone replacement therapy (HRT) to treat symptoms of post-menopause. However, it has also been connected to an increased risk of stroke and breast cancer (Huang et al., 2007, p. 148) in addition to several lifestyle diseases such as hypertension, reproductive and metabolic disorders (Gore et al., 2015). Estradiol is also tentatively linked to several neurological disorders as well as several behavioral disorders (MohanKumar et al., 2018). Studies have also shown that exposure to estradiol during prepubescence can lead to excessive rapid growth, delayed puberty in males and early puberty in females (U.S. Department of Health and Human Services, 2007). These health risks have led to increasing interest in minimizing contact with estradiol and finding methods for eliminating it from water streams and other means of exposure.

Estradiol is quite stable due to its four linked carbon rings. Three of the rings are single bonded while the last ring shows phenolic properties. 17β-Estradiol bears structural similarity with the other estrogen molecules, all of which have the same carbon ring skeleton and the phenolic ring which is quite sensitive to ozone exposure.

In order to eliminate the estrogenic activity of estradiol, the carbon rings that make up the majority of its structure would need to be ruptured through chemical reactions which will be discussed further in later sections. If that could be accomplished, then the estrogenic impact could be reduced if not eliminated. This elimination of estrogenic activity is corroborated by research into the byproducts of E2 oxidation and their estrogenic toxicity (source). After exposing E2 to advanced oxidation processes, the byproducts were examined using a mass spectrometer to identify the individual steps of the degradation pathways. When those intermediate compounds were examined using various assays and simulations, the compounds whose phenolic group was broken showed little to no estrogenic activity while the those with intact phenolic groups still displayed toxic effects.

*Figure 1.6: Simplified pathway for photodegradation of E2*

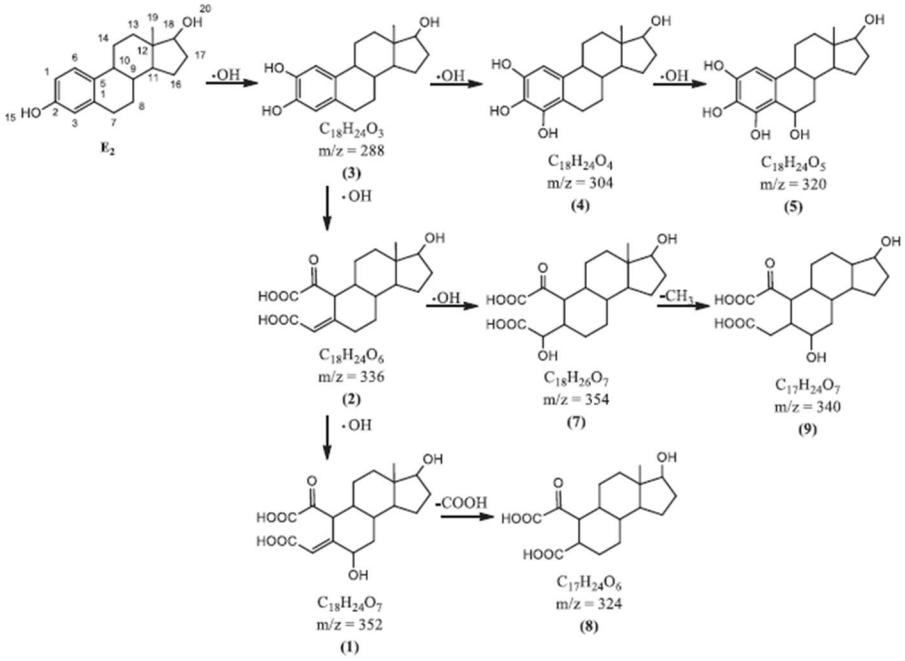



*Figure 1.7: predicted chronic and acute ecotoxicity of E2 and byproducts*

| Product | Fish 96h-$LC_{50}$ | *Daphnia* 48h-$LC_{50}$ | Green algae 96h-$EC_{50}$ | Fish ChV | *Daphnia* ChV | Green algae ChV | Reaction |
|---|---|---|---|---|---|---|---|
| 1 | | | | | | | $UV/O_3$ |
| 2 | | | | | | | $UV/O_3$ |
| 3 | | | | | | | All |
| 4 | | | | | | | $UV/TiO_2$ |
| 5 | | | | | | | $UV/O_3$ |
| 6 (E2) | | | | | | | All |
| 7 | | | | | | | $UV/H_2O_2$ |
| 8 | | | | | | | $UV/H_2O_2$ and $UV/O_3$ |
| 9 | | | | | | | All |

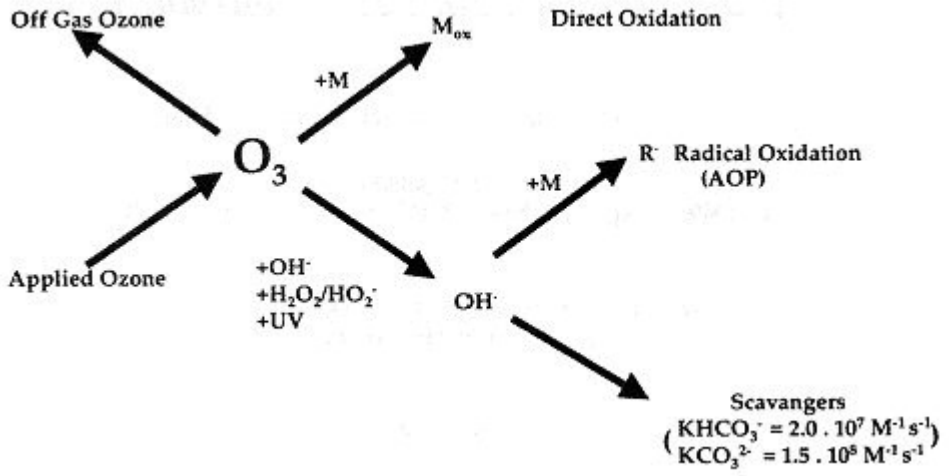
Very toxic
Toxic
Harmful
Not harmful

## Ozone Oxidation and Decomposition

Ozone exposure has two pathways for oxidizing organic compounds. The direct pathway, where the molecular ozone is what affects the contaminant compounds, is the main reaction in acidic conditions or when certain compounds inhibit the decomposition of

ozone. The radical pathway, so named by the hydroxyl radicals and other secondary oxidants that are produced by ozone decomposition, is the main reaction under basic conditions or when certain compounds promote ozone decomposition which increases the chain reaction.

*Figure 1.6: Ozone and dissolved solids reaction (*Ozone Reaction Mechanisms*, 2003)*




Research from Staehelin and Hoigne (1985, p. 1211) outlined a general model for these reaction pathways. In an aqueous ozonated solution, the ozone is either consumed reacting with a contaminant, become an ozonide ion radical ($\cdot O_3^-$) by electron transfer, or start decomposing. The decomposition reaction is initiated by a hydroxide ion ($OH^-$) reacting with an ozone molecule which forms one super oxide anion ($\cdot O_2^-$) and one hydroperoxyl radical ($HO_2\cdot$) in an acid-base equilibrium ($pK_a$=4.8). The $HO_2\cdot$ can further dissociate into a hydrogen ion ($H^+$) and another $\cdot O_2^-$.

$$O_3 + OH^- \rightarrow O_2^- \cdot + \mathrm{HO}_2 \cdot$$

$$HO_2 \cdot \leftrightarrow \mathrm{H}^+ + \cdot \mathrm{O}_2^-$$

The $\cdot O_2^-$ can further react with ozone molecules through electron transfer to form $\cdot O_3^-$.

$$O_3 + O_2\cdot^- \rightarrow O_3\cdot^- + O_2$$

The next step in the decomposition model is propagation. The $\cdot O_3^-$ reacts with $H^+$, called protonation, before decomposing into ·OH radicals. These radicals can then react with any contaminants in the solution. Some compounds, known as promoters, that the ·OH radicals can react to give off $\cdot O_2^-$ as a part of the reaction which can continue the chain reaction. Many other compounds that do react with ·OH radicals do not produce $HO_2\cdot/\cdot O_2^-$ and because of this typically terminate the chain reaction. These compounds are called radical catchers or inhibitors and include, formic acid, methanol, alkyl groups, t-Butyl alcohol, carbonate and bicarbonate ions, and phosphate ions. The entire reaction is shown below in Figure 2.x. This reaction is very pH dependent with some of the previous reactions having differing results in solutions with higher pH (Staehelin & Hoigne, 1982, p. 677).

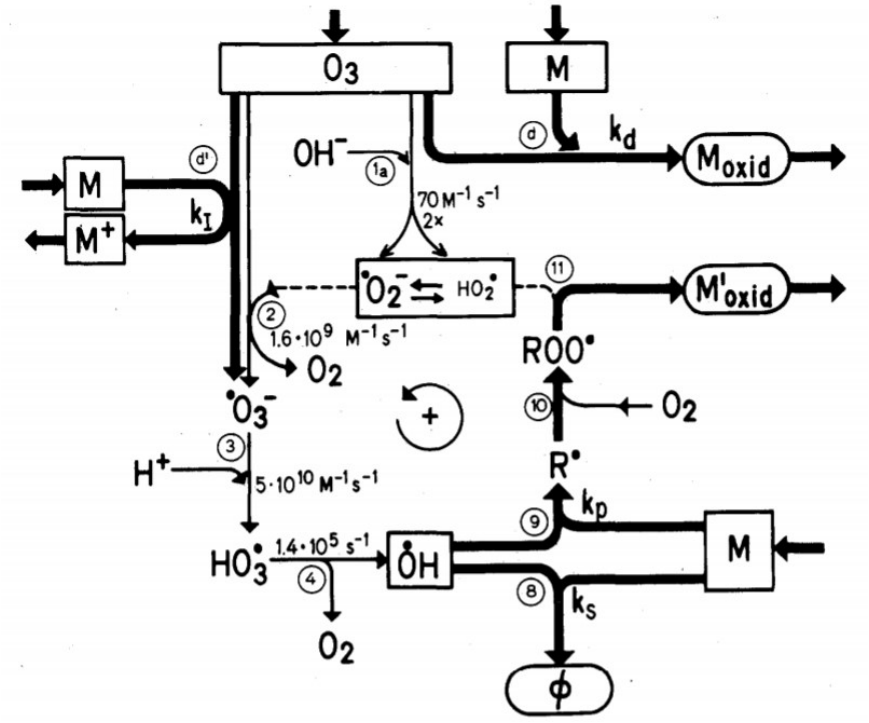
The addition of UV radiation to this set of reactions, acting as an AOP, changes the situation somewhat. UV light is capable of both forming more ozone, by splitting oxygen molecules in the air and that are given off as a part of the ozone decomposition process, and hastening the ozone decomposition process and creating more ·OH radicals (Ikemizu et al., 1987, p. 79). In an aqueous solution, ozone reacts with water in the presence of UV light to form hydrogen peroxide ($H_2O_2$) which can be further disassociated by UV radiation into 2 ·OH radicals.

$$O_3 + H_2O \xrightarrow{uv} H_2O_2$$

$$H_2O_2 \xrightarrow{uv} 2\ \cdot\mathrm{OH}$$

In addition, UV radiation with wavelengths shorter than 190nm can form hydroxy radicals directly from water and water vapor ("The Photolysis of Aqueous Systems at 1849 A I. Solutions Containing Nitrous Oxide," 1965, p. 308; Ung & Back, 1964, p. 754).

*Figure 1.8: Reactions of aqueous ozone in presence of solutes M which react with $O_3$ or $^{\cdot}OH$ (Staehelin & Hoigne, 1985, p. 1208)*




**How much ozone is enough?**

An important question to answer when trying to implement a prototype to commercial use is how efficient is the prototype? The efficiency of estradiol exposure has been explored previously, specifically how much ozone is required to fully oxidize the compound, both on its own and in combination with UV radiation. The study (Irmak et al., 2005, p. 59), which is explored in greater detail in chapter 2, found that using ozone alone 8.89 mols were required for each mol of estradiol. When UV radiation was

included, that requirement shrank to 6.64 mols per mol of estradiol. Estradiol has a molecular weight of 272.4 grams per mol while ozone has a molecular weight of 48 grams per mol. Therefore, in the best-case scenario of complete estradiol oxidation with the minimum of used ozone, a mass of ozone approximately 15-20 percent more than the mass of the estradiol would need to be used. In less ideal scenarios, the mass requirement can approach double the mass of the estradiol to be oxidized.

**Hydroxyl Radicals**

A hydroxyl radical is composed of a hydrogen atom bonded with an oxygen molecule and under most atmospheric conditions is the main form of oxidative capacity in the natural atmosphere (Gligorovski et al., 2015, p. 13079). It is highly reactive due to its ability to strip hydrogen atoms off other molecules to form water molecules. It can be formed naturally through solar irradiation of nitrate ions, nitrite ions, and chromophoric dissolved organic matter (CDOM). Due to this high reactivity, hydroxyl radicals are a subject of environmental research to remove, transform, or otherwise degrade organic and inorganic pollutants and contaminants as a part of water and wastewater treatment. Multiple techniques for forming and using hydroxyl radicals have been implemented including using hydrogen peroxide, ozone, UV radiation, and /$Fe^{III}$. The oxidative capabilities of hydroxyl radicals coupled with their relative ease of production makes them a key part of any advanced treatment method.

**How Ozone and Hydroxyl Radicals act on 17β-Estradiol**

The aromatic ring of 17β-Estradiol is susceptible to oxidative action both directly through action by ozone molecules and indirectly by the ·OH radicals that are formed by ozone

decomposition. There are two pathways for ozone oxidation of estradiol, but both involve attacking the aromatic phenol ring and have the same end products of dicarboxylic acids (Irmak et al., 2005, p. 59). Reactions with ·OH radicals lead to the production of the same compounds. Intermediate products can be detected using tandem mass spectrometry by their differing m/z ratios, a ratio of the compounds mass to their charge. These byproducts differ by where the ozone/**.**OH radical attached to the aromatic ring of the estradiol molecule. This difference in position leads to different polarity between molecules. Regardless, oxidative reactions will begin at the phenolic ring. As this specific section of its molecular structure is key for proper receptor binding, oxidative reactions to this structural group should at minimum reduce the possible estrogenic activity of the compound (Zhao et al., 2008, p. 5283). As exposure continues, more sites along phenolic ring will be acted on by ·OH radicals in addition to sites along the other aliphatic rings. These ring rupturing reactions can continue until the only byproducts left are $CO_2$ and $H_2O$ if exposure continues for a long enough period.

*Figure 1.9: Mechanism for direct reaction of ozone with the aromatic ring of estradiol (Irmak et al., 2005, p. 59)*

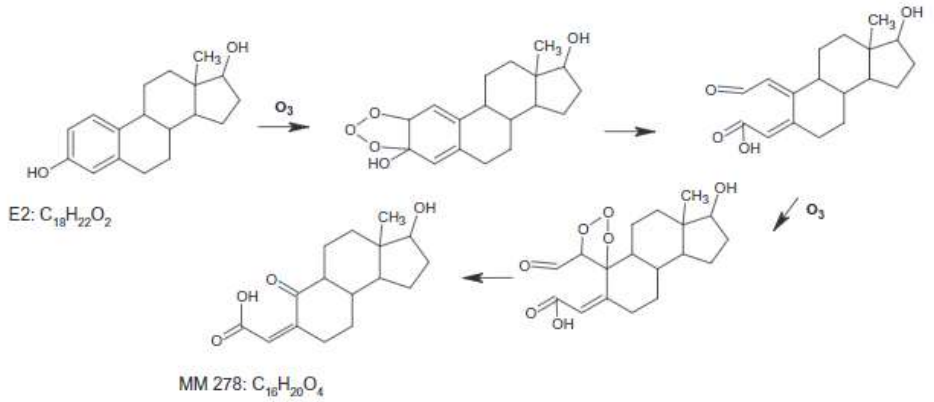
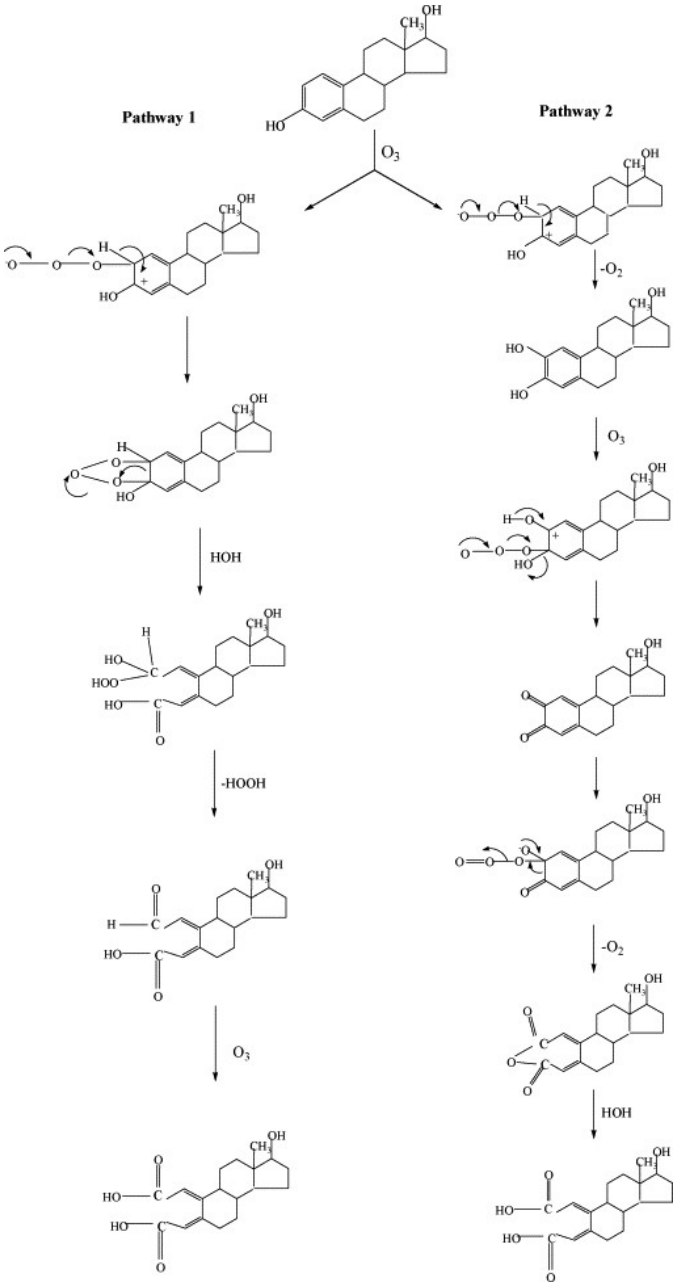


*Figure 1.10: Proposed pathway for the formation of the E2 byproduct with m/z 277 (Pereira et al., 2011, p. 1536)*



# Chapter 2

## Literature Review

In research from Huber et al. (2003, p. 1017), the authors investigated how conventional ozonation practices and "advanced ozone processes" affected several pharmaceuticals in a bench-scale environment. The pharmaceuticals in question, Bezafibrate, Carbamazepine, Diazepam, Diclofenac, 17alpha-Ethinylestradiol, Ibuprofen, Iopromide, Sulfamethoxazole, and Roxithromycin, were exposed to an ozone stock solution in order to determine rate constants for their reaction with the ozone, in excess of ozone and in excess of pharmaceuticals. Rate constants were also found for the compounds' reaction with OH radicals. This allowed the authors to create a model predicting the oxidation of the previously mentioned pharmaceuticals. They then used natural water with varying dissolved organic carbon content and alkalinity to simulate treatment conditions. Due to similar molecular features, such as phenol or amino groups, molecules of the same class are expected to have similar rates of oxidation. The experiment showed that ozone exposure is extremely effective for reducing concentrations in compounds that are sensitive to ozone exposure as shown in figure 2.1. Because of their similar structures and being in the same class of compound, it is expected that 17β-estradiol would be equally susceptible to ozone oxidation.

*Figure 2.1: Fast-reacting pharmaceuticals percent transformation (Huber et al. 2003)*

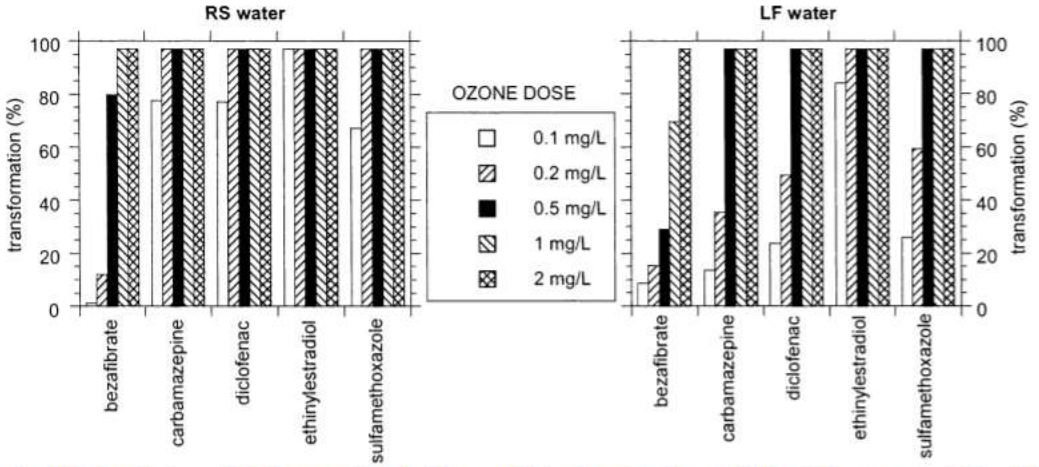
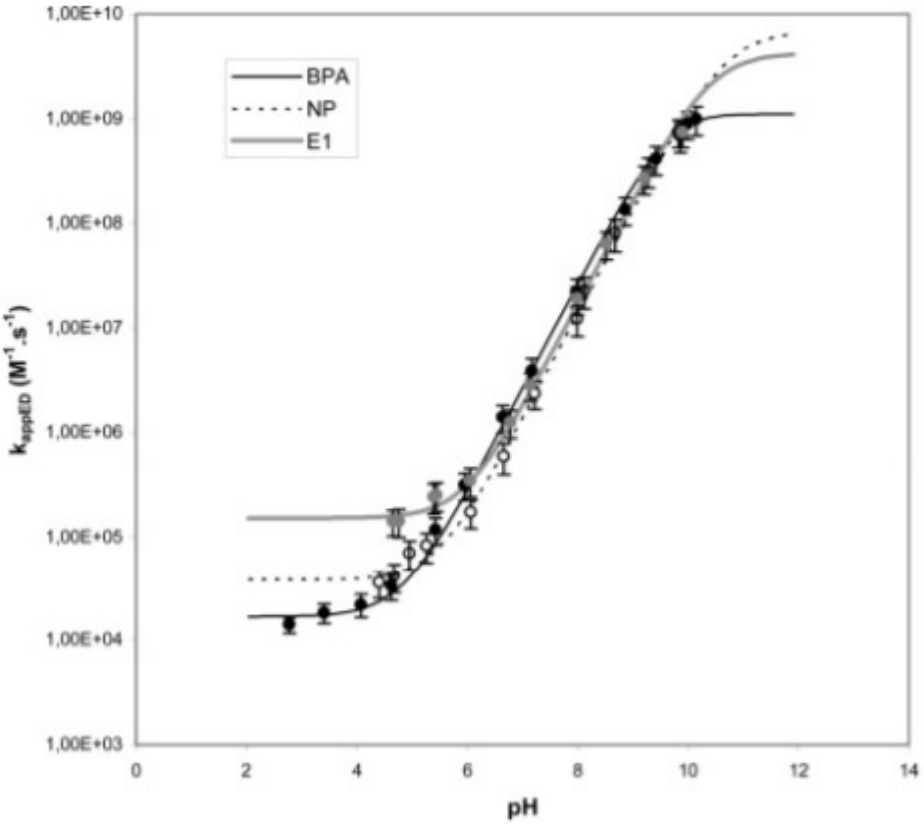



FIGURE 2. Oxidation of fast-reacting pharmaceuticals in RS water (DOC = 1.3 mg/L, alk = 4.1 mM) and LF water water (DOC = 3.7 mg/L, alk = 0.7 mM) as a function of the ozone dose. Experimental conditions: pH = 8, $T$ = 10 °C, $[\text{pharmaceuticals}]_0$ = 0.5 $\mu$M. Samples were measured after all ozone was consumed. Transformations up to 97−99% could be measured with the existing detection limits.

Deborde et al. showed similar results in their paper (2006, p. 4324). They selected 6 endocrine disruptors, 4-n-nonylphenol (NP), bisphenol A (BPA), 17alpha-ethinylestradiol, 17β-estradiol, estrone, and estriol due to their increasing occurrence in the environment. The compounds were dissolved in pure water and exposed to ozone. It was determined that all of the chosen compounds were highly reactive to ozone, needing 0.002 mg*min/L of exposure to achieve a removal efficiency greater than 95%. They also exposed the compounds to chlorine to determine how effective the current treatment process is in removing EDCs. Under the same conditions as the ozone exposure, chlorination processes required doses orders of magnitude larger than ozone in order to reach half the starting concentration, 65 mg*min/L for NP, 13 mg*min/L for BPA, and 6-7 mg*min/L for the hormones. They note that only hormones and BPA could be efficiently removed, greater than 90%, while ozone was effective for the elimination of all explored compounds. In addition, they noted that, while still very effective at neutral

pH, the ozone oxidation reaction was quite pH dependent for the second-order rate constants.

*Figure 2.2: pH dependence of second-order rate constants for ozone reaction (Deborde et al., 2005)*




In their paper, (Rosenfeldt & Linden, 2004, p. 5479), the authors explored the use of UV and an advanced oxidation process (AOP) using UV radiation and hydrogen peroxide to degrade three EDCs: BPA, ethinyl estradiol, and estradiol. They chose compounds due to their prevalence in the environment and the frequent human exposures that occur. They note that the combination of UV and hydrogen peroxide can oxidize many organic compounds found in raw water, including those that cause odors or bad tastes. Therefore, it could be viable as a tertiary water treatment both to oxidize possible EDC contaminants

but also to eliminate any remaining organic compounds that could be a problem. The authors used both monochromatic and polychromatic UV light to evaluate their effect on the EDCs in addition to adding hydrogen peroxide. Their monochromatic UV lamps outputted at a wavelength of 253.7 nm. The authors performed their experiment by exposing solutions spiked with concentrations of the previously mention EDCs with a UV fluence of 1000 $mJ/cm^2$ by itself for each type of lamp and then again with 15 mg/L of hydrogen peroxide. In addition, they performed tests to determine the quantum yield and the hydroxyl radical rate for their experimental design so they could create a model that could accurately predict EDC destruction in other experiments.

They noted that the compounds absorb UV radiation from wavelengths between 200-300 nm with minimal absorption occurring at approximately 250 nm. The absorption spectrum of the compounds and the emission spectrum of the two types of UV lamps that they used are shown in Figure 2.3. The authors note that the monochromatic lamp will likely do very little to oxidize the compounds due to the compounds absorbing a small fraction of the radiation from the emitted wavelength.

*Figure 2.3: Absorption and emission spectrum for bisphenol A, ethinyl estradiol, estradiol, and hydrogen peroxide charted alongside the emission spectrum of the UV lamps (Rosenfeldt & Linden, 2004, p. 5479).*

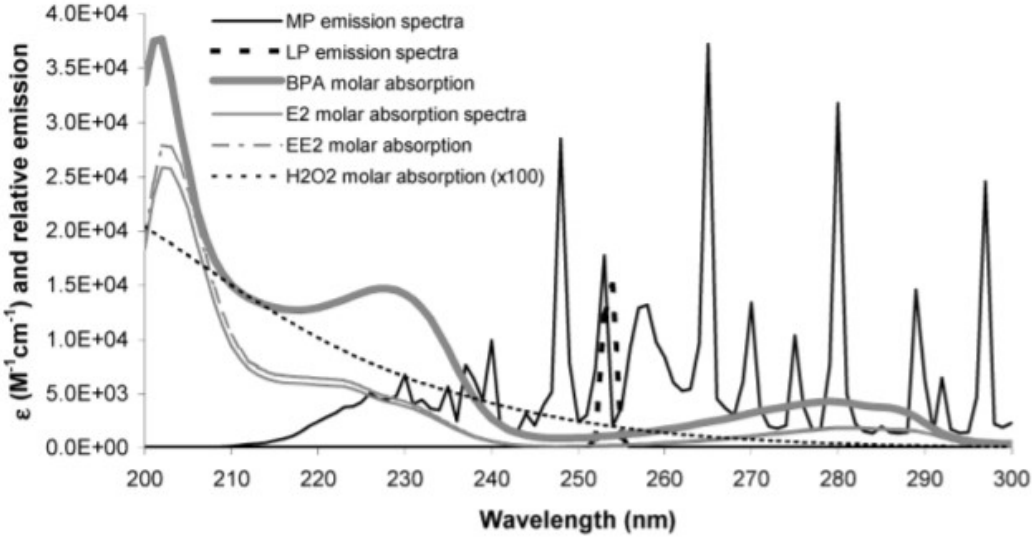
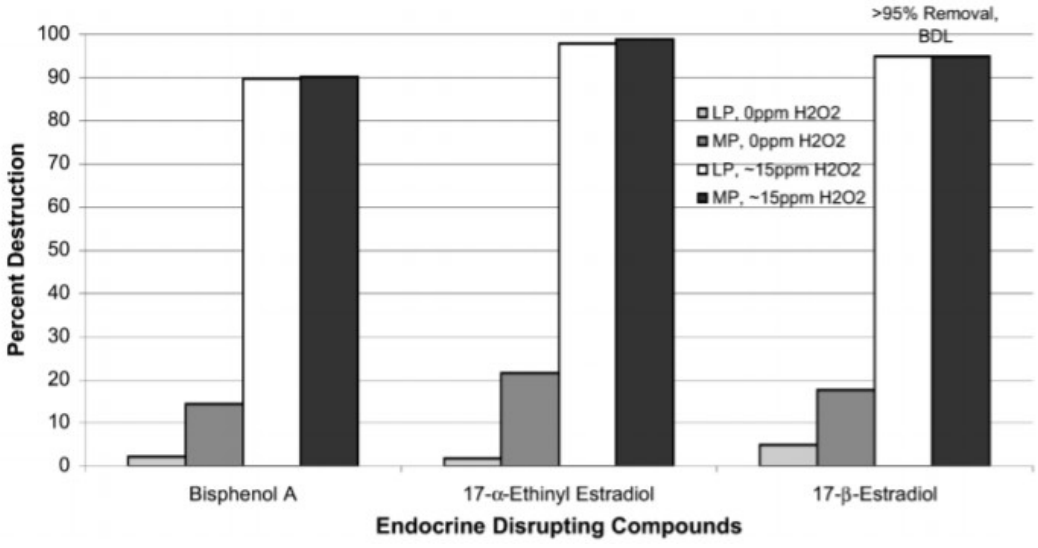



*Figure 2.4: Percent destruction of BPA, EE2, and E2 following UV fluence of 1000 mJ/cm$^2$ via direct photolysis (0 mg/L of $H_2O_2$) and UV/ $H_2O_2$ AOP (~15 mg/L of $H_2O_2$) (Rosenfeldt & Linden, 2004, p. 5479).*



They were correct with their supposition with the monochromatic lamp oxidizing 5% or less for all examined compounds. The polychromatic lamp performed better with 14.5%, 21.6%, and 17.7% moving from left to right on Figure 2.4. The inclusion of hydrogen peroxide drastically improves the degradation, to >90% removal for all compounds, with only a slight difference between the two types of UV lamps.

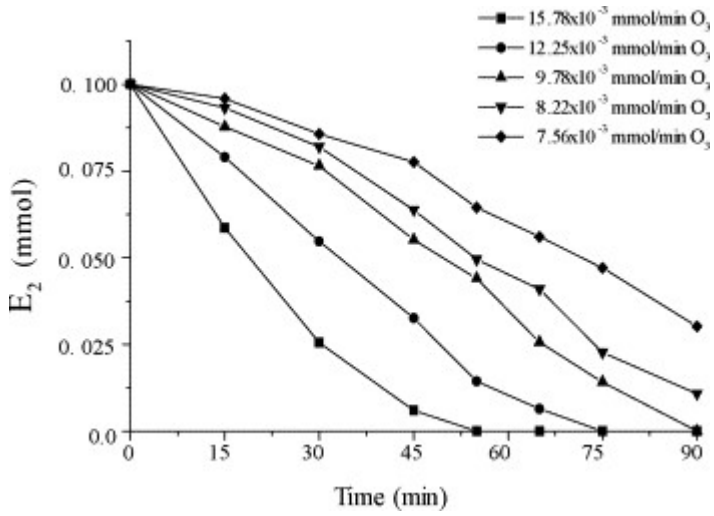
The research from Broséus et al. (2009, p. 4710) explored the effect of ozonation on a number of EDCs, pharmaceuticals and personal care products (PPCPs), and pesticides. The compounds that were chosen are, caffeine, trimethoprim, carbamazepine, naproxen, gemfibrozil, estrone, estriol, estradiol, 17α-ethinylestradiol, progesterone, medroxyprogesterone, norethindrone, levonorgestrel, cyanazine, deethylatrazine, and deisopropylatrazine. The detection of the compounds was accomplished by automated on-line solid phase extraction with liquid chromatography and tandem mass spectrometry to analyze the results. The bench scale experiments were performed with ultrapure water and filtered water from municipal water treatment plants. Samples were spiked with the compounds for concentrations in the ng/L range. Ozone was applied in 0 to 3 mg/L doses via injecting ozone stock solution into a batch continuously stirred glass reactor containing the water sample. After the dose was given, 4 mL aliquots were taken from the sample at regular intervals to check for residual ozone concentrations.

The steroid phenolic hormones, estrone, estradiol, ethnylestradiol, showed very high ozone reaction rate constants when compared to the other compounds, with a k value

greater than $10^6$ compared to the rest with a range from $558 \pm 9\ M^{-1}\ s^{-1}$ to $2215 \pm 76\ M^{-1}\ s^{-1}$. The rate constants for natural waters were found to vary by contaminant, with no significant impact for progesterone up to a 13.5% increase for northindrone. However, because they were found to be within the same magnitude as the kinetic rate constants for ultrapure water, the ultrapure water constants were deemed useful for approximately predicting oxidation in natural waters.

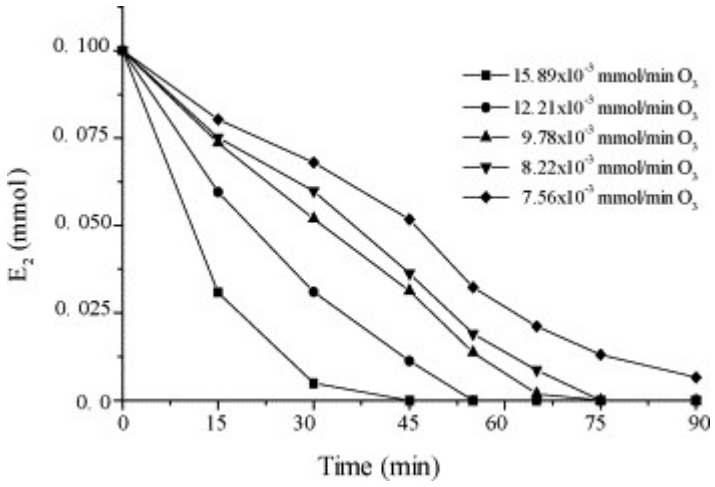
Research by Irmak et al. (2005, p. 59) showed the efficacy of using $O_3$ and $O_3$/UV oxidation for the removal of estradiol and BPA. Both compounds were treated in an aqueous medium at a starting concentration of 0.40 mM. Due to its low solubility in water, the estradiol solution was prepared by dissolving it in acetonitrile in addition to water. Acetonitril was used due to miscibility with water and its low reactivity with ozone. The BPA was dissolved directly into water. An ozone generator was used to produce ozone which flowed into a glass reactor. Samples were taken from the reactor at specific intervals and quenched in a mixture of sodium thiosulfate-sodium sulphite mixture to eliminate residual ozone and OH radicals. The UV lamp used was a 15W low-pressure mercury lamp. The oxidation tests were carried out at different ozone flow rates to determine its effect.

*Figure 2.5: E2 concentration vs time for different ozone doses (Irmak et al., 2005, p. 59).*




As shown in Figure 2.5, the lowest dose of ozone was not sufficient to fully remove the estradiol concentration. The doses that did fully remove the 0.1 mmol estradiol used 0.868, 0.919, and 0.880 mmol of ozone. As all these amounts are roughly equal, this shows that the ratio of ozone to estradiol for complete estradiol removal is approximately 8.89 mols of ozone per mol of estradiol oxidized. By applying UV in addition to ozone, the removal rate is shown to be higher. Of the five ozone doses, four of them completely oxidized the estradiol by the end of 90 minutes as shown in Figure 2.x. For complete removal of 0.1 mmol of estradiol, 0.715, 0.672, 0.655, 0.616 mmol of ozone were consumed for an average of 0.664 mmol. This gives an ozone to estradiol ration of 6.64 mols of ozone per mol of estradiol oxidized, a 22.5% reduction in ozone when UV is also applied compared to ozone alone.

*Figure 2.6: E2 concentration versus time for different ozone doses during $O_3$/UV application (Irmak et al., 2005, p. 59).*




Samples taken were run through a HPLC chromatogram and a mass total ion chromatogram. The MS showed fewer peaks due to its lower sensitivity. The peak corresponding to estradiol, seen at 23.035 and 22.88 minutes for HPLC and MS respectively, come after the peaks corresponding to the byproducts.

*Figure 2.7: HPLC chromatogram of ozonation sample of E2 (Irmak et al., 2005, p. 59).*

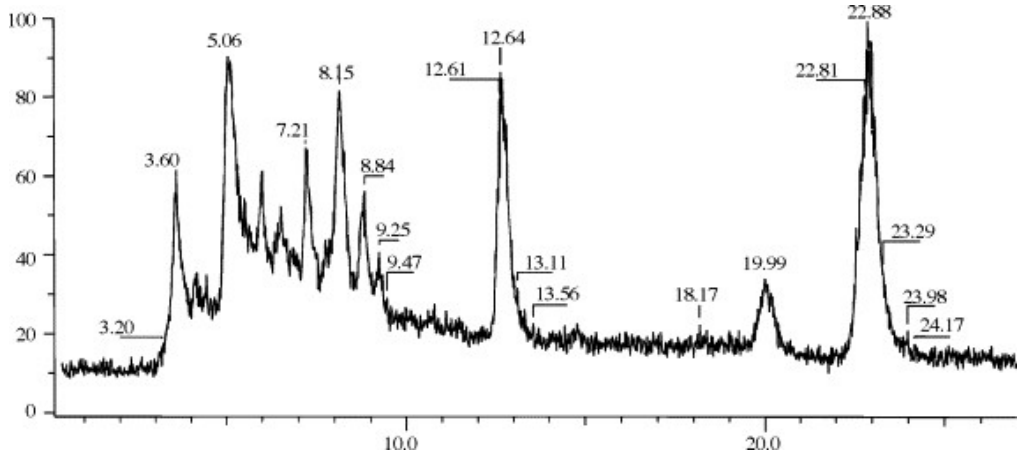
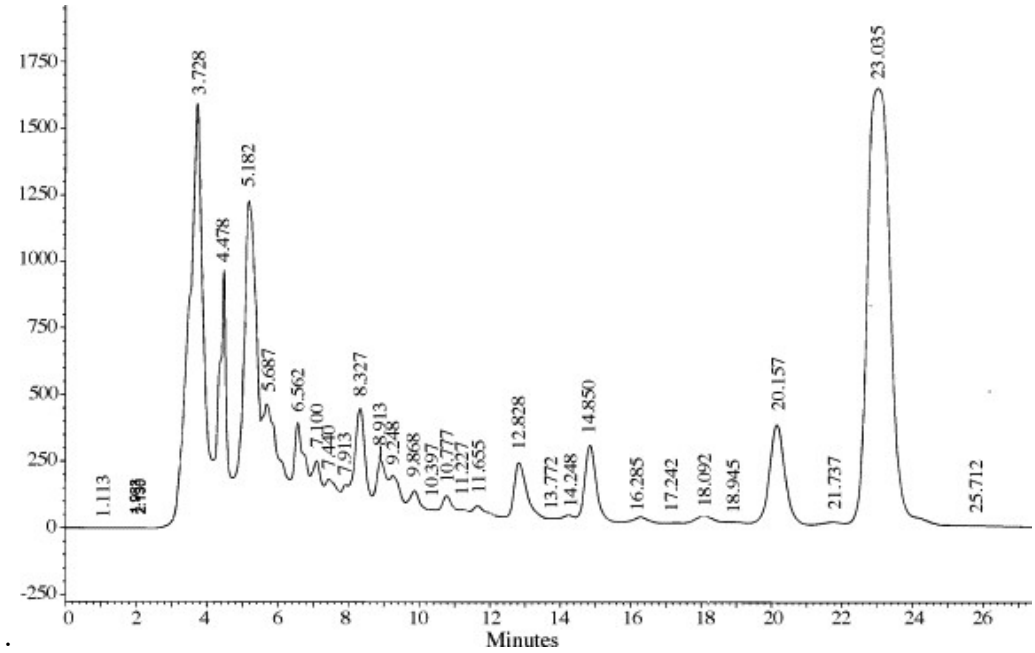



*Figure 2.8: Mass total ion chromatogram E2 ozonation sample (Irmak et al., 2005, p. 59)*

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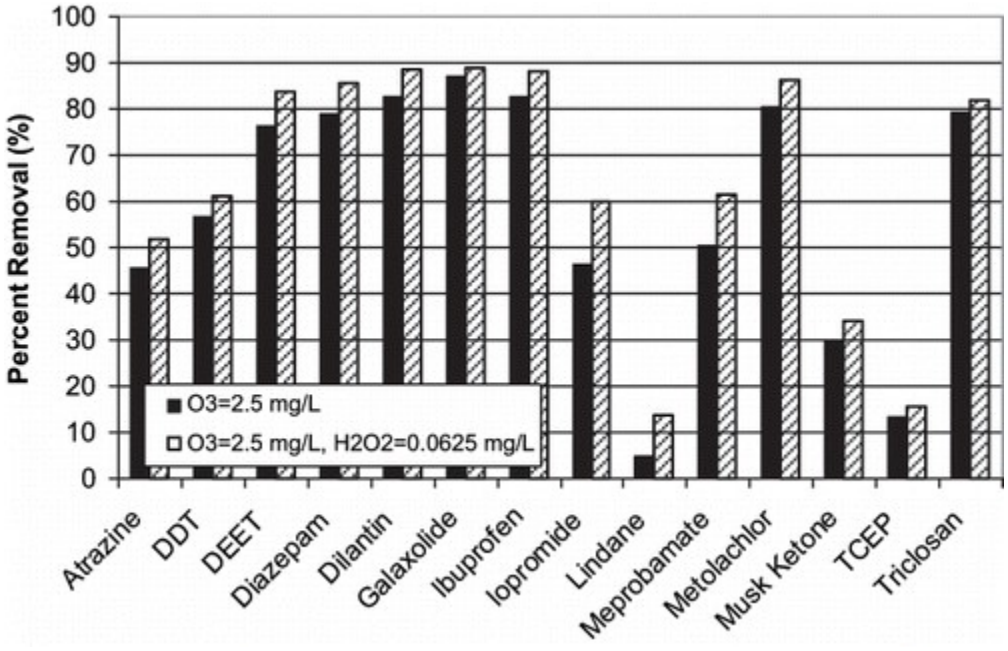
In research from Snyder et al. (2006, p. 451), the authors performed bench scale and pilot scale oxidative removal experiments on surface water spiked with target compounds and on wastewater effluent containing concentrations of target compounds. The authors targeted several EDCs and pharmaceuticals as a part of their study. For the benchtop experiments the authors took water samples from Lake Mead and spiked the samples with the target compounds to achieve concentrations between 100 and 300 ng/L. calculations were made to determine the dose of ozone required to meet USEPA regulations on concentration-time, approximately 0.8 min-mg/L for this experiment. 22 of the 36 targeted compounds were removed with an ozone does of 2.5 mg/L. The percent removal for the remaining compounds are shown in Figure 2.9

*Figure 2.9: Bench Scale removal of target compounds that were not removed below level of detection (Snyder et al., 2006, p. 451)*



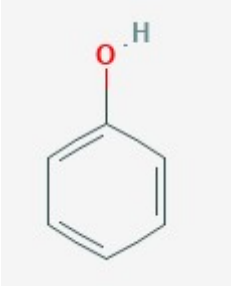

For the pilot-scale experiments, two testing systems with flow rates of 1.0 L/min and 23 L/min were used to conduct ozone and ozone/hydrogen peroxide oxidation experiments

on both wastewater effluent and pretreatment drinking water. The smaller of the two pilot scale systems, which the authors refer to as Bench-Top Pilot Plant (BTPP), consisted of a continuous flow ozone contactor, a 208 steel drum acting as a tank, and a peristaltic pump for controlling flow. Several chemical feed ports were installed to allow for injection of hydrogen peroxide. BTPP testing was performed with both wastewater and raw Colorado River water. The 170 L of tertiary treated wastewater that had not been disinfected. Residual ozone was measure at 2, 6, 10, 14, and 18 minutes. A human breast carcinoma bioassay was used to measure the estrogenicity of the wastewater experiments. The results of the bioassay were reported as estradiol equivalents. To evaluate the production of ·OH radicals, 70L of filtered tertiary treated waste was spiked with probe compound para-chlorobenzoic acid (pCBA). The residual ozone was determined to have decayed by the twelve-minute mark for all ozone doses. For the water ozone experiments, 13 of the 36 target compounds had a removal rate greater than 90% within the first two minutes of zone contact with a dose of 1.25 mg/L. The introduction of hydrogen peroxide resulted in small increase in removal for most compounds. For a few of the compounds removal rate was on average 15% lower using ozone and hydrogen peroxide in combination than with ozone alone. The wastewater contained concentrations of 17 of the 36 target compounds, 7 of which were removed below the level of detection by even the lowest evaluated dose of ozone of 4.9 mg/L. The bioassay returned significant estrogenicity for both the raw sewage that was measure and the tertiary treated effluent. Ozone exposure reduced the estrogenicity to below the level of detection for all ozone

doses which suggests that the byproducts formed by the oxidative reactions were not estrogenic in nature.

Phenol is a type of organic compound used in antiseptics, household products, resins, weed killers, and as an intermediate for industrial synthesis. The term phenol can refer to the specific compound phenol, or the family of organic compounds that has phenol as the simplest member (Wade, 2018). Phenols are characterized by a hydroxyl group attached to an aromatic ring.

*Figure 2.10: Depiction of the chemical structure of phenol (National Center for Biotechnology Information, 2020).*



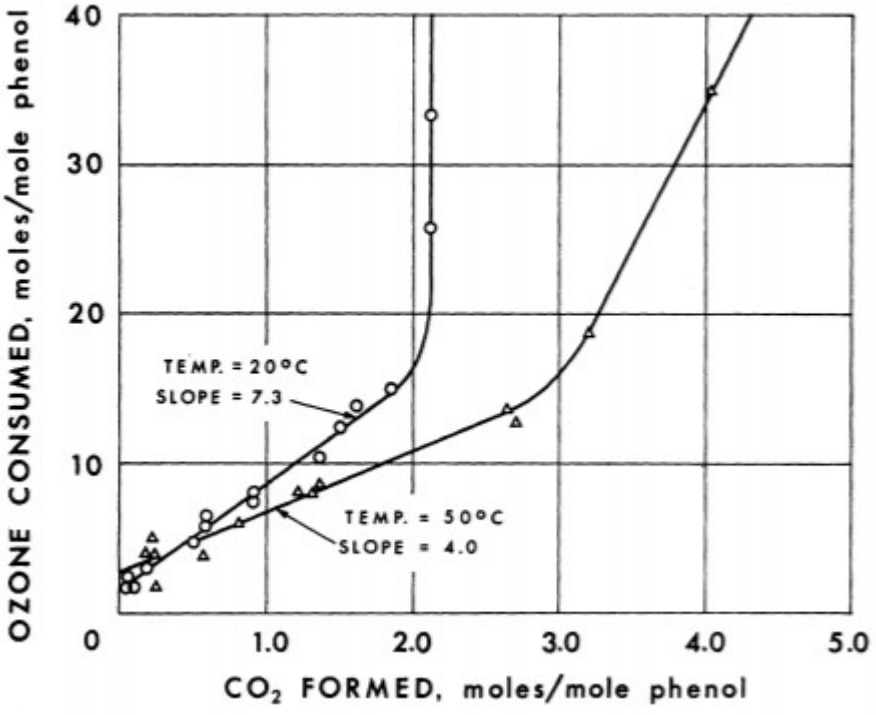

The molecular structure of estradiol bears resemblance to phenol, with an aromatic carbon ring with an attached hydroxyl group. Because of this structural similarity research into the oxidative process of phenol could be applicable for the removal and oxidation of estradiol. Several studies have explored the oxidation of phenol using ozone. In research from Li et al. (1979, p. 587), the authors showed that ozone rapidly reacted with the phenol in aqueous solution. The intermediate products were also rapidly oxidized by the presence of ozone with the final products of the reaction being Catechol, o-quinone, hydroquinone, oxalic acid, humic acid, and a dimer identified via MS.

Research by Eisenhaugher (1971, p. 207) sought to improve the efficiency of the phenol oxidation reaction and to gain a better understanding of the reaction pathway. Using an ozonation reactor, aqueous solutions of phenol with concentrations ranging from 50 mg/L to 300 mg/L were exposed to ozone at flow rates of 0.1 L/min to 0.5 L/min. The experiments were also run at several different pH levels, from 3.00 to 11.06 to observe how pH affected the reaction. During the experiment the ozone that was not consumed by the reaction and the carbon dioxide that was released by the reaction was monitored. The author found that the lower initial pH did not affect the reaction though he noted that the pH of the system rapidly decreased to a value of 3 to 3.5. At the highest initial pH level reaction rate nearly doubled while the pH of the system only decreased to a value of 9.9. The carbon dioxide released by the reaction indicates a complete oxidation of the phenol in solution and all its oxidative byproducts. The quantity of carbon dioxide if the reaction was occurring at peak efficiency can be determined by the following stoichiometric equation.

$$C_6H_6O + 14O_3 \rightarrow 6CO_2 + 3H_2O + 14O_2$$

If the reaction was occurring at 100% efficiency, then the conversion of one mole of phenol into 6 moles of carbon dioxide would require 14 moles of ozone, for 2.33 moles of ozone per mole of carbon dioxide. As shown in Figure 2.11 below, the efficiency of the two recorded runs are about 30% and 65%.

*Figure 2.11: carbon dioxide production (Eisenhaugher,1971, p. 207).*




At the time, the author was uncertain as to the exact mechanisms that reduced the phenol to just carbon dioxide. The oxidative pathways were relatively understood up to catechol and it was assumed that catechol broke down into o-Quinone as shown in Figures 2.12 through 2.14. Later studies into the degradation of phenol would more fully map out the reaction pathway shown in Figure 2.15.

*Figure 2.12: Formation of intermediate IV by the Baily mechanism (Eisenhaugher,1971, p. 207).*

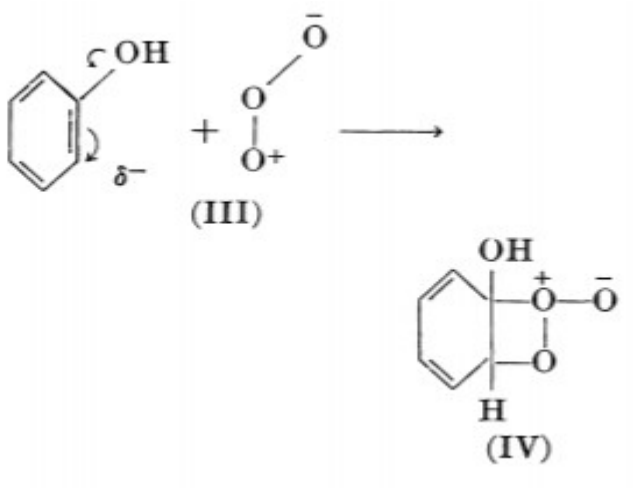



*Figure 2.13: Break down of intermediate IV into catechol (VI) (Eisenhaugher,1971, p. 207).*

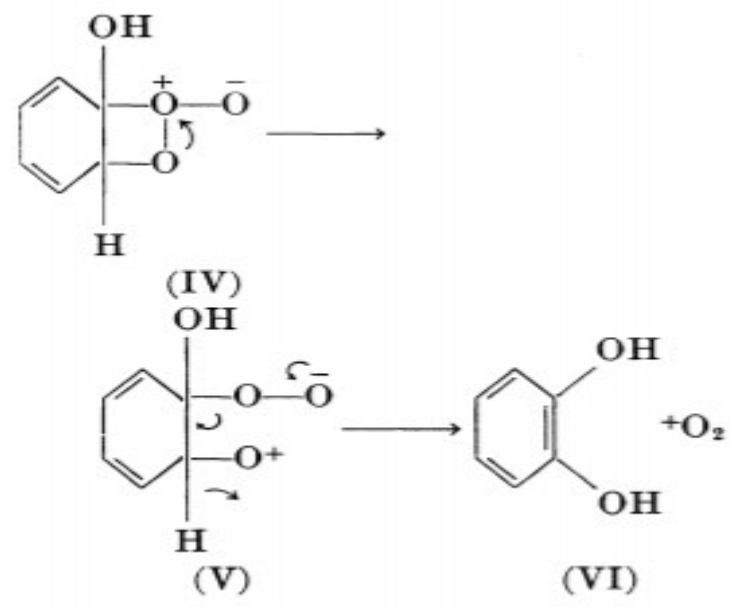


*Figure 2.14: Degradation of catechol (VI) to o-Quinone (X) (Eisenhaugher,1971, p. 207).*

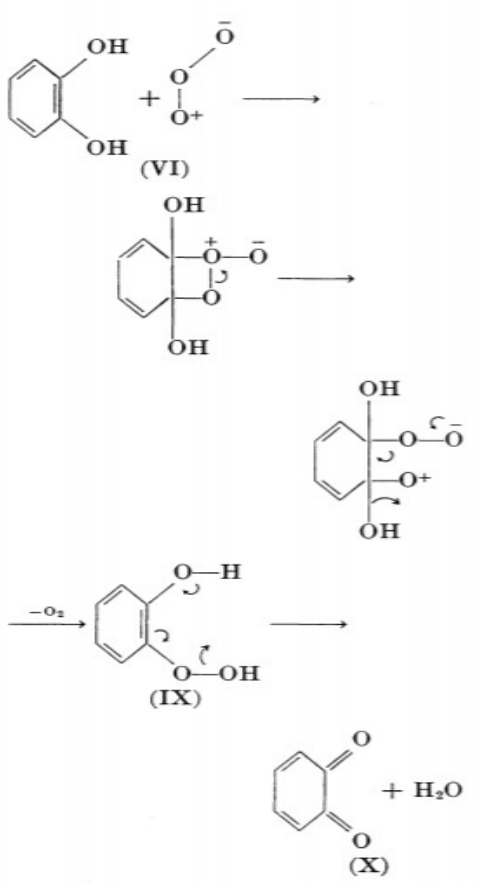
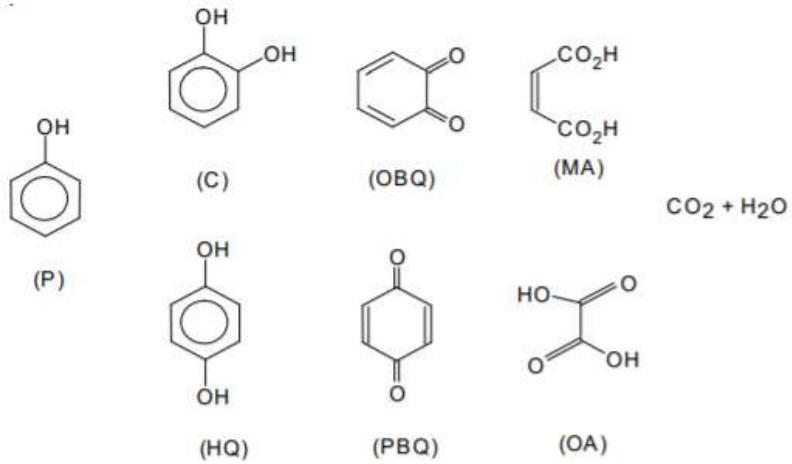



*Figure 2.15: Reaction pathway for phenol degradation (Turhan & Uzman, 2008, p. 260).*



# Chapter 3

## Materials and Methods

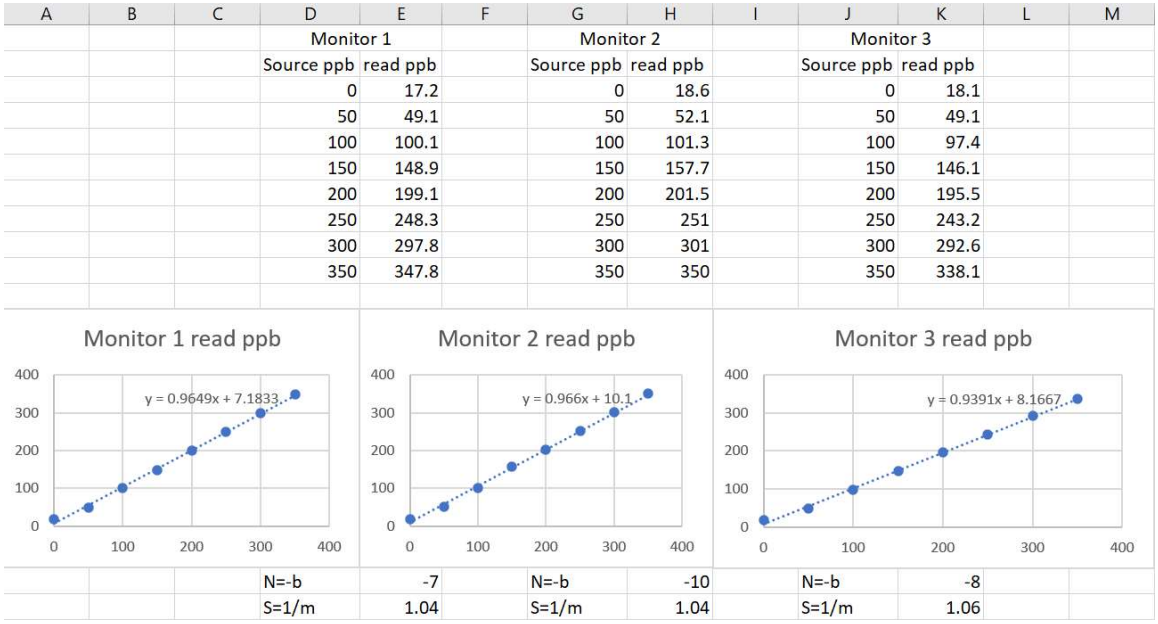
For this thesis, there are two portions of the experiment that have materials and methodology that need to be noted, those being the sample gathering and the testing portions. Both portions are laid out below.

### Ozone Monitor Calibration

In order to accurately measure the ozone in the headspace of the PIOx tank, an ozone monitor needed to be properly calibrated. To prevent unfortunate circumstances from hindering accurate measurements, multiple ozone monitors were calibrated for use. The software to automatically calibrate the monitors was not available so they had to be calibrated manually. In order to calibrate the monitors, the following items were needed: ozone monitors, nonreactive tubing, an ozone source, and a computational software such as Excel. The procedure for calibrating the ozone monitors are as follows. Navigate to the calibration menus for each monitor and zero out the N and S values. Navigating through the monitors' menu, have it output the 1-minute average value. Connect the monitors to the ozone source via nonreactive tubing in a well-ventilated area. Allow both the ozone source and the monitors to run for at least an hour before beginning calibration to warm up. Set the ozone source to output at least 5 different ozone concentrations and record the value the monitor gave for each concentration. Graph the concentration versus the recorded value for each monitor. Find the trendline equation for that data set. The calibration values are calculated from the trendline equation. N is equal to the negative of

the y intercept of the trendline while S is the inverse of the trendline's slope. An example of a calibration spreadsheet is shown below in Figure 3.1.

*Figure 3.1: Ozone monitor calibration spreadsheet*



| | Monitor 1 | | Monitor 2 | | Monitor 3 | |
|---|---|---|---|---|---|---|
| | Source ppb | read ppb | Source ppb | read ppb | Source ppb | read ppb |
| | 0 | 17.2 | 0 | 18.6 | 0 | 18.1 |
| | 50 | 49.1 | 50 | 52.1 | 50 | 49.1 |
| | 100 | 100.1 | 100 | 101.3 | 100 | 97.4 |
| | 150 | 148.9 | 150 | 157.7 | 150 | 146.1 |
| | 200 | 199.1 | 200 | 201.5 | 200 | 195.5 |
| | 250 | 248.3 | 250 | 251 | 250 | 243.2 |
| | 300 | 297.8 | 300 | 301 | 300 | 292.6 |
| | 350 | 347.8 | 350 | 350 | 350 | 338.1 |
| | N=-b | -7 | N=-b | -10 | N=-b | -8 |
| | S=1/m | 1.04 | S=1/m | 1.04 | S=1/m | 1.06 |



Sample Gathering

For the Sample Gathering portion of the experiment, the following materials were used: PIOx system, Water in excess, Estradiol concentrate, Methanol, Glassware, Pipettor, Containers for holding samples, ozone monitor. To create the Estradiol concentrate solution that was used to spike the system, 50 milligrams of powdered Estradiol concentrate with a purity of >96% was dissolved into 50mL of 200 proof methanol and enough deionized water to make the total volume 62.5 milliliters. This was done to both increase the viscosity so a pipettor might be used and to get the concentration to the intended level of 0.8 milligrams per milliliter.

Two separate methods were used in the taking of sample data. Both methods involved filling the tank of the PIOx system with approximately 200L of tap water which would be

spiked with 200 microliters of the Estradiol concentrate for an estimated final concentration in the tank of 800 picograms per milliliter. The solution would then be circulated through the tank with the PIOx system active for 15 minutes. The ozone in the headspace would be measured by the ozone monitor and the value recorded every time a sample was taken. Where the two methods differ is in when the concentrate would be applied and the timing of when the samples would be taken.

In the first method, the concentrate would be applied as the machine's water pump was on and circulating the water but before the UV light was turned on. The pump was allowed to circulate the mixture for a few minutes before the 0 minute sample was taken and the UV lamps were turned on. This was to allow the mixture to be thoroughly mixed for an accurate starting benchmark before the experiment began. Samples would then be taken at every odd minute up to the 15-minute mark.

In the second method, the PIOx system would be allowed to run with the UV lamps activated for several minutes until the peak concentration of ozone in the headspace of the tank was reached. The concentrate would then be added, along with starting the timing. Taking a sample at 0 minutes would not be feasible because either the estradiol concentration would not be mixed into the tank or while waiting for a thoroughly mixed sample the concentration would be decreasing without being measured. Samples would then be taken at 1, 3, 5, 7, 11, and 15 minutes. This modification to the methodology was to simulate a possible application of this piece of technology, that of a water treatment

plant. In a treatment environment it would be feasible that the system would be running constantly and therefore this modification would more closely resemble actual service conditions.

**Data Testing**

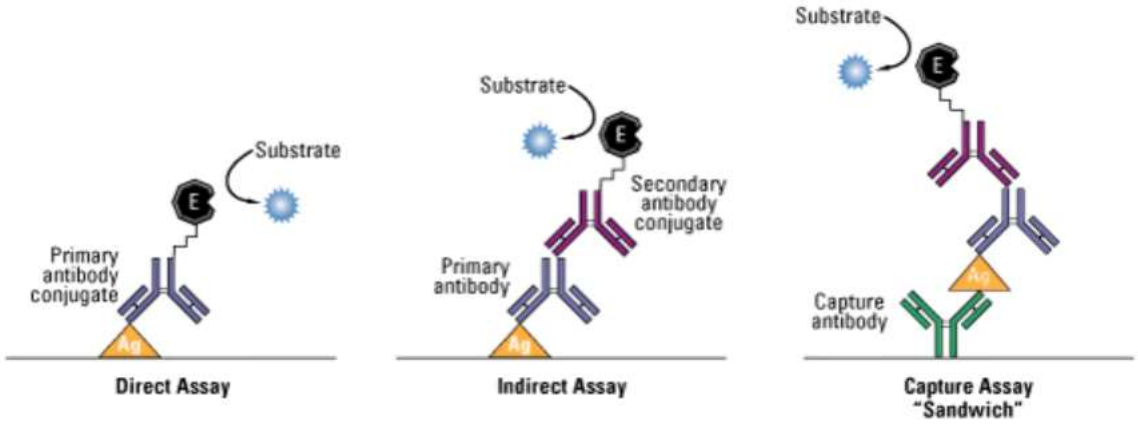
For the Data Testing portion of the experiment, the following materials were used:17β-Estradiol high sensitivity ELISA kit Enzo®, Deionized water, Pipettors for volumes between 5µL and 1,000 µL, Disposable beakers, Glassware, Microplate shaker, Microplate reader. The Estradiol ELISA kit contains the following items: Assay Buffer Low BSA, 17β-Estradiol Standard, Donkey anti-Sheep IgG Microtiter Plate, 17β-Estradiol Antibody, 17β-Estradiol Conjugate, Wash Buffer concentrate, pNpp Substrate, Stop Solution, Plate Sealer, Complete Assay Layout Sheet.

**ELISA Testing Format**

The means of testing the collected samples for this thesis project is via an enzyme linked immunosorbent assay (ELISA) which is a method that was designed for detecting a specific protein, peptide, antibody, or biomolecule in a complex solution. The assay works by preparing the surface of a well plate with antibodies and enzymes that bind to the substance that is trying to be quantified. The now prepared wells are then washed to remove anything that was not fixed to the surface before being incubated in a substrate to allow the reporter enzymes to react. The plate is then read by one of a few different methods depending on the substrate used to determine the concentration of the subject compound. This aspect of the process, the binding and immobilization of reagents is what allows ELISAs to be simple to design and perform. There are a few different methods for

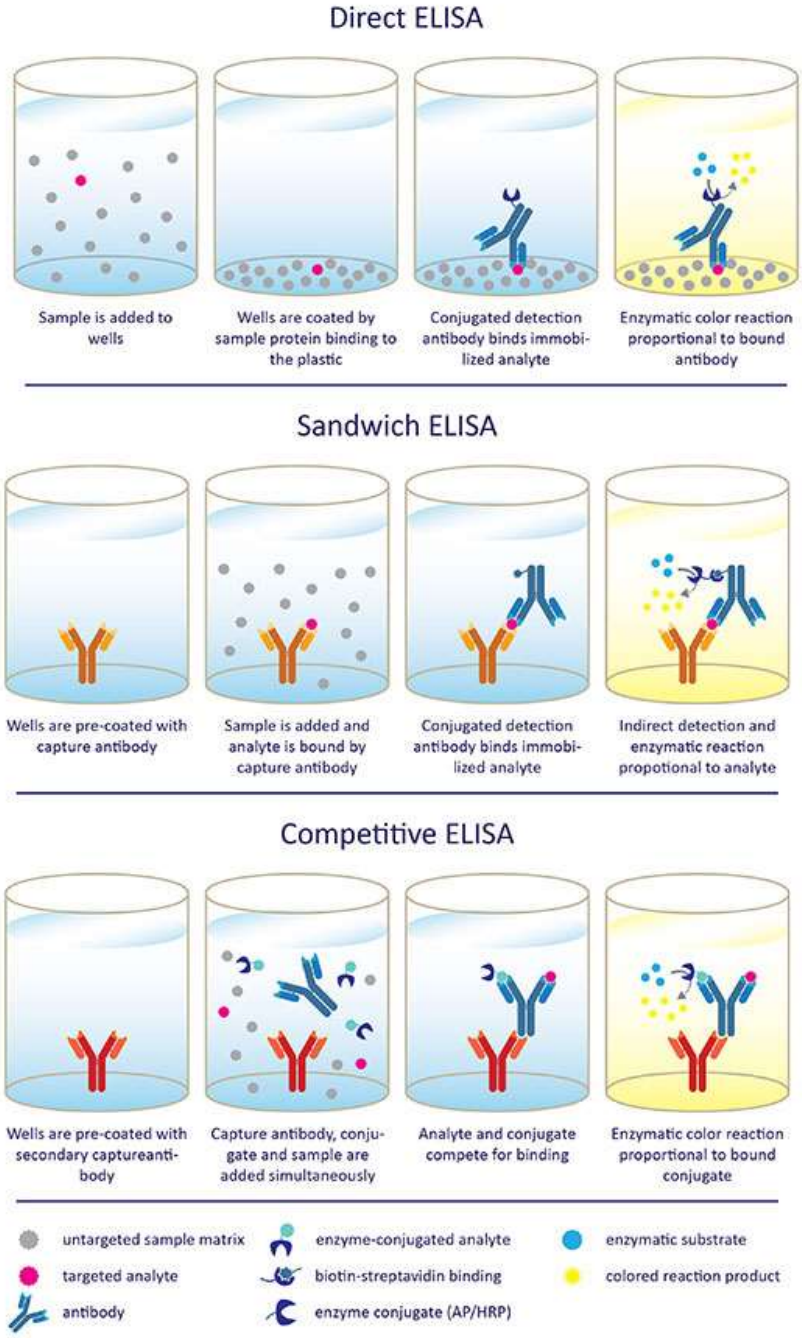
how the ELISA works as an assay. The first difference is in how the compound of interest is attached to the plate. The compound is either attached directly to the surface of the plate along with any other miscellaneous compounds that are in solution or indirectly by a capture antibody that is attached to the plate which only will grab the compound of interest while everything else is washed away. The second difference between these methods is the means of detection. While the method of attaching the antigen to the well plate is part of what defines the type of ELISA, the method of detection is largely what determines the level of sensitivity. Direct detection uses a primary antibody that directly attaches to the compound of interest and is labeled with a tag or reporter enzyme. Indirect detection uses a primary antibody that attaches to the antigen along with a secondary antibody labeled with the reporter enzyme that attaches to the primary antibody. Because multiple secondary antibodies can attach to the primary antibody, it increases the level of sensitivity for the assay. There are three different categories of ELISAs based off the previously mentioned method of capture and detection used. These categories are direct, indirect, or “sandwich”.

*Figure 3.2: Diagram of common ELISA formats (Overview of ELISA | Thermo Fisher Scientific - NL, n.d.)*




Direct assays use direct capture and direct detection, indirect assays use direct capture and indirect detection, and capture or "sandwich" assays use indirect capture and indirect detection which gives the method its moniker. Because of its sensitivity and reliability, most commercial ELISA kits utilize the "sandwich" method. There is one other type of ELISA that these categories do not cover, competitive ELISAs. Competitive ELISAs utilize indirect capture but differ in how the detection antibodies function. The antibody that captures the compound of interest can also bind with a conjugate that is added to each well. The detection enzyme reacts based on the quantity of bound conjugate. Because there is finite quantity of binding sites in each well, more conjugate bound to the antibodies means fewer antigens are bound. So, the colorimetric response is inversely proportional quantity of the compound of interest. The ELISA kit utilized in this thesis project is a competitive ELISA.

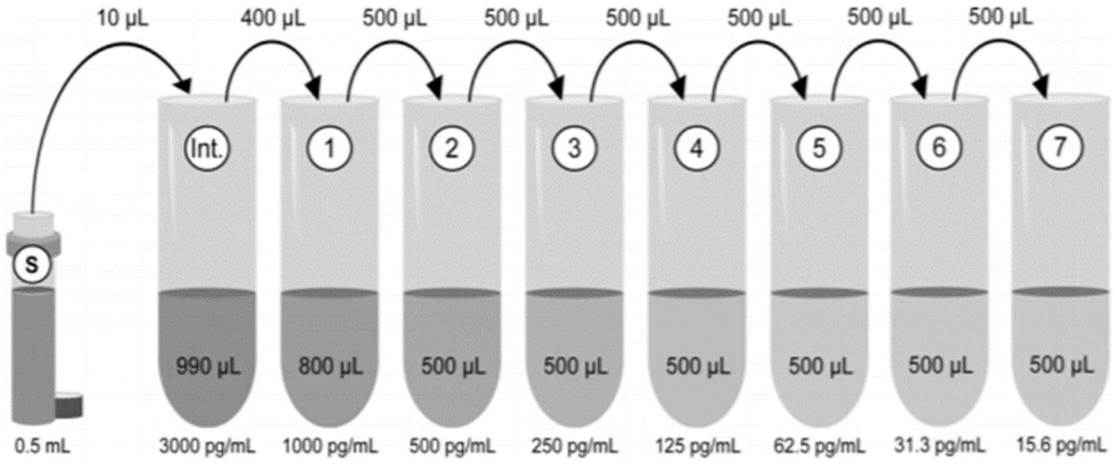
*Figure 3.3: Types of ELISA (Overview of ELISA | Thermo Fisher Scientific - NL, n.d.)*



**Assay Preparations**

Before the assay procedure, four pieces of preparation need to be accomplished. First, the reagents need to be allowed to warm to room temperature before use. Second, the wash buffer that is provided needs to be diluted one part buffer to twenty parts deionized water. Third, the assay record sheet should be filled out in order to ensure accurate record keeping and that there are enough wells for all the samples to be analyzed. All samples should be assayed in duplicate to minimized to effect of any possible contamination or mistake. An example of the assay record sheet from this experiment will be provided in the appendix. Finally, the 17β-Estradiol standards by dilution will need to be created.

*Figure 3.4: Creation of Estradiol Standards by Dilution (Enzo Life Sciences, 2015)*




Allow the 300,000 pg/mL 17β-Estradiol standard to come to room temperature and vortexed to ensure even concentration. Label seven 12 x 75 m tubes #1 through #7 and one tube "int". Pipet 990 µL assay buffer into tube "int" and 800 µL into tube #1. Pipet 500 µL assay buffer into tubes #2 through #7. Remove 10 µL from the stock vial and add to tube "int" and vortex thoroughly. Remove 400 µL from tube "int" and add to tube #1

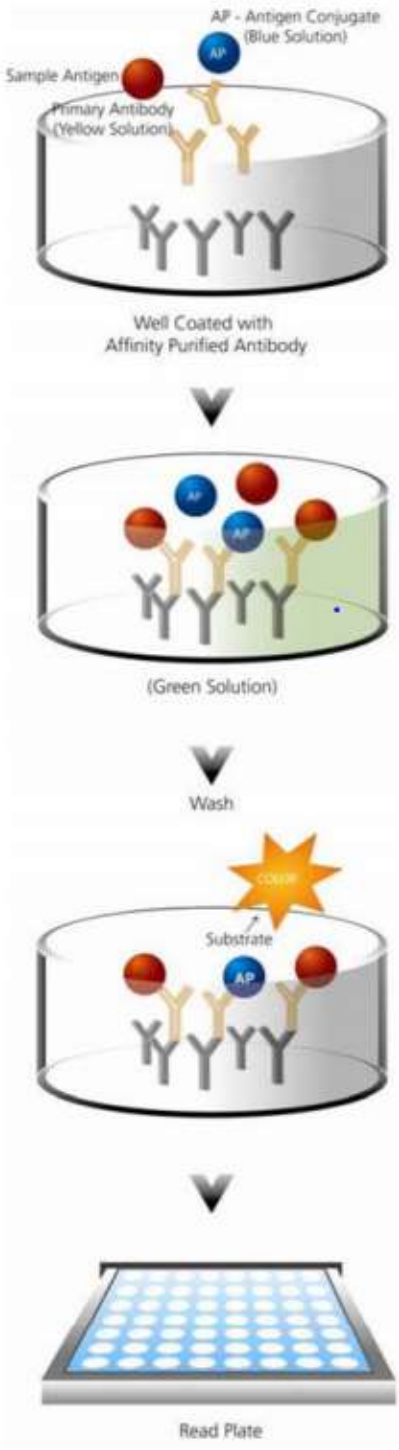
and vortex thoroughly. Remove 500 µL from tube #1 and add to tube #2. Vortex thoroughly. Continue this from tubes #3 through #7. These diluted standards should be used in an assay within 60 minutes of preparation. The concentrations of the diluted standards are shown in Figure 3.1 above.

**Assay Procedure**

1. Pipet 150 µL of the assay buffer into the NSB (non-specific binding) wells.
2. Pipet 100 µL of the assay buffer into the Bo (0 pg/mL standard) wells.
3. Pipet 100 µL of Standards #1 through #7 to the bottom of the appropriate wells.
4. Pipet 100 µL of the samples to the bottom of the appropriate wells.
5. Pipet 50 µL of the blue conjugate into each well except the TA and Blank wells.
6. Pipet 50 µL of the yellow antibody into each well except the Blank, TA, and NSB wells. Note: Every well used should be green in color except the NSB wells which should be blue. The Blank and TA wells are empty at this point and have no color.
7. Seal the plate. Incubate at room temperature with shaking (~500 rpm) for two hours.
8. Empty the contents of the wells and wash by adding 400 µL of wash buffer to every well. Repeat 2 more times for a total of 3 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
9. Pipet 5 µL of the blue conjugate (diluted 1:2) to the TA wells.
10. Add 200 µL of the substrate solution into each well.

11. Incubate for 1 hour at room temperature without shaking.
12. Pipet 50 µL stop solution into each well.
13. After blanking the plate reader against the substrate blank, read optical density at 405 nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

*Figure 3.5: Overview of ELISA process for thesis project (Enzo Life Sciences, 2015)*



The data from the plate reader would then be exported in an Excel format which would be used to make further calculations to ascertain the estradiol concentrations in each sample. While there is software that is capable of performing the following calculations, it was unable to be accessed when calculations were being performed.

**Evaluating the Raw Data**

The results that come from the plate reader are the optical density (OD) of each well in the plate. The substrate that is added to each well prior to the final incubation is clear at the start and turns yellow as the enzymes react with the substrate. The amount of signal, which equates to the darker the color, is inversely proportional to the amount of estradiol in the sample. However, those results are calculated in relation to the 4 control wells, Blank, Total Activity (TA), Non-Specific Binding (NSB), and zero standard binding ($B_{0)}$ which are laid out in duplicate in the first column as shown on the layout sheet in the appendix. The blank well, also referred to as a chromogen blank, is filled with only the substrate and the stop solution. Its purpose is to check the substrate's contribution to the OD of the samples, which can be a problem if the substrate is too old. TA wells are included to act as quality control and check the viability of the conjugate or the coated antibodies. NSB wells are used in competitive ELISAs to determine the background that is occurring to unspecific binding of the conjugated enzyme. It is found by not adding the capture antibody, instead allowing the conjugate to bind directly to the antibodies that precoated on the plate. It acts as a blank to be subtracted from OD of the samples and can be useful for determining the source of arbitrarily high results. Finally, the $B_0$ wells act as maximum possible value in a competitive ELISA. As only the conjugate and no samples

are added to the well, the conjugate does not face binding competition so maximum binding, and therefore maximum coloration, will be achieved. It is typically used in reference to percent bound (%B or $B/B_0$) where the conjugate binding of each sample is presented as a percentage of the maximum possible binding (*Which Controls to Use in ELISA Assays?*, 2020).

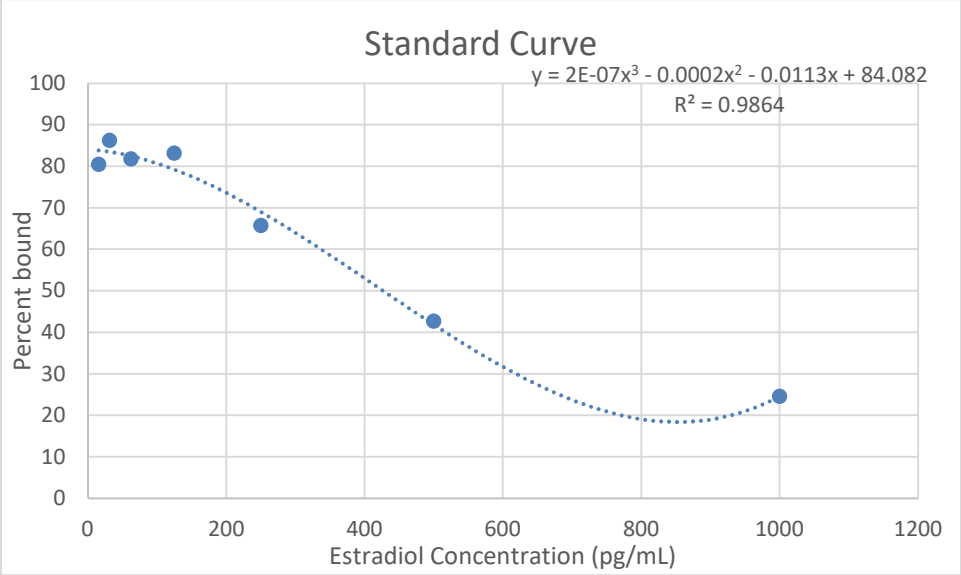
The manual calculation of results begins with averaging the duplicate for each sample and standard to get the average OD for that sample. Next, the average NSB OD value should be subtracted from all other average OD values to get the average net OD. This should remove any background values caused by the substrate. Finally, to find %B for each value divide average the net OD by the net $B_0$ OD. By plotting the %B versus concentration of 17β-Estradiol for the standards a standard curve can be established. The concentration of 17β-Estradiol of the sample unknowns could then be determined by interpolation. However, by finding the equation of the trendline and taking the inverse of it, that inverse equation could be used to directly calculate the concentration of 17β-Estradiol using the %B values.

# Chapter 4

## Results and Discussion

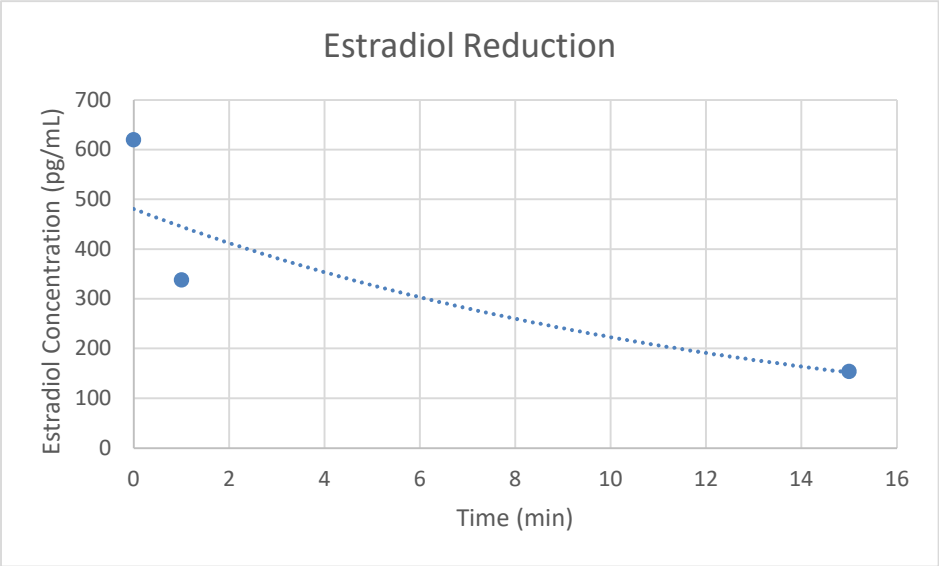
A total of 6 sample sets were obtained, an abbreviated initial set meant to ensure that concentration calculations were correct and that samples would be on the scale set by the estradiol standards, 3 sets using method one and 2 sets using method two. That truncated sample set used method one and consisted of only the samples taken at 0, 1, and 15 minutes. The sampling process went swiftly though the ozone monitor was not available at that time so there is no corresponding data for the ozone concentrations in the headspace. The ELISA itself was simple enough to complete though time consuming with the 3 hours of incubation required. In addition, it was noted that performing the ELISA on such a small number of samples was inefficient. The need to use 22 of the available 96 wells in the microplate for quality checks and data requirements meant more waste if the entire plate was not used at once. This was corrected for the later ELISAs. The standard curve for that run was more than acceptable as shown in Figure 4.1 with an $R^2$ of 0.9864.

*Figure 4.1: Standard Curve of estradiol concentration versus percent bound for trial run of experimental procedure.*




As shown in Figure 4.2, all the samples were within the level of detection, so the estimations that were made for the volume of the tank were reasonable. The initial concentration is a bit lower than expected at 620 pg/mL rather than the estimated 800 pg/mL but it is within the same order of magnitude with only a 22% difference between the two values. This could have been due to either a larger quantity of water in the tank than was estimated or an incomplete mixing which allowed for an area of lower concentration. Regardless, Figure 4.2 shows a clear downward trend over the 15 minute testing period with a removal rate of just over 75% at 15 minutes. These results confirmed previous assumptions as to the capability of the PIOx system for oxidizing estradiol and confirmed that the starting sample concentrations would be within the level of detection for the ELISA so the experiment could continue without adjustment.

*Figure 4.2 Estradiol concentrations versus time for trial run*




The first sample set of method one was taken by itself due to time constraints while the second and third sample sets were performed sequentially on the same day as the sample sets for method two. The ozone readings for that day of testing shown in figure 4.x indicated a decrease in ozone concentrations in the headspace. This decline in concentrations was later quantified The ELISA standards that were run alongside the samples showed good results with R2 values equal to 0.9669 and 0.9971 respectively. To determine the estradiol concentrations for the samples, the inverse of the Standard curve is needed, as mentioned in the previous chapter. This allows the value for percent bound to be plugged into the trendline equation to get its equivalent estradiol concentration. Finding the inverse of Figures 4.3 & 4.4 yields Figures 4.5 & 4.6. The trendlines of those figures then were used to calculate the concentrations of estradiol for all the sample sets.

*Figure 4.3: Estradiol concentration versus percent bound for set 1 of method one*

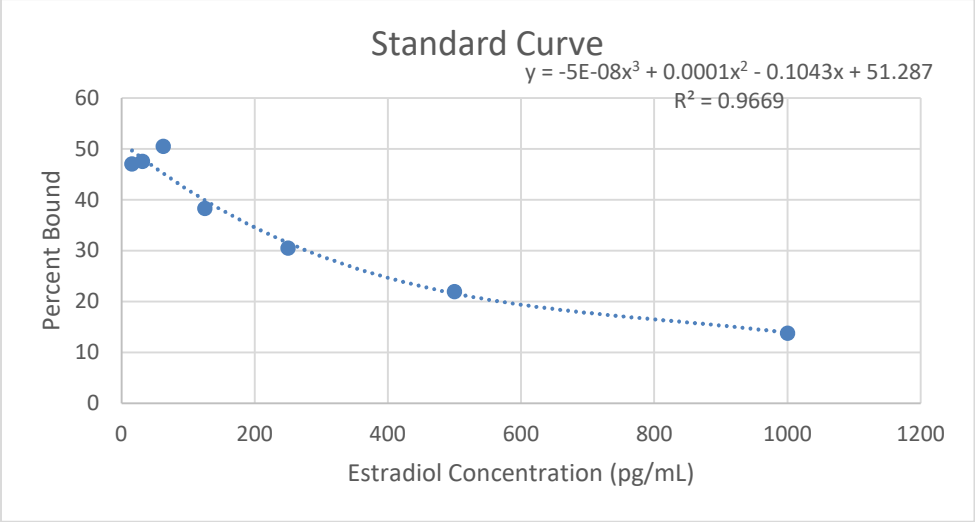



*Figure 4.4: Estradiol concentration vs percent bound for sets 2 & 3 for method 1 and all sets for method 2.*

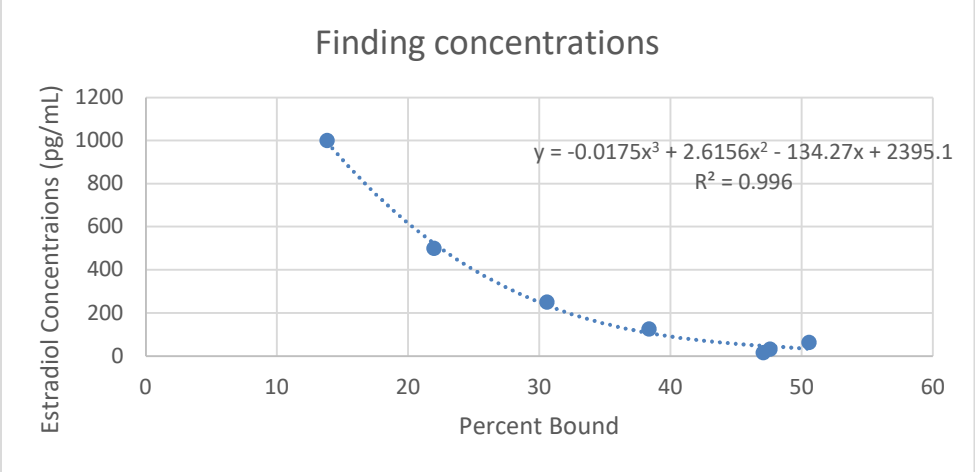


*Figure 4.5: Inverse of Figure 4.3 for calculation of estradiol concentration in samples*

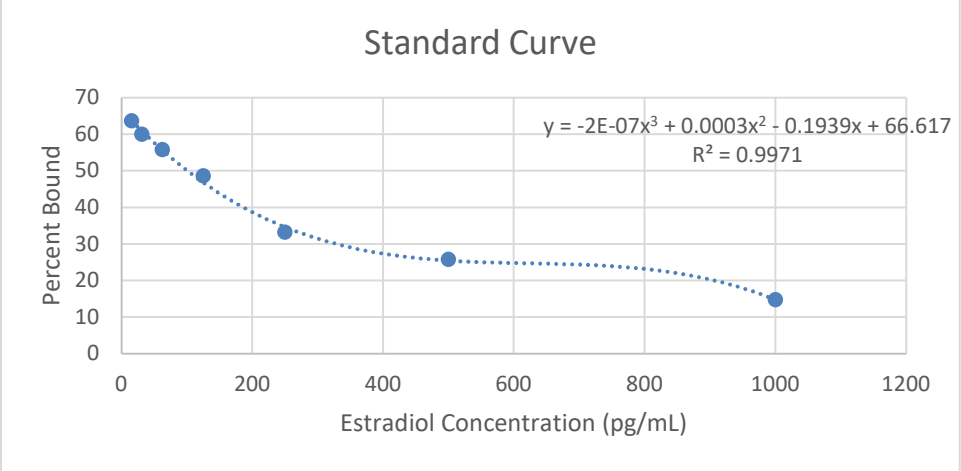



*Figure 4.6: Inverse of Figure 4.4 for calculation of estradiol concentration in samples*

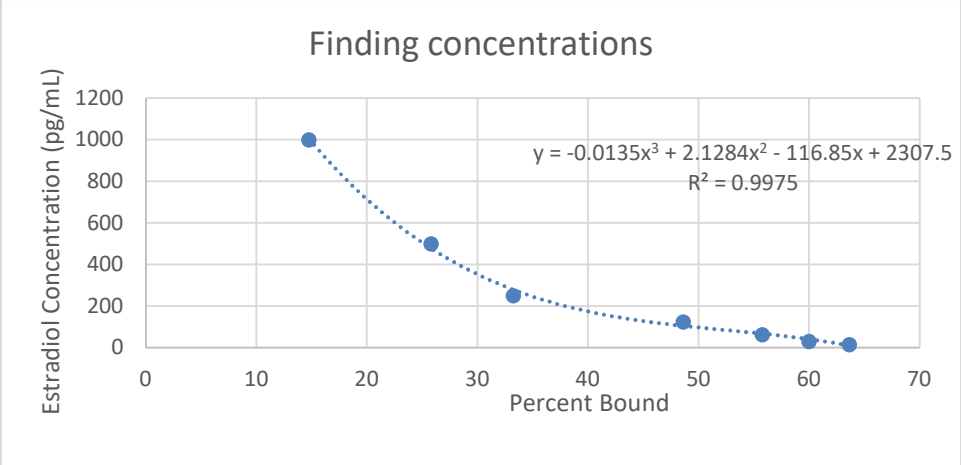


*Figure 4.7: Estradiol concentration versus time for first sample set of method 1*

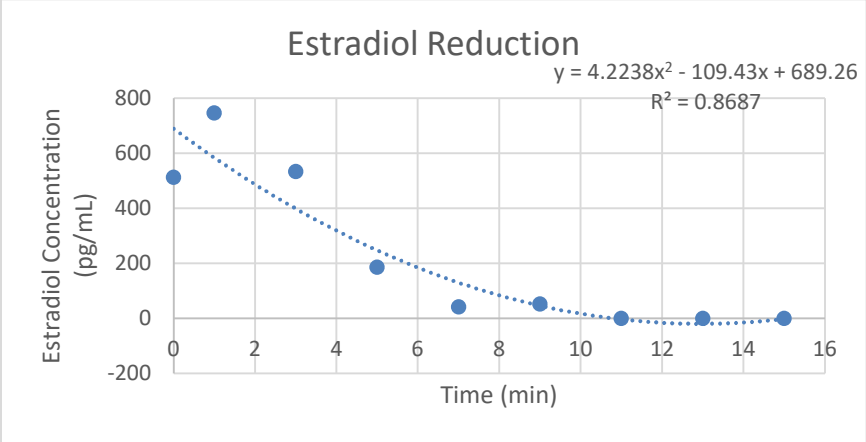
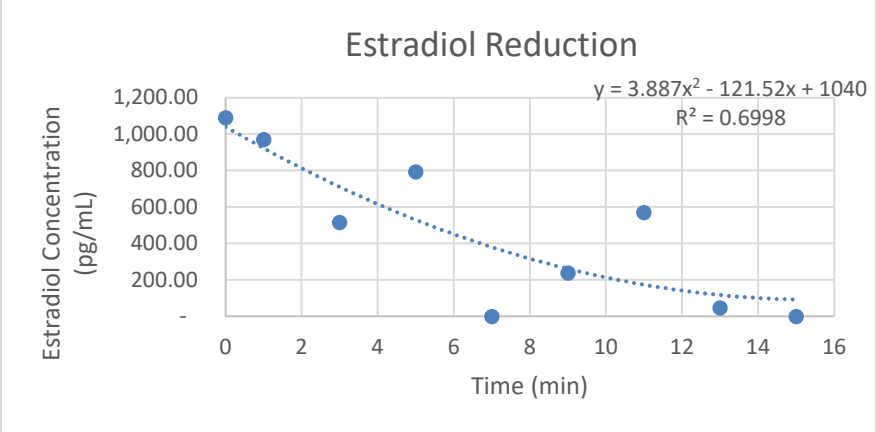



*Figure 4.8: Estradiol concentration versus time for second sample set of method 1*




*Figures 4.9: Estradiol concentration versus time for third run of method 1*

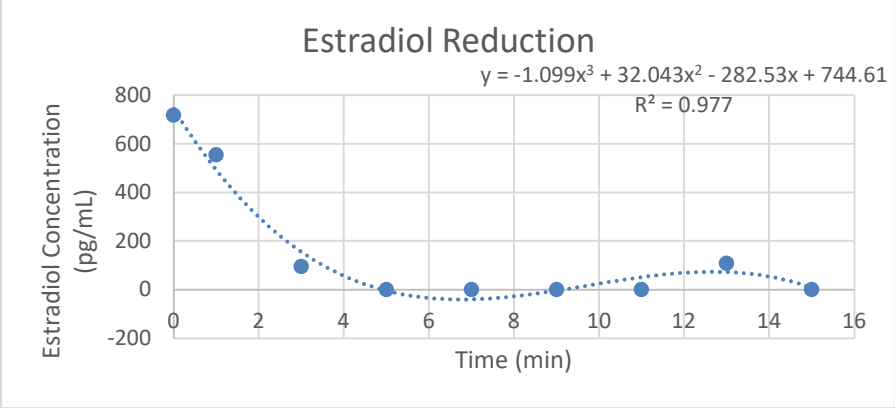
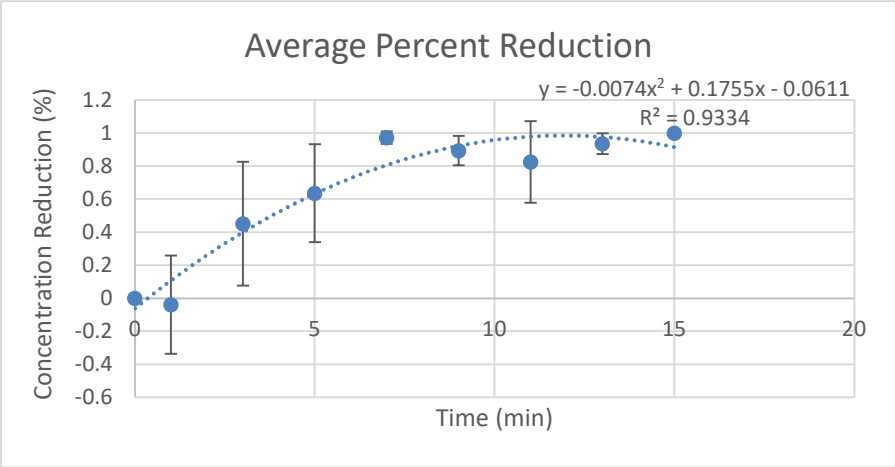


*Figure 4.10: Average percent reduction of experimental method 1*




The starting concentrations varied significantly between all three runs despite allowing for more than adequate mixing. This variation indicates an error either in the measuring of the concentrate for spiking the tank, in the creation of the concentrate, or in the filling of the tank with water. The sample sets are still able to be aggregated into a single result using percent reduction, but it does indicate a need for more precise measurement. All 3 sample sets had concentrations below the level of detection for the ELISA, at 14 pg/mL by 15 minutes. This is a significant improvement in removal efficacy over what was suggested by the trial run. Figure 4.10 has significant variation for the first several data points with the error bars shrinking as time continues. The deviation from the trend line may be due to incomplete mixing leading to pockets of higher or lower concentrations. It may also be due to how exactly the samples were collected, either taking the sample immediately or by allowing the possibly stagnant and unmixing water pooling in the spigot to run out for a few seconds before taking the sample.

Ozone readings were recorded from the ozone monitor connected to the headspace for every sample that was taken for the second and third sample sets of method one and for all sample sets of method two. The recorded ozone readings are shown in Figure 4.11. The readings show a clear downward trend over time which was not immediately explainable. In order to ascertain if the decline in concentration was due to the oxidative reactions taking place or due to some other source, a series three runs of the PIOx system without estradiol were completed. These dry runs were held for a total of one hour with ozone readings taken at 0, 1, 2, 3, 4, 5 minutes and every fifth minute after that. The results of those three dry runs are shown in Figures 4.12 & 4.13.

*Figure 4.11: Ozone in headspace*

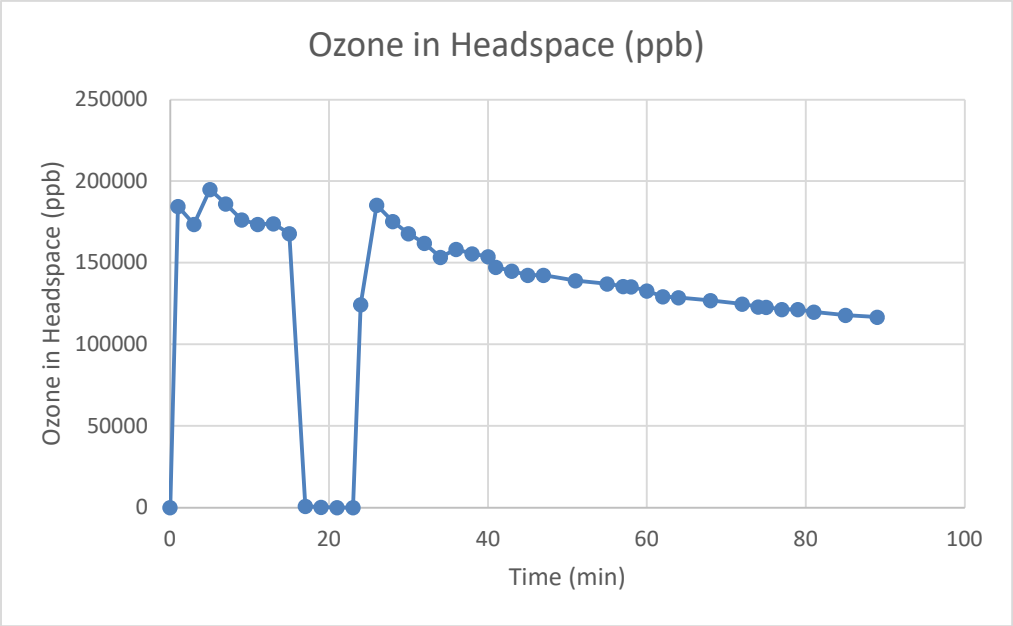


*Figure 4.12: Dry run ozone readings*

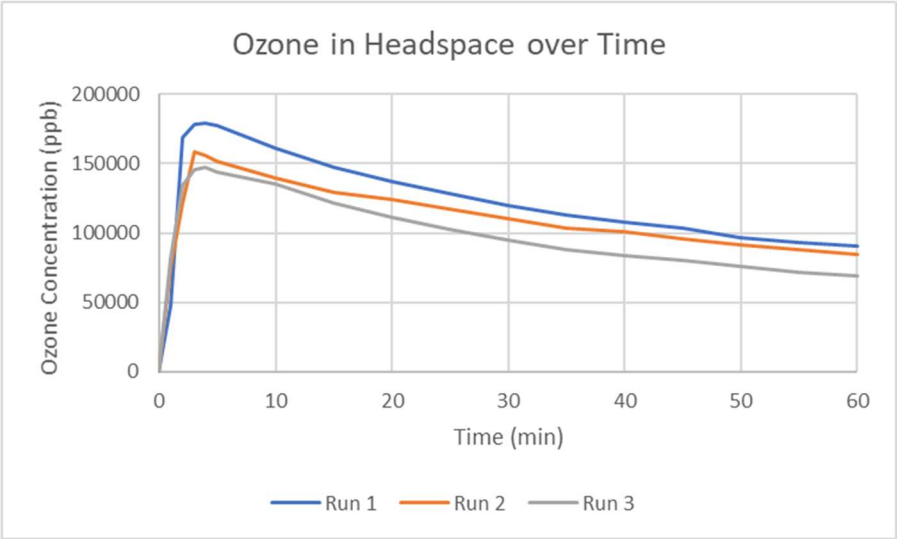



*Figure 4.13: Percent of maximum ozone remaining*

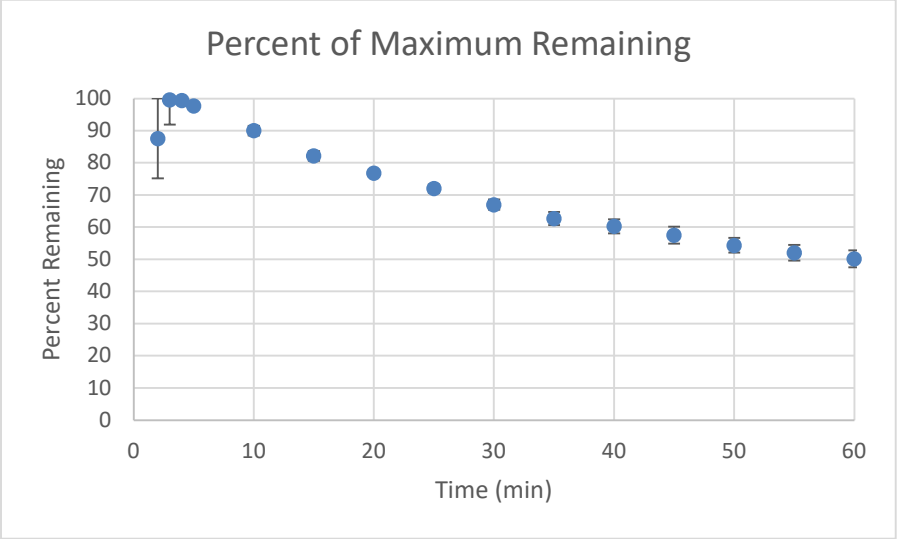


*Figure 4.14: Estradiol concentration versus time for first sample set of method 2*

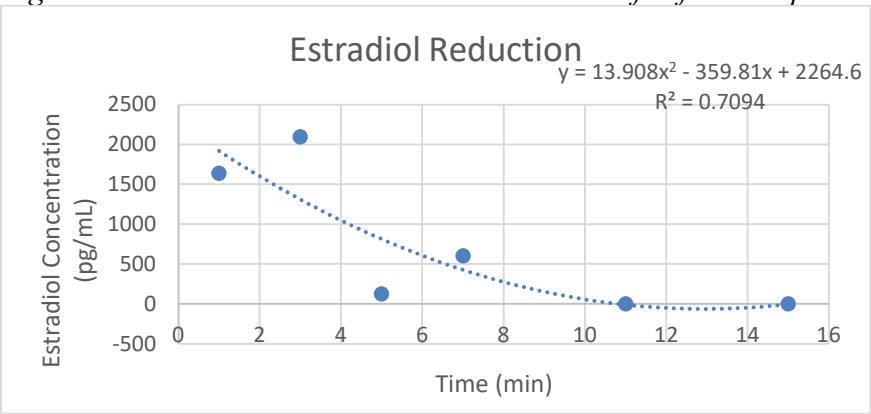
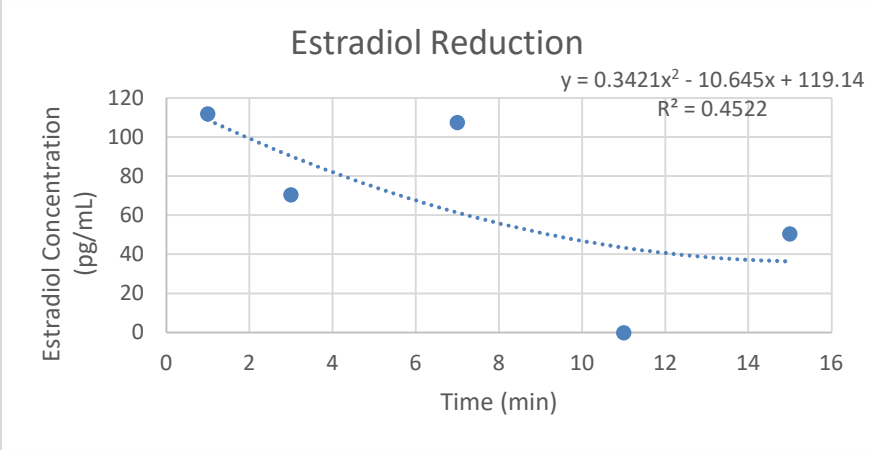
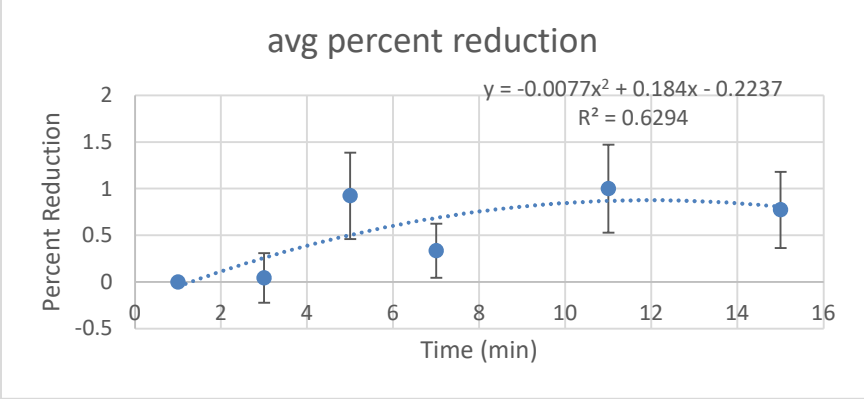



*Figure 4.15: Estradiol concentration versus time for second sample set of method 2*




*Figures 4.16: Average percent reduction versus time for experimental method 2*



The method two samples were less successful, with significantly more deviation from the trend. Only one of the two runs achieved concentrations below level of detection within the 15-minute testing time. This is almost certainly an effect of the lack of time given to ensure homogeneous concentrations leading to pockets of higher estradiol concentrations. This could be alleviated by increasing the length of exposure to ensure elimination.

The viability of the PIOx system can only be measure when compared to other studies and systems. The study by Irmak et al. (2005, p. 59) carried out similar oxidation experiments on estradiol using a combination of UV and ozone exposure. As noted previously the authors exposed 0.1 mmol of estradiol, equivalent to 27.24 mg, to several ozone flow rates from $15.89 \times 10^{-3}$ mmol/min to $7.56 \times 10^{-3}$ mmol/min. For the ozone/UV process this consumed 0.664 mmol of ozone, equivalent to 31.872 mg, to completely eliminate the estradiol concentration. This led to an "efficiency" of 6.64 moles of ozone required to consume 1 mole of estradiol. While the circumstances for this project were less controlled than theirs, owing to the inability to precisely gauge ozone production, it is still a reasonable comparison. For these experiments, 200 µg of the concentrate, which had a concentration of 0.8 mg/mL, was used to spike the system before each test. This equates to 0.16 mg of estradiol or $5.9 \times 10^{-4}$ mmol in the system. The one hour dry run of the system showed an ozone concentration from 160,000 ppb to 80,000 ppb. The ozone monitor samples directly from where the UV lamps are situated so it can be assumed that the volume for that concentration is localized around the lamps as well. The contact chamber around the lamps has a volume of approximately 134 $in^3$ or $2.2 \times 10^{-3}$ $m^3$.

Converting from ppb to $mg/m^3$ and then multiplying by the chamber volume gives a total production over one hour of 8.77 mg of ozone and a production rate of 0.146 mg/min or $3.0x10^{-3}$ mmol/min. Over the 15 minute testing period, the samples would be exposed to $4.56x10^{-2}$ mmol of ozone. If all of the produced ozone reacted with the estradiol, this would give an ozone/estradiol ratio of 77.29. The calculations are shown below in Figure 4.17.

*Figure 4.17: Spreadsheet calculations of PIOx ozone production*

| | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| 1 | | ozone (ppb) | | | | | | | |
| 2 | Time (min) | Run 1 | Run 2 | Run 3 | Avg | ppm | 1 ppm=2.14 mg/m^3 | mg | |
| 3 | 0 | 10.0 | 13.6 | 13.5 | 12.4 | 0.012 | 0.026 | 0.000 | |
| 4 | 1 | 47220.0 | 75268.9 | 82335.0 | 68274.6 | 68.275 | 146.108 | 0.322 | |
| 5 | 2 | 168855.6 | 121636.3 | 134884.0 | 141792.0 | 141.792 | 303.435 | 0.668 | |
| 6 | 3 | 178088.5 | 158444.3 | 146046.2 | 160859.7 | 160.860 | 344.240 | 0.758 | |
| 7 | 4 | 178846.6 | 155941.3 | 147119.9 | 160635.9 | 160.636 | 343.761 | 0.757 | |
| 8 | 5 | 177808.3 | 151738.0 | 143999.6 | 157848.6 | 157.849 | 337.796 | 0.743 | |
| 9 | 10 | 161199.1 | 139742.3 | 135101.8 | 145347.7 | 145.348 | 311.044 | 0.685 | |
| 10 | 15 | 147294.9 | 129633.0 | 121222.9 | 132716.9 | 132.717 | 284.014 | 0.625 | |
| 11 | 20 | 136818.9 | 124531.1 | 111082.3 | 124144.1 | 124.144 | 265.668 | 0.585 | |
| 12 | 25 | 128826.7 | 117265.6 | 103103.1 | 116398.5 | 116.398 | 249.093 | 0.548 | |
| 13 | 30 | 120049.1 | 110075.0 | 94764.9 | 108296.3 | 108.296 | 231.754 | 0.510 | |
| 14 | 35 | 112821.5 | 103330.3 | 88094.5 | 101415.4 | 101.415 | 217.029 | 0.478 | |
| 15 | 40 | 107531.7 | 100755.7 | 84088.7 | 97458.7 | 97.459 | 208.562 | 0.459 | |
| 16 | 45 | 103236.7 | 95454.6 | 80372.5 | 93021.3 | 93.021 | 199.066 | 0.438 | |
| 17 | 50 | 96386.5 | 91192.4 | 76042.2 | 87873.7 | 87.874 | 188.050 | 0.414 | |
| 18 | 55 | 93084.3 | 87658.6 | 71917.0 | 84220.0 | 84.220 | 180.231 | 0.397 | |
| 19 | 60 | 90277.7 | 84334.5 | 68914.3 | 81175.5 | 81.176 | 173.716 | 0.382 | |
| 20 | | | | | | | | | |
| 21 | | Volume | | | | | Total production | 8.76724 | mg |
| 22 | in^3 | 134.3031 | | | | | Production Rate | 0.146121 | mg/min |
| 23 | m^3 | 0.0022 | | | | | | 0.003044 | mmol/min |

This ratio is significantly higher than the ratio obtained by the authors and even further away from the theoretical minimum ratio. The most likely reason for the discrepancy is the lack of precise dosages, the inability to determine how much of the ozone goes unreacted due to the system constantly producing more ozone. The ozone production rate is an underestimation as ozone concentrations in the water were unable to be accurately

measured by ad hoc colorimetric tests. In addition, the ozone could diffuse into the headspace of the main tank which could partially explain the consistent decline in ozone concentrations during the tests.

There are a few different ways this set of experiments could be improved which could also lead to improved efficiencies for the PIOx system. A secondary ozone monitor drawing samples from the headspace of tank after the contact chamber could be installed as a means of possibly determining the quantity of unreacted ozone. This would hinder the system's ability to run with a completely full tank but would aid in accurately measuring important metrics.

A carbon dioxide monitor could also be installed as another metric of estradiol elimination. In addition, a carbon dioxide monitor would give an indication as to the extent of the degradation of the intermediate products. As shown by (Eisenhaugher,1971, p. 207), the oxidative process of phenol has carbon dioxide as a byproduct at several steps from the rupturing of aromatic ring and the stripping of the carbon atoms from its structure. As estradiol has a phenolic group, it is reasonable to assume that the oxidation of estradiol would lead to carbon dioxide also being a byproduct.

A final improvement to the system could be the inclusion of a polychromatic medium pressure UV lamp rather than the predominantly monochromatic UV lamp that is currently a part of the PIOx system. As shown previously in research by (Rosenfeldt &

Linden, 2004, p. 5479), a polychromatic UV lamp would allow for significantly more oxidation by direct photolysis due to the increased emission band allowing for more absorption and an increase in ozone production if the wavelength is in the 100-200nm range.

# Chapter 5

## Conclusions and Recommendations

The PIOx system proved to be quite effective in the idealized scenario of the Method one with removal below level of detection within 15 minutes. However, Method two shows that the system has some difficulties handling the sudden introduction of contaminants similar to that of a treatment environment with full reduction not occurring within the 15-minute testing window. When compared to other similar oxidative processes, PIOx is less efficient requiring more ozone to oxidize estradiol though that can be partially explained by the inability to accurately measure unreacted ozone. Overall the system is capable of eliminating estradiol at concentrations equivalent to environmental concentrations within a reasonable timeframe. It is reasonable to assume that it would be equally capable in oxidizing other ozone sensitive compounds in a similar manner.

The system could be improved by installing a carbon dioxide monitor and an additional ozone monitor to allow for observation of waste ozone and byproducts of the oxidative process. Because the PIOx system functions as continuous flow stirred tank reactor when operating in its original capacity, it could be implemented in a similar manner in a water or wastewater treatment plant as a tertiary treatment. Proper installation of the system could allow for longer exposure periods which would increase the likelihood of completely eliminating estradiol and other EDCs.

One significant hurdle that will need to be overcome to allow for widescale implementation of the PIOx system and other AOPs is the expense both in time and costs for testing contaminant concentrations. To accurately measure the concentration of estradiol specifically or EDCs in general, either an assay for each contaminant would need to be purchased, which would be costly and tedious, or a detection technique like HPLC or MS would need to employed which requires expensive equipment. Water and wastewater treatment plants run multiple tests on a daily basis to ensure the contaminant standards are being met and that any unusual spikes of regulated contaminants can be compensated for. If EDCs are to be included in that list of regulated compounds, then quicker and less expensive detection methods need to be explored to ensure that standards can be met.

The heart of the PIOx system is just the UV bulbs and the sleeve to create the micron foam. Therefore, scaling the system is just a matter of more UV bulbs, larger or more numerous sleeves, and higher capacity pumps that are capable of outputting at the required pressure to maintain the microfoam. There are other considerations that need to be explored before commercial implementation. How the size of any dissolved solids in solution may affect the design life of the PIOx system needs further exploration, in addition to how the bulbs' capability degrades under commercial conditions. Maintenance protocols for cleaning the metal screen would need to be established in addition to exploring how the system design could be modified to expedite the refurbishment or replacement of the screen and bulbs.

Further research is needed to determine what other EDCs the PIOx system is capable of removing. PIOx is behaving similarly to other UV/O3 AOPs but tests should be conducted using other EDCs to determine the capabilities of the system and where its limits are. Should the system maintain its similarity to other AOPs, PIOx could have the largest impact in more rural settings and smaller communities due to its more modular nature. Small scale applications would require minimal resizing allowing relative ease in set up.

There are other assays and methods of detection that provide near real-time results. These methods, which include an immuno-polymerase chain reaction and the use of estradiol imprinted nanoparticles and atomic force microscopy, are the subjects of research papers and are not commercially available. Reaching out to the authors of these projects would be useful to the continued exploration and application of the PIOx system to wastewater and water treatment.

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Appendix A. Plate Layout Sheet for ELISA Kit



• 17β-ESTRADIOL PLATE LAYOUT:

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 Blank | A2 Std 1 | A3 Std 5 | A4 M1R2 | A5 M1R2 | A6 M1R3 | A7 M1R3 | A8 M1R3 | A9 M2R1 | A10 M2R2 | A11 M2R2 | A12 M2R3 |
| | | | T1 | T9 | T0 | T7 | T15 | T7 | T3 | T15 | T7 |
| B1 Blank | B2 Std 1 | B3 Std 5 | B4 M1R2 | B5 M1R2 | B6 M1R3 | B7 M1R3 | B8 M1R3 | B9 M2R1 | B10 M2R2 | B11 M2R2 | B12 M2R3 |
| | | | T1 | T9 | T0 | T7 | T15 | T7 | T3 | T15 | T7 |
| C1 TA | C2 Std 2 | C3 Std 6 | C4 M1R2 | C5 M1R2 | C6 M1R3 | C7 M1R3 | C8 M2R1 | C9 M2R1 | C10 M2R2 | C11 M2R3 | C12 M2R3 |
| | | | T3 | T11 | T1 | T9 | T9 | T11 | T5 | T1 | T11 |
| D1 TA | D2 Std 2 | D3 Std 6 | D4 M1R2 | D5 M1R2 | D6 M1R3 | D7 M1R3 | D8 M2R1 | D9 M2R1 | D10 M2R2 | D11 M2R3 | D12 M2R3 |
| | | | T3 | T11 | T1 | T9 | T1 | T11 | T5 | T1 | T11 |
| E1 NSB | E2 Std 3 | E3 Std 7 | E4 M1R2 | E5 M1R2 | E6 M1R3 | E7 M1R3 | E8 M2R1 | E9 M2R1 | E10 M2R2 | E11 M2R3 | E12 M2R3 |
| | | | T5 | T13 | T3 | T11 | T3 | T15 | T7 | T3 | T15 |
| F1 NSB | F2 Std 3 | F3 Std 7 | F4 M1R2 | F5 M1R2 | F6 M1R3 | F7 M1R3 | F8 M2R1 | F9 M2R1 | F10 M2R2 | F11 M2R3 | F12 M2R3 |
| | | | T5 | T13 | T7 | T11 | T3 | T15 | T7 | T3 | T15 |
| G1 Bo | G2 Std 4 | G3 M1R2 | G4 M1R2 | G5 M1R2 | G6 M1R3 | G7 M1R3 | G8 M2R1 | G9 M2R2 | G10 M2R2 | G11 M2R3 | G12 |
| | | T0 | T7 | T15 | T5 | T13 | T5 | T1 | T11 | T5 | |
| H1 Bo | H2 Std 4 | H3 M1R2 | H4 M1R2 | H5 M1R2 | H6 M1R3 | H7 M1R3 | H8 M2R1 | H9 M2R2 | H10 M2R2 | H11 M2R3 | H12 |
| | | T0 | T7 | T15 | T5 | T13 | T5 | T1 | T11 | T5 | |

Kit Lot No. 032002 Exp. Date 30 Jun 2021 Date Oct 29 2020 Tech. A Pearson

1st Incub.: Start Time _____ Temp. _____ Notes: _____

End Time _____ Temp. _____

2nd Incub.: Start Time _____ Temp. _____

End Time _____ Temp. _____

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