Bioremediation of 17β-estradiol, an endocrine disrupting chemical, by the oyster mushroom, *Pleurotus ostreatus*

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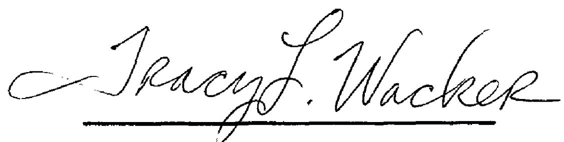
Approved By



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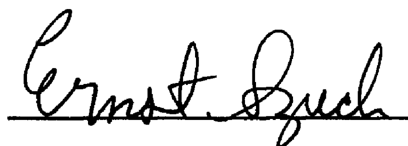

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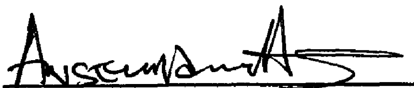

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ABSTRACT

This project investigated the abilities of *Pleurotus ostreatus* to sequester and break down the naturally occurring steroidal hormone 17-β estradiol through bioremediation in an aquatic environment. Pure fungal cultures were transferred to straw and wood substrate and colonized substrate was transferred to aquaria containing concentrations of estradiol in moving water. Samples were analyzed using High Performance Liquid Chromatography (HPLC-UV). Results were inconclusive due to the detection limitation of the HPLC-UV system utilized and the adsorption of 17β-estradiol onto components in the aquarium. In a separate experiment, water samples of the Flint River Watershed, were sent to Arizona Laboratory for Emerging Contaminants for analysis through GC/LC/MS. This analysis showed levels of 17α-ethynyl estradiol well above those that caused morphological and behavioral changes (40 ng/L) in fish during in vitro experiments. The results of the water sampling also included BPA, a chemical which mimics steroidal estrogens. Another experiment measured the dry weight of P. ostreatus when exposed to 17β-estradiol (mixed in acetone) for 18 days. There was not a significant difference in dry weights of *P. ostreatus* grown with or without estradiol. The final experiment involved uptake and adsorption of 17β-estradiol using a human serum ELISA kit after 8 days of exposure to estradiol. Results were inconclusive due to organic contaminants. Growth of mycelium was observed in all in vitro experiments. This could indicate *P. ostreatus* was not inhibited by 17β-estradiol and may be able to use it as a carbon source.

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Chapter 1: Introduction

Bioremediation is the use of biological organisms such as plants or microbes to aid in removing hazardous substances from a contaminated site. Bioremediation has been effective in removing bacterial contaminants from water treatment facilities, radioactive elements from contaminated soils, and petroleum from ocean spills. The technique can break down toxins and thereby improve the health of the afflicted ecosystem. Several studies have shown the benefits of using fungi as bioremediators (mycoremediation). Some examples include reduction of dioxins, anthracenes, and pesticides into less harmful by-products (Gadd 2001). Mycoremediation has shown great promise because basidiomycetous fungi, unlike plants and microbes can break down very complex organic compounds.

Mycoremediation

Pleurotus ostreatus, commonly known as the oyster mushroom, is a white-rot fungus. *P. ostreatus* belongs to the Class Basidiomycetes and is a saprotrophic species. These fungi are ecologically important because of their ability to biodegrade wood and other plant material. They are useful in mycoremediation because they are easily grown on straw, wood, and common mycological media.

In the degradation process there are three key groups to the decomposing process; primary, secondary, and tertiary. Primary decomposers include two functional groups, white-rot and brown-rot fungi. Brown-rot fungi are predominantly members of the Basidiomycota and degrade cellulose. These fungi have to deal with a sub-par substratum

depleted of simple sugars and nitrogen via parasites, bacteria, and 'sugar fungi.' They must hydrolyze the cellulose molecules into cellobiose and then glucose. After the cellulose is degraded the main material left is lignin (a heterogenous polyphenolic polymer) which is degraded by the white-rot fungi. There are few fungi that can decompose this complex material and all of them belong to Basidiomycota (Moore-Landecker 1996). Lignin degradation during secondary metabolism provides access to wood polysaccharides without a net energy gain (Pointing 2001). The fungi require some other carbohydrate, because the lignin-decomposing fungi are not capable of using lignin as the sole carbon source (Moore-Landecker 1996). The enzymes associated with the breakdown of lignin are: lignin peroxidase (LiP), manganese peroxidase (MnP), laccase (Lac), and versatile peroxidase (VP). What is significant about these enzymes is that they have a highly non-specific substrate range. White-rot fungi are capable of "in vitro transformation or mineralization of a highly recalcitrant organopollutants with structural similarities to lignin" (Pointing 2001). Both *P. ostreatus* and *Phanerochaete spp.* are primary decomposers and white-rot fungi that show mycoremediation promise.

Phanerochaete chrysosporium can transform TNT (Trinitrotoluene), an explosive RDX (hexa-hydro-1,3,5-trinitro-1,3,5-triazine), and persistent and toxic PAHs (polynuclear aromatic hydrocarbons; constituents of creosote). Pentachlorophenol (PCP) is an organic compound used in wood preservation, as a pesticide and fungicide. Fungi that produce large amounts of laccase enzyme, such as *P. ostreatus*, are able to degrade PCP (Ramesh and Pattar 2010). Several species of fungi are also able to convert insoluble metal compounds (from certain oxides and phosphate compounds) into soluble forms. The fungi are able to excrete protons and various metabolites including organic

acids. One advantage of fungi in mycoremediation is their ability to leach high levels of toxic metals in low or high pH environments. Most leaching applications that use bacteria find they are unsuitable candidates for bioremediation because of their inability to survive in elevated pH environments (Sayer and others 1995).

Terrestrial and aquatic ecosystems generally contain relatively low concentrations of bioavailable organic carbon and other nutrients. The ability of fungi to grow in soils is thought to be limited by the presence of low levels of available carbon (Fomina and others 2003). Starvation plays a key role in the growth of mycelium. Mycelial growth is favored by a monosaccharide (such as glucose or fructose). Mycelium plays an important role in breaking down plant remains. Maximum decomposition will occur only as long as there is an abundant supply of nitrogen and carbon compounds and other essential nutrients (especially phosphorus) present in the substratum or soil (Moore-Landecker 1995). During the colonization of solid substrates, hyphal extension provides a means of exploring new regions for fresh nutrients, whereas branch formation enables full utilization of the substrate already colonized. Under nutrient-poor conditions, branching of hyphae are sparser, and in nutrient-rich conditions extensive branch formation is utilized to fully take up available substrate (Fomina and others 2003).

Endocrine Disrupting Chemicals (EDCs)

An endocrine disrupter has been defined as "an exogenous substance or mixture that alters function (s) of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or subpopulations." (Vos and others 2000) The aquatic environment has been termed "the ultimate sink" for natural and man-made chemicals (Sumpter 1998). EDCs are constantly entering the aquatic environment through agricultural runoff from animal feeding operations, pulp mills, and sewage treatment effluent (Salierno and Kane 2009). EDCs are comprised of pharmaceuticals, personal care products, surfactants, various industrial additives and numerous chemicals purported to be endocrine disrupters. The total list includes 900 chemicals (IEH 2005). These EDCs can be found in sewage effluent in low concentrations (μg/L to ng/L) but have elevated estrogenic potencies. The low concentrations make them difficult to detect using analytical instruments. In addition, each compound requires different analytical methods using different techniques, extraction, and equipment. Therefore, there is a need for a low-cost procedure that works for detection of all EDCs and a low-cost method of eradicating them. Due to their hydrophobic properties, estrogens are considered "persistent under aerobic conditions" (Cajthaml and others 2009). An experiment studying 17α-ethynyl estradiol (EE2) in aquifer material found it was resistant to biodegradation and remained unchanged and persistent under aerobic and anaerobic conditions (Ying and others 2004). Zuehlke and others (2004) hypothesized that estrogenic steroids should be adsorbed in the aquatic sediments and would unlikely be found in subsoil and groundwater. However, several positive detections of estrogens were found in ground water and drinking water in Germany (Zuehlke and others 2004).

The major route of exposure for humans and animals is through diet. Ingestion of EDCs leads to bioaccumulation and biomagnification, especially for species at the top of the food chain.

Estrogens direct the development of the female phenotype in embryogenesis and during puberty by regulating gene transcription and, thus, protein synthesis. It also induces the production of gonadotropins, which in turn, induces ovulation. EE2 is an oral contraceptive and is synthesized from 17β-estradiol (E2). E2 is excreted from humans and other animals and is the major estrogen secreted by the premenopausal ovary. EE2 is considered a carcinogenic risk to humans because estrogens can stimulate the growth of human breast cancer cells (Cajthaml and others 2009). Studies in humans strongly suggest that the administration of estrogens is causally related to an increased incidence of endometrial carcinoma and there is evidence that this is similar for all estrogens. Bisphenol A (BPA) and certain pesticides are considered EDCs due to their ability to bind to estrogen receptors. BPA is produced in large quantities for the production of polycarbonate plastics and epoxy resins. Polycarbonate plastics have many applications including use in food and drink packaging, e.g. water and infant bottles, compact discs, impact-resistant safety equipment, and medical devices. Epoxy resins are used as lacquers to coat metal products such as food cans, bottle tops, and water supply pipes. Some dental sealants and composites may also contribute to BPA exposure (Bucher and Shelby n.d.). In general, the reproductive physiology of vertebrates is similar and reproduction involves the hypothalamus, pituitary, and gonads.

Mammals release GnRH (gonadotrophin-releasing hormone) to the pituitary gland, which secretes gonadotrophic hormones (GTH) then signaling the gonads to produce steroid hormones (Mills and Chichester 2005).

There is the possibility that chronic low-level exposure to estrogenic chemicals even after maturity can have similar effects in humans as demonstrated in other animals. A model for human exposure to steroidal estrogens is the use of Diethylstilbestrol (DES) from 1948 to 1971, and prescribed by physicians to prevent spontaneous abortions. Daughters of mothers who took DES suffered from reproductive organ dysfunction, abnormal pregnancies, and a reduction in fertility, immune system disorders, and periods of depression. (Colborn and others 1993)

In fish, the secretion of GnRH can change due to environmental cues. (Dawson 1998) Fish species vary between viviparous and oviparous and sex determination can be genetic or phenotypic based on temperature. Fish species can be dioecious or hermaphroditic or may be sequentially hermaphroditic (male to female or female to male) (Mills and Chichester 2005). All of these factors make fish sensitive to environmental changes and therefore, good environmental health indicators. In the aquatic environment, EDCs become available to fish through aquatic respiration, osmoregulation, and maternal transfer of contaminants in lipid reserves of eggs. Also, additional routes are through contact with contaminated sediments and ingestion of contaminated food (Mills and Chichester 2005). An indicator of exposure to EDCs in fish is vitellogenin. Vitellogenin is a phospholipoglycoprotein produced in the liver of female fish, which is regulated by circulating estrogen (Salierno and Kane 2009). If it is being produced by males or juvenile fish then high concentrations of EDCs could be to blame.

Reaction to EDCs varies with the species of fish and its reproductive physiology, water temperature, migratory behavior, prior history of EDC exposure, and type of EDC exposure. Several characteristics of exposure to EDCs include: reduced, absent, or inhibited egg production; decreased male sexual behavior; all female fish; males with testis-ova; reduced testicular growth; and even mortality (Mills and Chichester 2005) Exposure to EE2 and BPA causes reduced egg production, reduced hatching, males with testis-ova, and high male mortality (Metcalfe and others 2001). Exposure to EDCs can cause behavioral and morphological changes. These changes can affect the reproductive fitness of the species and the decline could possibly lead to extinction.

Wastewater Treatment

Currently, the conventional waste water treatment plants (WWTP) are not effective in the elimination of newly identified contaminants due to lack of regulations and monitoring of these EDCs. It is extremely important to remove EDCs from wastewater, therefore preventing contamination of ground water and drinking water. Current technologies focus on nanofiltration and reverse osmosis membranes (Bolong and others 2009). However, many different factors influence the efficiency of these technologies and they are expensive. Therefore, there must be additional treatment processes at the wastewater treatment plants before the effluent enters the waterways.

The aim of this project was to create an inexpensive method to remediate contaminated water using a natural bioremediator, *P. ostreatus*. *Phanerochaete sordida* (another white-rot fungus) decreased the natural steroidal hormone, estrone, in a low-nitrogen and high-carbon culture medium. (Tamagawa and others 2006).

My study attempts to replicate the results with a key difference, low-carbon culture medium was used to encourage exploration by the mycelia for additional nutrition sources. It was hypothesized that *P. ostreatus* placed in a low carbon environment could use 17β-estradiol as a sole carbon source. It would then utilize the 17β-estradiol and remediate the aquatic pollutant from the environment. Experiment one focused on water quality sampling of the Flint River. Experiment two was an aquatic bioremediation project which focused on the ability of *Pleurotus ostreatus* to sequester and break down the naturally occurring steroidal hormone 17-β estradiol in an aquatic environment. Experiment three measured the dry weight of P. ostreatus when exposed to 17β-estradiol (mixed in acetone) for 18 days. The final experiment involved analysis of *P. ostreatus* uptake and adsorption of 17β-estradiol using a human serum ELISA kit after 8 days of exposure to estradiol.

Chapter 2: Water Quality Sampling

Methods and Materials

Water Sampling

Water samples of one liter were collected in eight (1 liter) amber glass sterilized bottles using a Kemmerer water sampling device. Samples were collected at two locations: site one was Montrose, MI with samples taken upstream and downstream from the wastewater treatment plant; site two was Flushing, MI with samples taken upstream and at the point source from the wastewater treatment plant (Table 1). The first water samples were taken on August 24, 2010 and stored at 4°C from site 1 at 3:46 p.m., site 2 at 4:18 p.m., site 3 at 4:48 p.m., and site 4 at 5:05 p.m. On August 25, 2010, samples were taken at site 1 at 10:45 a.m., site 2 at 11:10 a.m., site 3 at 11:40 a.m., and site 4 at 11:55 p.m. Samples from both days were packed on ice on August 25, 2010, and sent overnight to Arizona Laboratory for Emerging Contaminants. Processing of samples began when received on August 26, 2010. GC/LC/MS was used to analyze samples for 17α-ethynyl estradiol and BPA (Appendix 1). Data were analyzed using Kruskal-Wallis test.

Table 1: Location of water sampling sites including city, GPS location, distance to wastewater treatment plant, and elevation.

Sites	*GPS Location*		*Distance to WWTP*	*Elevation*
Site 1 Montrose, MI	*N 43° 14' 7.2"*	*W 83° 51' 56.2"*	*2.1 km Downstream*	*618 ft.*
Site 2 Montrose, MI	*N 43° 10' 22.6"*	*W 83° 52' 26.3"*	*4.9 km Upstream*	*624 ft.*
Site 3 Flushing, MI	*N 43° 04' 01.9"*	*W 083° 51' 44.1"*	*Point Source Direct Drain into Flint River*	*654 ft.*
Site 4 Flushing, MI	*N 43° 03' 36.2"*	*W 083° 51' 15.9"*	*1.9 km Upstream*	*679 ft.*

Results and Discussion:

Results for 17α-ethynyl estradiol were significant ($p<0.01$, K: 20.94, df=3) when comparing Montrose downstream and Flushing point source to Montrose upstream and Flushing upstream (Figure 1). Results for BPA were significant ($p<0.05$, K: 9.26, d=3) when comparing Montrose downstream and Montrose upstream to Flushing point source and Flushing upstream (Figure 2).

Montrose, MI, has a population of approximately 1600 and Flushing, MI, has a population of approximately 8000. Concentrations of 17α-ethynyl estradiol in all samples are well above those that cause morphological and behavioral changes (40 ng/L); indicating a need for further studies on wildlife populations in the Flint River Watershed. BPA has been linked to morphological changes in testes of several fish species and are caused by concentrations as low as 10 μg/L levels (Mills and Chichester 2005). In this study, results for BPA are lower than 10 μg/L, however, there is cause for concern for WWTP that service larger populations on the Flint River. Larger samples need to be taken and the river needs to be monitored on a frequent basis, especially in larger populated areas.

Figure 1: Comparison of 17-α-ethynyl estradiol in water samples taken from different sampling sites along the Flint River and analyzed by GC/LC/MS. There is a significant difference between Montrose Downstream and Flushing Point Source compared to Montrose Upstream and Flushing Upstream (Kruskal-Wallis, $p<0.01$, K: 20.94, df=3)

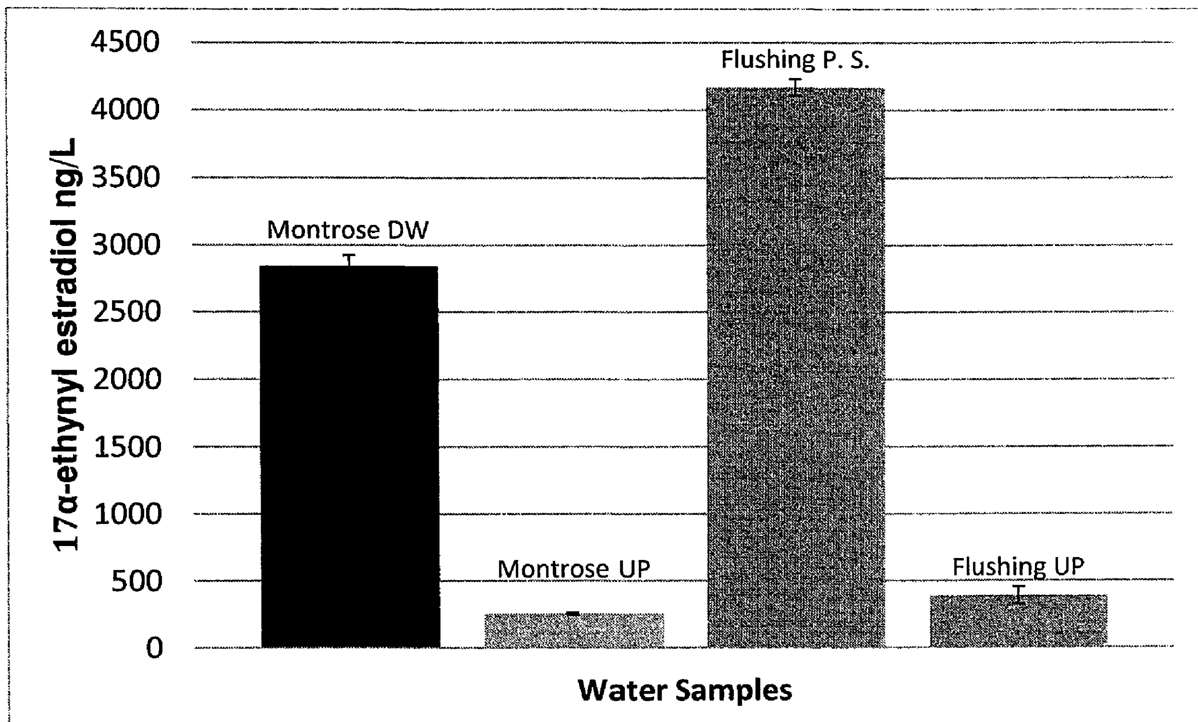
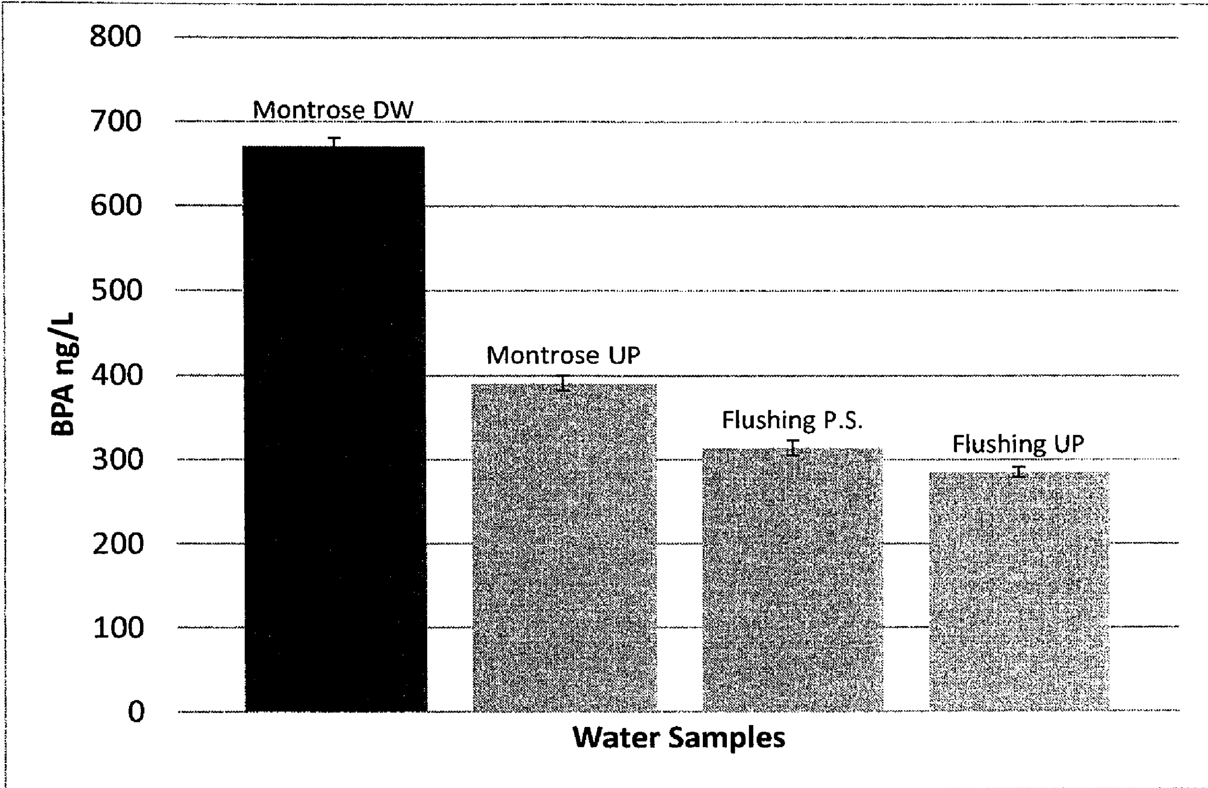


Figure 2: Comparison of Bisphenol A in water samples taken from different sampling sites along the Flint River and analyzed by GC/LC/MS. There is a significant difference between Montrose Downstream and Montrose Upstream compared to Flushing Point Source and Flushing Upstream (Kruskal-Wallis, $p<0.05$ K: 9.264 df=3)



Chapter 3: Mycoremediation Project

Methods and Materials:

Fungal Species, media and culture conditions

Pleurotus ostreatus samples were acquired from a commercial source and cultured on malt extract agar (MEA) amended with Gentamycin (200 mg/20 ml MEA Sigma-Aldrich). Cultures were subsampled weekly until a pure culture was obtained. Rye Grain (800 cm^3) with water (400 ml) was autoclaved for 20 minutes in a 1500 ml Erlenmeyer flask. Three plugs approximately 0.05 cm^2 of *P. ostreatus* were aseptically transferred to rye grain and incubated at 20°C for 6 weeks with shaking of flask every three days to encourage evenness of mycelial growth.

Two different substrates, cereal straw (400 g) and Poplar wood shavings (400 g), were autoclaved (121°C; 20 psi) for 60 minutes, soaked in water overnight, and then transferred to spawn bags ("12 x 24", "Fungi Perfecti®") the following day. They were then drained of excess water and autoclaved again (121°C; 20 psi) for 30 minutes. One tenth of the inoculated rye grain was placed inside each spawn bag to inoculate the cereal straw and poplar wood and sealed with an impulse sealer. (Stamets and Chilton 1983). Spawn bags were incubated at 20°C for 10 weeks. The remaining grain was placed in an autoclaved (121°C; 20 psi) sealed bag for use in the repeat experiment.

Aquarium set-up

Eight 20 gallon aquaria were filled with twelve liters of distilled water. Inside each aquarium is a 15.24 x 30.5 cm galvanized hardware cloth box and plastic tubing with 2.54 cm spaced holes connected to an air supply. Packets of charcoal fiberglass screen (13.2 x 26.4 cm) were filled with one of the following: 100 g of straw inoculated with fungus, 100 g of wood inoculated with fungus, 100 g of cereal straw or poplar wood without fungus. Packets were added to the appropriate aquaria and labeled: Tank 1-Control Wood, Tank 2-Control Straw, Tank 3 (1)-Wood inoculated with *P. Ostreatus*, Tank 4 (2)-Wood Inoculated with *P. Ostreatus*, Tank 5 (3)-Wood inoculated with *P. Ostreatus*, Tank 6 (1)-Straw inoculated with *P. ostreatus*, Tank 7 (2)- Cereal Straw inoculated with *P. ostreatus*, Tank 8 (3)-Straw inoculated with *P. Ostreatus*. Each tank was inoculated with 12 ml of 17β-estradiol stock solution (100 mg/ml 17β-estradiol and water). Water levels were maintained at 12 liters; room temperature was maintained at 18° C. Air supply was maintained at a pace that created small bubbles to mimic a small natural stream and tanks were covered with plastic to prevent evaporation.

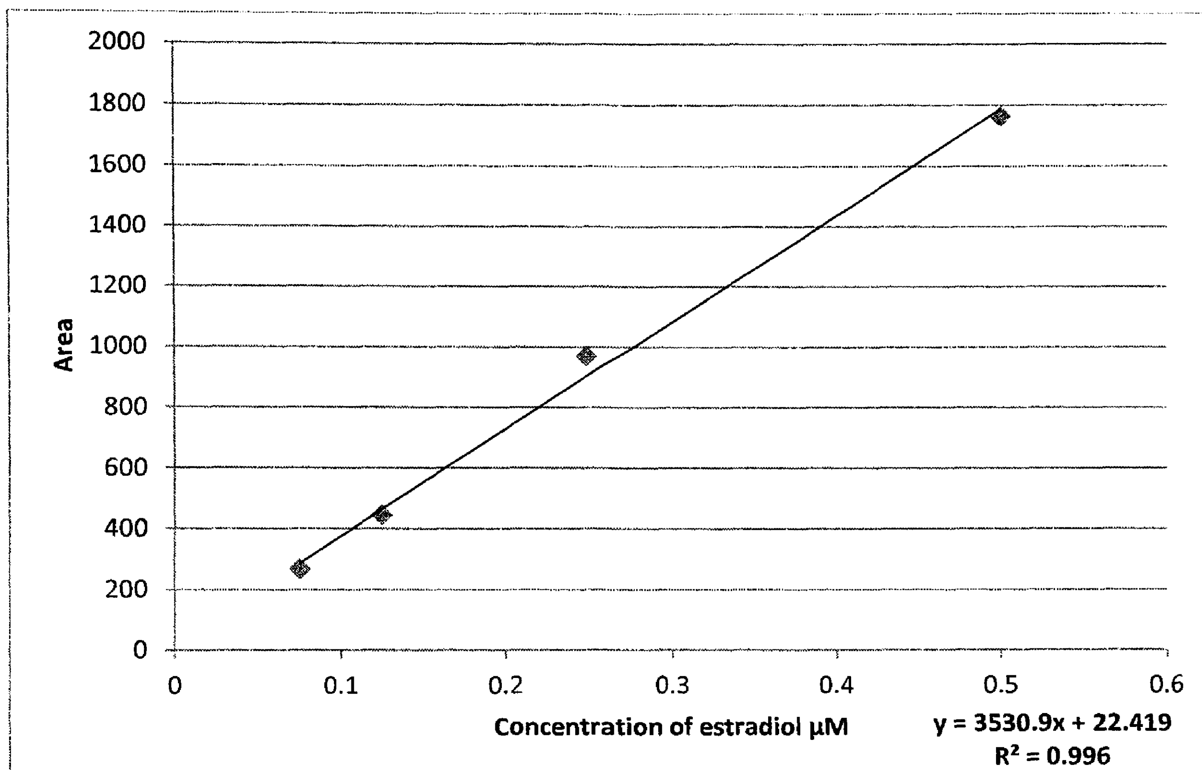
Water samples were taken from the middle of the front of the tank on day six and day twelve using a sterile pipetman (three 1.0 ml subsamples) and stored at 4°C. The experiment was replicated once.

Detection Method

Samples were analyzed using an Agilent 1200 series High Performance Liquid Chromatography (HPLC). A standard curve was produced using a stock solution of 50 mg of 17β-estradiol in 100 ml MeOH. One ml was then added to 783 μL in a 5 ml flask and then diluted with mobile phase.

The standard curve was established using 0.075, 0.25, 0.125, and 0.5 μM concentrations (Figure 3). One ml of sample was then added to a HPLC vial and placed in an autosampler. A Waters Corporation XBridge^{Tm} phenyl-hexyl column (3.5 μm, 2.1 x 150 mm) was used for separation using an isocratic mobile phase of 60% methanol/acetone (95:5) and 40% water with a flow rate of .3 ml/min, an injection volume of 10 μL, at a detection of 225 nm (Havlikova and others 2006). Retention time on this column is approximately 6.7 minutes.

Figure 3: *Calibration curve for 17β-estradiol (HPLC) in mobile phase at 0.075, 0.125. 0.25. and 0.5 μM concentrations.*



Results and Discussion:

Pleurotus ostreatus showed positive mycelial growth in the aluminum bags after six days and all aquaria containing fungus inoculated packages were fruiting. *P. ostreatus,* was not inhibited by the steady flow of water and the saturation of either substrate. This was an important result because this non-aquatic species was able to survive in abnormal conditions (water and 17β-estradiol stock), which may indicate its use in other applications.

The stock solution was prepared in water, which was recommended, before it was realized these are extremely hydrophobic compounds, and dosing could not be assumed to be accurate. Aquaria had large amount of water as well as different materials in the tanks (PVC, hardware cloth, aluminum mesh, wood or straw). Xenobiotic-transforming fungi need an additional carbon source because they cannot utilize the substrates as an energy source for growth. Therefore, lignocellulosic wastes such as corncobs, straw, and sawdust are used as nutrients to enhance pollutant degradation (Gadd 2007). This could cause hydrophobic compounds to adhere to the material instead of circulating in the water. A calibration curve was obtained for 17β-estradiol and is shown in Figure 3. However, the samples analyzed were below the detection limit of the HPLC-UV system.

Future studies of E2 samples at such low concentrations would require additional techniques such as GC-MS (gas chromatography- mass spectrophotometry), LC-MS (liquid chromatography- mass spectrophotometry), LC-MS/MS (liquid chromatography-mass spectrophotometry- mass spectrophotometry), or GC/LC/MS (gas chromatography-liquid chromatography- mass spectrophotometry). These instruments can process very low concentrations but sometimes still require pre-concentration steps. Unfortunately, we do not have the aforementioned equipment on our campus and must rely on outside laboratories, which is very expensive. This also requires extensive funding that isn't available at this time.

Chapter 4: Growth of *Pleurotus ostreatus* in 17β-estradiol amended medium

Methods and Materials

Pure cultures of *Pleurotus ostreatus* were maintained on MEA plates and subcultured bi-weekly. Sixteen 250 ml flasks were prepared with 100 ml of Kirk's medium (Appendix 2) and autoclaved for 20 minutes (121°C; 20 psi); after cooling all flasks were inoculated with 4 mm diameter plugs of *P.ostreatus*. Eight experimental flasks were inoculated with 25 µL of 17β-estradiol stock solution (4 mg of 17β-estradiol per 1 ml of acetone). Cultures incubated for 18 days at room temperature on an orbital shaker at 125 rpm and were vacuum filtered onto Whatman 4 filter paper, dried for 24 hours at 46°C, and weights recorded. Aqueous samples (1.8 ml) were taken from each flask and stored at 4°C to be analyzed by ELISA (Chapter 5). Data were analyzed using a paired t-test (Appendix 3).

Results and Discussion:

After 18 days both experiment one and experiment two did not generate significant results. It was a concern that the acetone solvent could influence the growth of *P. ostreatus*. In the duplicate experiment 25 µL of acetone was added to the control flasks. Results showed no significant difference with the acetone added. In Chapter 5 an ELISA test was used to try to quantify the uptake of 17β-estradiol by *P. ostreatus*. Unfortunately, the ELISA test results were inconclusive due to unreliable results from the standards (Appendix 4). Further research should focus on finding a solvent that is more compatible with *P. ostreatus*. A larger sample size is also needed.

Table 2: In experiment 1 there is no significant difference between the control (*P. ostreatus* in medium) and the treatment (*P. ostreatus* in medium with .001 mg/ml 17β-estradiol in acetone). In experiment 2 there is no significant difference between the control (*P. ostreatus* in medium with 25 µl acetone) and the experiment (*P. ostreatus* in medium with .001 mg/ml 17β-estradiol in acetone).

	Control *(P. ostreatus in medium)* **Mean ± S.D.**	**Treatment** *(P. ostreatus in medium with 0.001 mg/ml 17β-estradiol in acetone)* **Mean ± S.D.**
Experiment 1	*68.5mg ± 14.4 mg*	*56.7 mg ± 12.6 mg*
Experiment 2 *(with acetone added to control)*	*83 mg ± 67.3 mg*	*65.3 mg ± 9.34 mg*

Chapter 5: Determination of utilization of 17β-estradiol by *Pleurotus ostreatus* or adsorption to substrates by ELISA

Methods and Materials

Cultures of *P.ostreatus* were maintained on Sabouraud Dextrose Agar and transferred weekly. Sixteen 250 ml flasks were prepared with 100 ml Kirk's Medium. All flasks were inoculated with 25 μl of 17β-estradiol stock solution (4 mg of 17β-estradiol per 1 ml of acetone). Treatments included: the control (estradiol only), 500 mg of cereal straw, 500 mg of poplar wood shavings, or a single plug of *P. ostreatus* (four flasks of each treatment). Cultures were incubated at room temperature (20°C) on an orbital shaker (125 rpm) for eight days. At the end of 8 days, samples (50 μl) were analyzed using Cayman Chemical Company Estradiol EIA kit (Catalog 582251). Absorbance values were obtained at 405 nm by a Biolog Microplate Reader.

Results and Discussion:

ELISA kit 1 did not process correctly and read as out of range readings. ELISA kit 2 faced the same difficulties and although several readings were observed, the standards were read as out of range. A standard curve could not be completed (Appendix 4) and data were deemed to be unreliable. In both cases it is speculated that either organic contaminants (impurities in the nanopure water) or possibly user error is to blame. There may be a need to purify samples and standards through activated carbon filter cartridges or other organic scavengers and the kit may work through modification.

Also, tested in this experiment, was the ability of 17β-estradiol to adsorb onto cereal or straw substrate. Had the ELISA kit worked there would have been a way to quantify the results. Also, a multichannel pipette was not used and could have provided more accurate results. In addition, a plate washer could have assisted with the final washing of buffer solution and may have curbed the out of range readings from organic contaminants. Neither of these items is available at the University of Michigan-Flint. This kit was chosen because of its ability to bind to 17β-estradiol in a wide range of conditions and its ease of use. The enzyme is highly stable under the assay conditions, has a wide pH range, and is not inhibited by common buffer salts or preservatives. Also, most biological fluids such as plasma, urine, tissue culture, supernatants, etc., can be assayed for steroids directly upon dilution with EIA buffer. To the best of my knowledge, the ELISA kit has not been used for detection of 17β-estradiol in mycological media. There are field kits available for the detection of 17β-estradiol in water samples typically used by departments such as the Environmental Protection Agency (EPA). These field kits are very expensive and also require very specific equipment such as multi-channel pipette and a reliable microplate reader. The Cayman estradiol ELISA kit was designed for health care settings, but with further testing could prove to be a less expensive alternative to the field kits.

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Appendix 1:

Water quality sampling: Includes site location, sample bottle number, day sample was taken, average, and standard deviation for 17α-ethynyl estradiol and Bisphenol A.

			Concentration (ng/L)			
			17α-ethynyl estradiol		*Bisphenol A*	
Name	*Sample #*	*Day*	*Avg*	*Stdev*	*Avg*	*Stdev*
Montrose DW	*1*	*1*	*1523.56*	*100.97*	*522.14*	*2.82*
Montrose DW	*5*	*2*	*1316.97*	*65.18*	*819.10*	*16.56*
Montrose UP	*2*	*1*	*232.45*	*11.81*	*379.80*	*6.25*
Montrose UP	*6*	*2*	*275.75*	*5.38*	*401.96*	*3.07*
Flushing P.S.	*3*	*1*	*4518.15*	*25.88*	*349.32*	*11.80*
Flushing P.S.	*7*	*2*	*3816.56*	*94.40*	*278.65*	*6.26*
Flushing UP	*4*	*1*	*370.44*	*104.85*	*294.75*	*8.48*
Flushing Up	*8*	*2*	*408.78*	*24.73*	*274.36*	*3.09*

Appendix 2:

Kirks's Medium (Tien and Kirk 1988)

1 Liter Distilled Water

5 g Glucose

1 g KH_2PO_4

0.5 g $MgSO_4 . 7 H_2O$

0.2 g NH_4NO_3

0.1 yeast extract

0.01 g $CaCl_2$

1 mg $CuSO_4.5H_2O$

1 mg $FeSO_4 . 7 H_2O$

Appendix 3:

Results from experiment1: There is no significant difference between the control (*P. ostreatus* in medium) and the treatment (*P. ostreatus* in medium with .001 mg/ml 17β-estradiol in acetone).

Flask	*Control (grams)*	*Experiment (grams)*	*d*	d^2
1	*.0953*	*.0508*	*.0445*	*.00198*
2	*.0716*	*.0532*	*.0184*	*3.39 e -4*
3	*.0574*	*.0718*	*-.0144*	*2.074 e -4*
4	*.061*	*.0450*	*.0160*	*2.56 e -4*
5	*.0496*	*.0529*	*-.0033*	*1.09 e -5*
6	*.0719*	*.0744*	*-.0025*	*6.25 e -6*
7	*.0612*	*.0659*	*-.0047*	*2.21 e -5*
8	*.0798*	*.0393*	*.0405*	*.00164*
			Total	**Total**
			-.0945	**.00446109**

N=8

$$t= \frac{.0945}{\sqrt{\frac{(8\,x\,.00446109)-.00893025}{8-1}}} = \frac{.0945}{.0618} = 1.52844 \text{ at 7 df}$$

p (0.05) = 2.365

P (0.01) = 3.499

Results from experiment 2. In experiment 2 there is no significant difference between the control (*P. ostreatus* in medium with 25 µl acetone) and the treatment (*P. ostreatus* in medium with .001 mg/ml 17β-estradiol in acetone).

Flask	*Control (grams)*	*Experiment (grams)*	*d*	d^2
1	*.0613*	*.0803*	*-.0190*	*3.61 e -4*
2	*.2486*	*.0642*	*.1844*	*.03400*
3	*.0530*	*.0650*	*-.0120*	*1.44 e -4*
4	*.0697*	*.0610*	*.0087*	*7.57 e -5*
5	*.0624*	*.0703*	*-.0079*	*6.24 e -5*
6	*.0511*	*.0476*	*.0035*	*1.23 e -5*
7	*.0661*	*.0704*	*-.0043*	*1.85 e -5*
8	*.0517*	*.0639*	*-.0122*	*1.49 e -4*
			Total	**Total**
			-.1412	**.0348229**

N=8

$$t = \frac{.1412}{\sqrt{\frac{8\,x\,.0348 - .0199}{7}}} = \frac{.1412}{.1922} = .735 \text{ at 7 df}$$

p (0.05) = 2.365

P (0.01) = 3.499

Appendix 4:

	1	2	3	4	5	6	7	8	9
Calc OD A	.421 Blk	.038 S1	.038 S1	.454 C1	.418 C3	.476 W2	.552 Sw 1	.676 Sw 4	.505 F2
Calc OD B	.424 Blk	Out S2	Out S2	.498 C1	.437 C4	.486 W2	.557 Sw 1	.454 Sw 4	.573 F3
Calc OD C	.452 NSB	1.608 S3	1.688 S3	.627 C1	.491 C4	.812 W3	.532 Sw 2	.482 Sw4	.437 F3
Calc OD D	.831 NSB	2.327 S4	2.105 S4	.465 C2	.525 C4	.547 W3	.518 Sw2	.575 F1	.465 F3
Calc OD E	Out B_0	Out S5	2.307 S5	.541 C2	.575 W1	.537 W3	.527 Sw 2	.528 F1	.499 F4
Calc OD F	Out B_0	Out S6	Out S6	.449 C2	.535 W1	.523 W4	.554 Sw 3	.494 F1	.507 F4
Calc OD G	Out B_0	Out S7	Out S7	.457 C3	.472 W1	.486 W4	.595 Sw 3	.526 F2	.483 F4
Calc OD H	Out TA	Out S8	Out S8	.578 C3	.625 W2	.464 Sw 1	.582 Sw 3	.528 F2	.499 F4

Results from ELISA kit 2

LEGEND:

Blk: Blanks

NSB: Non-specific binding; measure of lowest binding

B_o: Maximum binding

TA: Total activity; specific activity of the radioactive tracer

S 1-8: Standard samples in order of least binding to most binding.

C 1-4: Control samples triplicated numbers designate flask

W 1-4: Samples containing wood, triplicated, numbers designate flask

Sw 1-4: Samples containing straw, triplicated, numbers designate flask

F 1-4: Samples containing *P. ostreatus*, triplicated, numbers designate flask