MONITORING THE REMOVAL OF ESTROGENIC ACTIVITY IN WASTEWATER TREATED BY A PILOT-SCALE CONSTRUCTED WETLAND USING THE YEAST ESTROGEN SCREEN

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A thesis submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Department of Environmental Sciences and Engineering in the Gillings School of Global Public Health.

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ABSTRACT

Kathleen McDermott: Monitoring the Removal of Estrogenic Activity in Wastewater Treated by a Pilot-Scale Constructed Wetland using the Yeast Estrogen Screen
(Under the direction of Howard Weinberg)

Wastewater spiked with chemicals representing persistent contaminants was applied to a pilot-scale constructed wetland containing a locally sourced substrate on which a biofilm had developed. The system utilized aerobic, hypoxic, and aerobic columns, and was evaluated for its removal of estrogenic activity from wastewater using the yeast estrogen screen, as a function of five different dosing intervals. The removal of activity, as measured by estrogen equivalents (EEQ), decreased as the length of the dosing interval increased, with an 8 hour dosing interval achieving the highest removal at 99.9%. For all dosing studies, higher removals in activity were achieved by employing the columns in series than was achieved by any one column individually. Moreover, it was found that hypoxic conditions resulted in a higher removal of EEQ, suggesting that a resequencing of the full scale system might improve the finished water quality.

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4-NP	4-Nonylphenol
AF1	Activator Function One
AF2	Activator Function Two
ATZ	Atrazine
BEQ	Biological Equivalents
CAFF	Caffeine
CBZ	Carbamazepine
CPRG	Chlorophenol-red- β-D-galactopyranoside
CW	Constructed Wetland
DBD	DNA Binding Domain
DDT	dichlorodiphenyltrichloroethane
DEET	*N,N*-Diethyl-*m*-toluamide
DES	Diethylstelbesterol
E2	17-β-estradiol
EC50	Effective Concentration at which 50% of the maximum effect is observed
EDC	Endocrine Disrupting Compound
EE	17-α-ethinylestradiol
EEF	Estrogen Equivalent Factor
EEQ	Estrogen Equivalents
ER	Estrogen Receptor
ERE	Estrogen Response Element

EV50	Effective Volume at which 50% of the maximum effect is observed
HF	Horizontal Flow
LBD	Ligand Binding Domain
LCMS	Liquid Chromatography with detection by Mass Spectrometry
LGW	Laboratory Grade Water
JLBC	Jordan Lake Business Center
SOP	Standard Operation Procedure
SPE	Solid Phase Extraction
TCS	Triclosan
TOC	Total Organic Carbon
USEPA	United States Environmental Protection Agency
VF	Vertical Flow
VFCW	Vertical Flow Constructed Wetland
WWTP	Wastewater Treatment Plant
YES	Yeast Estrogen Screen

CHAPTER 1: BACKGROUND

1.1 Environmental Relevance of Endocrine Disruption

The human endocrine system is responsible for chemical signaling and the maintenance of homeostasis. Chemical signaling occurs through the synthesis and transport of endogenous hormones that control the growth, development, and metabolism of the body. In humans, the endocrine system is composed of several glands including the hypothalamus, pituitary gland, adrenal glands, thyroid, parathyroid, pancreas, and the gonads (i.e. testes and ovaries). These seven glands are responsible for synthesizing and secreting endogenous hormones into the blood stream where they disperse, bind to hormone specific receptors, and initiate tissue and cell-specific biological effects.

Certain exogenous chemicals are capable of mimicking endogenous hormones, causing alterations in normal endocrine signaling and potential adverse biological effects, a process known as endocrine disruption. According to the Society of Toxicology, the generally accepted definition for endocrine disruption is "an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in the endocrine function" (Society of Toxicology, 2010). Endocrine disruption has been shown to cause deleterious effects in wildlife. Notable extreme examples include the rapid decline of high level bird species due to dichlorodiphenyltrichloroethane (DDT) exposure detailed in Rachel Carson's *Silent Spring,* and feminization of male fish in heavily polluted waters (Carson, Lois, Darling, Wilson, & Lear, 2003; Kabir, Rahman, & Rahman, 2015). Endocrine disruption remains relevant in the human context as well. Of particular concern for the public is disruption of estrogen signaling, which

has been linked to various cancer and reproductive health endpoints (Diamanti-Kandarakis et. al., 2009). A prospective human cohort study has demonstrated a link between *in utero* exposure to DDT and increased risk of breast cancer (Cohn et. al., 2015). The estrogen disruptor diethylstilbestrol (DES), which was prescribed to pregnant mothers to prevent miscarriage, is an even starker example of the potential health effects of estrogen disruption. It is estimated that between five and ten million mothers were prescribed DES before it was removed from markets due to increased incidence of clear-cell carcinoma, anatomical malformations of the reproductive tract, and decreased fertility in daughters exposed *in utero* (Cohn et. al., 2015; Henley & Korach, 2006; Soto & Sonnenschein, 2015).

While many of the aforementioned examples represent the effects of high and direct doses of chemicals, there is rising concern about chronic low level human exposure to endocrine disrupting compounds (EDCs) because their presence at low levels in the environment has been shown to demonstrate adverse developmental, reproductive, neural and immune health effects in mammals (Yang, Kim, Weon, & Seo, 2015). In fact, most research has focused on the negative effects of EDCs on sexual development and reproduction caused by interference with the estrogen receptor (ER) because the outcome is readily identifiable and represents a sensitive health issue for the general public (Henley & Korach, 2006). Inappropriate ER signaling can lead to increased risk of hormone-dependent cancer, impaired fertility, as well as abnormal fetal growth and development (Diamanti-Kandarakis et. al., 2009). Moreover, current epidemiological evidence also points to an association between increased environmental exposure to exogenous EDCs and increased incidence of such biological endpoints (Vandenberg et. al., 2012).

Emerging contaminants are defined by the United States Environmental Protection Agency (USEPA) as “chemicals or materials characterized by a perceived, potential, or real threat to

human health or the health of the environment or by a lack of published health standards" (US EPA, 2016). Many of these contaminants are suspected estrogen disruptors and are of particular concern because the consequences of their presence in surface waters remain largely unknown. Moreover, they are present in the environment in complex mixtures and while many individual compounds have been studied in depth for their estrogen disrupting capabilities, there is not yet enough information regarding mixtures of such compounds. In order to better protect human and environmental health from the impact of EDCs, evaluation of chemical mixtures in environmental samples using effective screening tools is required.

1.2 Mechanisms of Estrogen Signaling

The biochemical messages of the estrogen system are mediated through activation and repression of the estrogen receptor, a member of the steroid nuclear receptor family. Steroid nuclear receptors are also referred to as transcription factors due to their role in regulating target gene transcription (Smirnova, 2015). They are globular oligomeric proteins that exhibit complex three dimensional conformations which are critical to their biological function. For the estrogen receptor there are two subtypes, the ERα and the ERβ, which are very similar but have key structural differences that explain their different ligand affinities and target genes (Shanle & Xu, 2011). Estrogen receptors can mediate biochemical changes in target cells and tissues through genomic and nongenomic estrogen signaling. The genomic pathway consists of ligand-dependent modulation of gene expression through activation of the receptor. The nongenomic pathway is less readily understood but is thought to occur via ligand binding to membrane bound receptors, which can be either of the ERα or ERβ subtypes or another receptor type entirely, such as a G-protein coupled receptor. This pathway causes cellular signaling cascades that lead to rapid estrogen signaling independent of gene activation, transcription, and translation.

There are four main components of each receptor: the N-terminus (free amino terminus) containing the activator function 1 (AF1), the central DNA binding domain (DBD), a hinge region, and the ligand binding domain (LBD) containing the free carboxy terminus of the polypeptide and a second activator function (AF2) (Smirnova, 2015). The N-terminus is the most variable segment of the protein and its major role is housing the AF1, which is responsible for recruiting important cofactors and coregulators, in addition to playing a crucial role in tethering DNA to the receptor. The AF1 and AF2 regions are used to up- or down-regulate subsequent DNA binding, depending on cell type and the availability of coregulators near the ligand-receptor complex (Shanle & Xu, 2011). The most highly conserved region of the receptor is the DBD as it is required for proper recognition and binding of estrogen response elements (EREs) – a series of unique palindromic base pair repeats that are found upstream of target genes and help signal the transcription start site. The LBD is the region in which compatible ligands (i.e. an endogenous hormone or an exogenous EDC) bind to the receptor, acting as the initiating step of ligand-dependent receptor signaling. Finally, the hinge region connects the highly conserved LBD and DBD, acting as a spacer region and an interface for dimerization of the receptor complex (Heldring et. al., 2007).

In three dimensions, the LBD of the ER, also known as the receptor's active site, exists as a wide pocket in the surface of the protein. The arrangement of the independently folding alpha helices of the receptor protein creates a "wedged-shaped" molecular scaffold that maintains a large cavity in the protein's LBD in which a substrate can bind (Brzozowski et. al., 1997; Shanle & Xu, 2011). Binding of the LBD to a compatible ligand causes a shift in the conformation of the receptor structure that can either promote (agonism) or prevent (antagonism) subsequent dimerization and DNA binding (Shanle & Xu, 2011). Once an agonist binds to the LDB of the

receptor, the bound receptor must dimerize with a second receptor before it can translocate to the nucleus and bind EREs upstream of target genes. The presence of two ER subtypes means that the ER can form a heterodimer (an ERα and ERβ complex) or a homodimer (a complex of two identical ERs), depending on the intracellular availability of the two receptor subtypes. Agonists and antagonists bind at the same site in the LBD but induce distinct conformations in the transactivational domain of the LBD that either allows for continued signal transduction (agonism) or halting of the signaling pathway (antagonism) (Brzozowski et. al., 1997).

There are several important characteristics of a potential ligand that determine whether it will act as an agonist or an antagonist. In the case of the ERα, X-ray crystallography of a receptor bound with the endogenous hormone 17-β-estradiol (E2) shows there are three critical hydrogen bonding interactions between E2 and the amino acids Glu353, Arg394, and His524 (or Glu305, Arg346, and His475 in the ERβ**)** (Shanle & Xu, 2011)**.** The series of hydrogen bonding, in conjunction with strong hydrophobic interactions between the aromatic rings of E2 and nonpolar amino acids in the binding pocket, stabilizes the ligand-receptor complex, allowing for a conformational shift in alpha helix 12. Helix 12 flips over, forming a cap on the binding pocket and activating the AF2, which is critical to downstream DNA binding and gene transcription (Brzozowski et. al., 1997; Smirnova, 2015). When bound to an ER antagonist, helix 12 protrudes from its neighboring helices in a conformation that prevents dimerization and halts the signaling pathway.

When discussing receptor mediated gene transcription in the context of endocrine disruption, it is important to note that the ERs have a sizeable binding cavity within the face of the protein (Brzozowski et.al., 1997). As a result, even though the system of hydrogen bonds and hydrophobic interaction that bind ligand to receptor allows for great specificity in the binding of

endogenous hormone to receptor, other molecules that possess similar nonpolar and hydrogen bonding moieties can exhibit binding as well. Such compounds are capable of binding to the receptor, although often at a much lower binding affinity than endogenous hormones, influencing gene transcription.

1.3 Bioassays Used to Evaluate Estrogen Disruptors

Chemical methods for analyzing environmental samples containing mixtures of emerging contaminants are inadequate for assessing their endocrine activity (Kinnberg, 2003; Leusch, 2008; Osman, AbouelFadl, Krüger, & Kloas, 2015). For many emerging contaminants, their potential to act as an EDC is unknown; individual chemicals may cause biological effects at concentrations below analytical detection limit, and in mixtures that effect may be different than its effect individually (Leusch, 2008). In mixtures, synergism can occur where the overall effect is larger than the sum of the effects of the individual components, or antagonism can occur, where the overall effect is smaller than predicted from the sum of the effects of each component. Thus, attempts to determine the endocrine activity of environmental samples using chemical analysis can lead to an underestimation or overestimation of the true activity. As a result, there has been a movement towards using *in vitro* bioassays as screening tools for measuring activity in environmental samples due to their ability to capture total estrogenic activity without requiring prior knowledge of the specific chemical composition of the sample (Jia et. al., 2015; Kinnberg, 2003; Krein et. al., 2012; Leusch, 2008; Soto, Maffini, Schaeberle, & Sonnenschein, 2006). Moreover, it has been demonstrated that integration of information from chemical analysis and bioassays can provide a more complete understanding of estrogenic activity of environmental waters. In this manner, bioassays can be used to screen for estrogenic exposures, identify point sources of EDCs, and even monitor the efficiency of water treatment operations (Soto, Maffini,

Schaeberle, & Sonnenschein, 2006). There are three major classes of bioassays that are commonly used to evaluate potential estrogen disruptors: competitive binding receptor, cell proliferation, and reporter gene assays.

Competitive receptor binding assays are a common non-cell based assay used to assess the ability of a compound or sample to bind to the receptor's LBD (Soto, Maffini, Schaeberle, & Sonnenschein, 2006). These assays test the first event in the complex signal transduction pathway of ligand-dependent receptor-mediated endocrine function from which all downstream signaling initiates (or terminates) (Murk et. al., 2002). These assays utilize purified ER protein that are co-exposed to a native ligand (E2) and sample of interest and its ability to inhibit the binding of E2 is monitored. These assays quantify the ability of a sample to compete for the receptor's active site, but do not give information about biological mechanism of action (i.e. agonism or antagonism) (Murk et. al., 2002). While these assays are required for absolute confirmation of a compound as an estrogen disruptor, they are time consuming and require highly trained workers and specialized equipment, making them poor choices for environmental screening tools (Kinnberg, 2003). In addition, these non-cell based assays do not account for cellular uptake of a given compound and, thus, may overestimate risk for compounds whose physical and chemical properties prevent them from crossing cell membranes.

The next set of bioassays commonly used are cell proliferation assays, which test the ability of a compound to increase proliferation of human breast cancer (MCF-7) cells. Cells are cultured in media containing proliferation inhibiting factors, and the ability of a compound to overcome these inhibitory factors and induce proliferation is measured. While these assays are sensitive, they have been shown to be less estrogen specific, as there are many other processes independent of ER binding that can cause increased proliferation in some MCF-7 cell lines (Kinnberg, 2003).

The final group are reporter gene assays, which can be conducted using either mammalian or yeast cells stably transfected with a reporter plasmid (and in the case of yeast cells, stably transfected with the plasmid and the gene for human ER). EREs are places upstream of the transcriptional start site of the reporter gene, ensuring that the transcription of the gene is dependent on binding of an agonistic compound to the ER, subsequent dimerization, and binding of the dimer to the ERE. Thus, it is possible to model the dose dependent transcription of the reporter gene across a dose dilution curve of sample concentration. Reporter gene assays are popular for environmental analysis because of their higher throughput, greater sensitivity and specificity of mode of action (i.e. genomic ER signaling) (Jia et al., 2015). Cell based reporter gene assays have been shown to better correlate with *in vivo* study results than ER binding and cell proliferation assays, likely due to their ability to account for toxicokinetics at the cellular level and their higher mode of action specificity (Kinnberg, 2003). The sensitivity of reporter gene assays depends on the cell type employed, with yeast based assays having lower sensitivity than those employing mammalian cells. However, yeast cells have shown to perform well on highly polluted water and, thus, can be applied to wastewater matrices with a lower risk of cytotoxicity (Leusch et. al., 2010). While mammalian reporter gene assays are more sensitive with lower detection limits than yeast based assays, mammalian cells are much more difficult to culture and are less resilient when exposed to highly polluted samples (Kinnberg, 2003; Leusch et al., 2010). Although yeast cells contain a cell wall that might prevent cellular uptake and, thus, underestimate estrogenic activity, they are a low cost, higher throughput option (Soto, Maffini, Schaeberle, & Sonnenschein, 2006). As a result, yeast based reporter gene assays are a very useful screening tool that can be used to evaluate wastewater.

As with any bioassay, there are some disadvantages of using yeast based assays to evaluate

estrogen activity. *In vitro* assays involve single cells and do not accurately represent some important toxicological processes that determine how a compound will affect an entire organism, namely the concepts of absorption, distribution, metabolism and elimination. At the organismal level, compounds may be endocrine active but may be metabolized into a compound that is not active prior to exhibiting biological effects of disruption, making it of less physiological relevance. In addition, an organism may be exposed to a compound but it may not be bioavailable to the cells in which it has an effect. As a result, cellular response will not always imply higher-level effects, but they are a prerequisite for effects at the organismal or population level (Escher et. al., 2014). Moreover, from a precautionary public health standpoint the potential to cause adverse effects is still a "critical assessment endpoint" (Escher et. al., 2014). So while cell-based reporter gene assays cannot replace *in vivo* testing, they can provide hazard information for screening and prioritization (Judson et. al., 2013).

1.4 Environmental Exposures of EDCs

Estrogen mimics are thought to enter the aquatic environment largely through municipal and industrial sewage discharge into surface waters (Kinnberg, 2003; Schenck et. al., 2012). Urban and agricultural run-off also contribute to elevated levels of EDCs in waterways (Kabir, Rahman, & Rahman, 2015). Major potent estrogen species include natural and synthetic steroid hormones (used as birth control and treatment for menopausal disorders) such as estrone, E2, and 17-α-ethinylestradiol (EE). Common low-potency estrogen disruptors include the surfactant degradate nonylphenol (released from homes and the dry cleaning industry), flame retardants, polychlorinated biphenols (industrial production), phthalates (found in personal care products), natural phytoestrogens, pharmaceuticals, and pesticides (Céspedes et. al., 2004; Kabir, Rahman, & Rahman, 2015; Murk. et.al., 2002). Moreover, environmental samples typically contain

mixtures of these low-potency disruptors which are present in the low μg/L range (Crain et. al., 2007; Gunnarsson et. al., 2009; Luo et. al., 2014).

In the field of toxicology there is a central theme of "the dose makes the poison," which essentially means that exposure to low doses of chemicals is less of a concern than high doses simply due to their concentration. However, this same concept cannot be readily applied to the area of endocrine disruption because EDCs can have effects at low doses, often below chemical detection limits, that are not predicted by their effects at high doses (Vandenberg et. al., 2012). Given that circulations of endogenous hormones are so low, even low doses of endocrine active compounds can cause dramatic changes in hormonal homeostasis and have widespread effects that are different or even opposite of the effects of the hormone at high doses. Furthermore, there is a nonlinear relationship between ligand concentration and the number of bound receptors such that in the low dose range a 10-fold increase in hormone (or EDC) can cause a 9-fold increase in receptor occupancy, whereas the same increase at high doses is associated with only a 10% increase. As a result, moderate changes in ligand concentration in the low-dose range can produce substantial changes in receptor occupancy and generate significant biological effects (Vandenberg et. al., 2012). There has also been strong, reliable, and reproducible evidence from several studies that confirm the presence of low concentrations of EDCs and other chemicals in human tissue and fluids, as well as in environmental samples (Crain et. al., 2007). In addition, there are numerous epidemiologic analyses that repeatedly find associations between the observed concentrations in human samples and disease endpoints. As a result, even low levels of estrogenic species pose a potential threat to human and wildlife health.

1.5 Wastewater Treatment and Estrogenic Activity Removal

The occurrence of micropollutants, many of which are estrogenic, in wastewater treatment plant (WWTP) influent falls in the 0.1 to 10μg/L range, but some widely used chemicals such as ibuprofen, atenolol, caffeine, and nonylphenol have mean occurrence values much higher (Luo et. al., 2014). Removal efficiencies after conventional treatment, which typically includes primary or physical treatment (sedimentation) followed by secondary (biological) treatment to reduce the water's biochemical oxygen demand, depend on the physicochemical properties of the compound and typically range from 12.5-100% (Deblonde, Cossu-Leguille, & Hartemann, 2011). After treatment, the concentration of many micropollutants are in the range of 1ng/L to 1μg/L, but many compounds persist, including nonylphenol, triclosan, caffeine, and atenolol, at concentrations above 1μg/L.

The occurrence and removal of estrogenic activity during wastewater treatment tracks with the removal and persistence of micropollutants. Total estrogenic activity (expressed as estrogen equivalents (EEQ see section 1.7) of WWTP influents have been reported to range from 0.6-154ng/L. Conventional wastewater treatment plants utilizing activated sludge as their biological treatment can reduce estrogenic activity considerably (typical effluent EEQ fall below 25ng/L), but EEQ of up to 150ng/L in effluents have been reported in the U.S. (Kinnberg, 2003; Liu, Kanjo, & Mizutani, 2009). As a result, physiologically relevant levels of estrogenic activity persist even after conventional treatment (Pawlowski et. al., 2004; Schenck et.al., 2012; Westerhoff, Yoon, Snyder, & Wert, 2005). These effluents then contribute to surface water contamination and pose a threat to wildlife, which is susceptible to potent endocrine disruptors such as E2 and EE at levels as low as 0.1 to 10ng/L (Céspedes et. al., 2004). Moreover,

contamination of surface waters by WWTP effluent with estrogenic activity results in chronic, low dose exposures to fish and wildlife, the consequences of which are largely unknown.

Given the incomplete removal of many micropollutants during conventional wastewater treatment, tertiary treatment is often included in municipal WWTPs. A review of 14 WWTPs showed that tertiary treatment processes using activated carbon, advanced oxidation processes, and nanofiltration achieve higher and more consistent removal than conventional WWTPs (Luo et. al., 2014). Several studies have shown that optimized biological treatment or other more advanced technologies can also increase the efficiency of the removal of estrogens (Liu, Kanjo, & Mizutani, 2009; Völker et. al., 2016). Other studies have shown that advanced treatment with ozone was capable of removing the estrogenic effects on fish exposed to WWTP effluent as monitored via hepatic estrogen responsive genes (Gunnarsson et. al., 2009). Unfortunately, advanced treatment has a high cost and is typically only employed when higher quality water discharge is necessary (e.g. for water reuse) (Ávila et. al., 2014; Luo et. al., 2014). Advanced treatment with chemicals such as ozone and chlorine introduces concerns about the potential hazardous substances that are produced as byproducts (Gunnarsson et. al., 2009; Koh et. al., 2008). As a result, exploring low cost tertiary treatment processes capable of increasing removal of estrogenic compounds without the use of additional chemicals becomes of increasing interest.

One such option that has emerged as a low cost, low maintenance tertiary treatment to remove persistent micropollutants and, therefore, reduce estrogenic activity prior to discharge into the environment is the constructed wetland (CW) (Ávila et. al., 2014). This term refers to nonconventional water treatment systems (typically onsite at the source of wastewater production and storage) that use natural processes (sorption, biodegradation by microbes, photolysis etc.) to remediate wastewater (Gross, Montgomery-Brown, Naumann, & Reinhard,

2004). Subsurface vertical flow (VF) wetlands undergo intermittent dosing of wastewater, which then percolates through the sand/gravel bed. Vertical flows are often employed in series to alternate periods of dose and rest, which allows for greater mineralization of deposited solids during resting phases. Due to the intermittent dosing, VFs allow for the development of pockets of aerobic and anaerobic conditions. Horizontal flows (HF) are continuously fed and, therefore, saturated as the water flows horizontally through a vegetated gravel bed. As a result, horizontal flows are predominantly anaerobic in nature. Combining vertical and horizontal flow wetland design improves overall treatment efficiency as it allows for periods of both anaerobic and aerobic degradation, facilitating additional contaminant removal than CWs employing only VF or HF (Vymazal, 2011). CWs are effective in treating polluted waters from a wide range of domestic, industrial, and agricultural operations (Matamoros, Caselles-Osorio, Garcia, & Bayona, 2008; Vymazal, 2011). Wetland treatment had removal efficiencies ranging from 67% to 100% in a study looking at the removal of pharmaceuticals and alkylphenol ethoxylate metabolites, some of which may be possible EDCs (Gross, Montgomery-Brown, Naumann, & Reinhard, 2004). Moreover, it has been shown that constructed wetlands are capable of removing many types of pharmaceutical and personal care products, though the processes are typically not optimized for this objective and little is known about their potential for removing estrogenic activity (Conkle, White, & Metcalfe, 2008; Vymazal, 2011).

A vertical flow constructed wetland (VFCW) can be piloted in the laboratory using large stainless steel columns, packed with porous rock material (typically chosen based on locally sourced materials). In order to pilot a VFCW undergoing intermitted dosing – where there is a set time interval between applications (i.e. dosing) of a volume of wastewater – several columns are used in series with varying levels of oxygenation to mimic the development of pockets of

aerobic and anaerobic conditions in a full scale VF wetland. In order to simulate real world conditions, a wastewater matrix that has undergone some level of treatment is typically used. As a result, this water is cleaner than a typical wastewater influent and it becomes necessary to spike in target compounds in order to elevate their levels so that chemical analysis can track their fate. For the pilot-scale CW used in this particular study, seven target emerging contaminants have been chosen: atrazine (an herbicide), 4-nonylphenol (a surfactant degradant), triclosan (an antibiotic), carbamazepine (an antiepileptic drug), caffeine (a stimulant), E2 (a steroid hormone), and *N,N*-Diethyl-*m*-toluamide (DEET) (a pesticide). These are chemicals that are commonly found in domestic wastewater, and as mentioned previously, undergo different rates of removal during treatment due to their different physical-chemical properties. As a result, they represent large classes of contaminants and can be used as surrogates for their removal. Moreover, many of these compounds have biochemical activity, including estrogenic activity, making their removal of particular interest if the treated water is to have a wider reuse potential.

1.6 Use of the Yeast Estrogen Screen to Monitor Wastewater Remediation

As mentioned previously, the use of reporter gene assays is becoming increasingly more common to evaluate environmental samples for estrogenic activity. Recombinant yeast assays, specifically the yeast estrogen screen (YES), take advantage of the role ERs play as transcription factors to assess the estrogenic capabilities of samples. In the YES assay, Saccharomyces cerevisiae yeast cells have been stably transfected with the gene for the human ERα in addition to the receptor promoted *lac-Z* plasmid. An ERE is placed upstream of the *lac Z* gene, which ensures that the expression of the reporter protein, β-galactosidase, is controlled by the activation of the corresponding ERα via binding of an agonistic ligand. The reporter protein is secreted into the growth media, which contains the chromogenic substrate chlorophenol red-β-D-

galactopyranoside (CPRG). As depicted in Figure 1, β-galactosidase catalyzes the hydrolysis of the galactosidic bond in CRPG (yellow), releasing chlorophenol-red, which causes the growth media to turn red depending on the degree of β-galactosidase production.

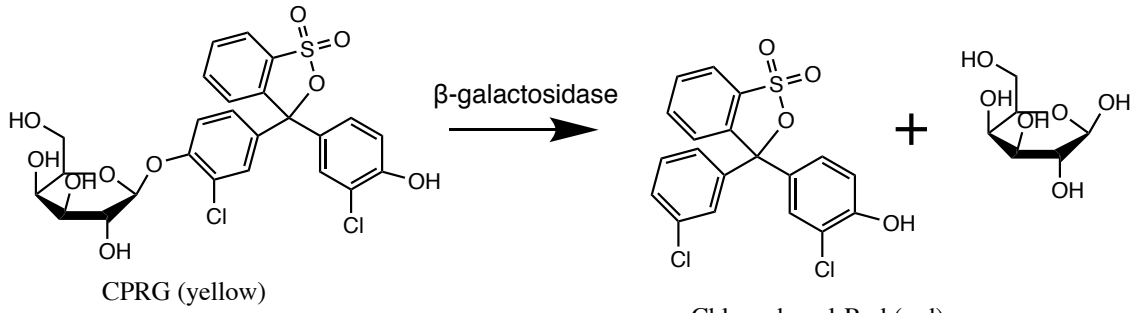

Figure 1. Cleavage of the galactosidic bond in CPRG with the enzyme B-galactosidase

Spectrophotometric detection of chlorophenol-red thus serves as a proxy for gene expression and receptor activation (Routledge & Sumpter, 1996). The assay is conducted in a 96-deep well plate, which allows for an eleven-point concentration dilution series to which the yeast is exposed. A plot of the absorbance against the analyte concentration in each well represents the dose-dependent production of reporter gene for a given sample, as shown in Figure 2 for E2. An effective dose 50% (EC50), which represents the concentration at which the dose response curve reaches 50% maximum response, is calculated for each curve. The EC50 value acts a surrogate for the potency of a receptor agonist and is an important measure that can be used to compare activity across chemicals and samples.

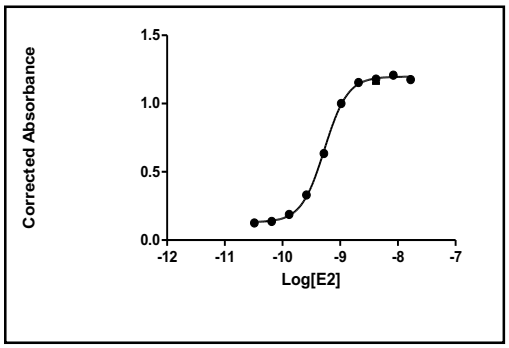

Figure 2. Dose-response curve for 17-β-estradiol (E2), the positive control for the YES assay.

The YES assay is particularly applicable to the assessment of endocrine activity in environmental samples and has received attention as a rapid and relatively simple screening tool in laboratory and field experiments (Pawlowski et. al., 2004; Routledge & Sumpter, 1996). In addition, the assay is viewed as a useful screening tool to prioritize future *in vivo* studies and targeted chemical analysis for indicating potential ecological risk from hormone activities (Krein et. al., 2012). Of particular note is the ability to use the YES assay in testing environmental samples for their potential as estrogenic agonists and antagonists (Saunders, Higley, Hecker, Mankidy, & Giesy, 2013; Völker et. al., 2016).

The most common method of preparing aqueous environmental samples for bioassay analysis is solid phase extraction (SPE). Environmental samples typically contain mixtures of natural and synthetic hormones and low-potency disruptors at low concentrations and, therefore, it is necessary to concentrate them for subsequent instrumental and bioassay analysis (Cespedes et. al., 2004, Leusch et. al., 2006). Several studies have used the YES assay to test the endocrine activity of solid phase extracts of Rhine and Nile River samples, as well as wastewater treatment discharge (Osman, AbouelFadl, Krüger, & Kloas, 2015; Pawlowski et. al., 2004). Pawlowski et. al., (2004), determined that estrogenic activity calculated from chemical analysis resulted in much lower values of activity than were produced using the YES assay in Rhine River SPE extracts.

1.7 Integrating Chemical and YES Analysis to Evaluate Mixtures of EDCs

As mentioned previously, a major benefit of using the YES assay is the ability to capture total estrogenic activity of environmental samples without requiring knowledge of the individual chemical mixture components. This can be done using the concept of biological equivalents (BEQs) (Jia et.al., 2015). BEQs allows for the representation of complex mixtures in terms of the

endogenous hormone that is known to activate a given biological pathway. For hormone-dependent estrogen receptor-mediated signaling, the endogenous hormone is E2 and, thus, individual chemical and mixture activity can be reported as estrogen equivalents (Jia et.al., 2015, Leusch et. al., 2006; Leusch et. al., 2010). Using this concept, it is possible to evaluate the activity of individual chemicals by calculating a potency factor that relates the activity of the EDC to the activity of E2, which can then be multiplied by the concentration of the EDC to represent how much estrogen would need to be present to produce the same biological effect. For environmental samples, this concept is particularly useful when the identity of the sample components is unknown. An EEQ can be calculated for the sample by comparing the volume of solid phase extract that produces a given response to the amount of E2 that would be needed to produce that same response (Leusch et. al., 2010). This concept is essentially answering the question "if all the observed estrogen activity of a mixture were caused by E2, how much E2 would be present in that mixture?"

EEQ can also be used in conjunction with chemical analysis if the potency of the individual chemical analytes has been quantified. To do so requires the assumption that the chemicals exhibit additivity. Assuming additivity, a predicted EEQ can be determined by first calculating the EEQ of each individual chemical in the mixture as described above, and summing those values to represent the predicted activity of those components in a mixture. It is possible to then compare predicted EEQ values with bioassay derived EEQ values to understand what percentage of the detected activity can be explained by the primary estrogenic analytes in the sample. In addition, comparing predicted and bioassay-derived EEQs may allow for greater insight into potential mixture effects occurring within the sample (e.g. additivity, antagonism, synergy).

1.8 Research Objectives

The aim of this project was to accomplish the following objectives

1. Use the YES assay to determine the estrogenic activity of influent and effluent from an optimized pilot-scale CW wastewater treatment method.
2. Evaluate the changes in the estrogenic activity of wastewater through the pilot-scale CW unit processes.
3. Determine how the predicted estrogenic activity of wastewater, derived from chemical analysis at various time points of treatment, compares to the activity measured using the YES assay for different dose intervals.

The specific project objectives were achieved by:

i. Evaluating the 7 target chemicals (atrazine, estradiol, 4-nonylphenol, DEET, carbamazepine, triclosan, and caffeine) for their estrogenic activity individually and in mixtures.

ii. Evaluating the estrogenic activity of the wastewater entering the pilot-scale CW prior to treatment.

iii. Using the concept of estrogen equivalents to monitor changes in the estrogenic activity of CW-treated wastewater as it moves through the various stages of the pilot-scale wetland for each dose interval.

iv. Using data from chemical analysis and bioassay-derived data to compare the predicted and observed estrogenic activity of wastewater samples.

CHAPTER 2: METHODS

2.1 YES Procedure

The Yeast Estrogen Screen follows the methods of Routledge and Sumpter (1996) and the entire procedure is presented in Appendix A with one modification. YES colonies are streaked on solid agar plates prepared from Ura-Trp liquid media instead of solid agar plates prepared from growth media because Ura-Trp is a differential and selective media that helps prevent contamination of plates by other microbes. The assay is conducted in a 96-deep-well plate with samples run in duplicate rows, such that three chemicals can be tested on each plate with the final chemical tested being the assay's positive control, E2. On the first day of the assay a single yeast colony isolate is transferred to a 50mL falcon tube containing 7mL of growth media, and is allowed to grow in an incubator-shaker for 48 hours at 30°C and 200rpm. On the third day of the assay, the YES suspension is diluted with additional growth media to an optical density between 0.15- 0.20 using a UV-VIS plate reader set to 620nm (a measure of turbidity and, therefore, a surrogate for yeast cell density). The YES dilution is then spiked with a 0.01g/mL solution of CPRG prepared in laboratory grade water (LGW), such that the ratio of CPRG to growth media is 10μL/mL. LGW was prepared using a Dracor Water System unit (Durham, NC) which removes residual disinfectants, reduces the total organic carbon (TOC) to < 0.2 mg/L C and removes ions to 18 MΩ. LGW that is used to make the 10% ethanol solution, the CPRG solution, and all chemical and sample dilutions is also filtered using sterile Corning filter systems to prevent microbial contamination of the assay after several instances during the

early part of this research of contamination interfering with the assay results. Using a multichannel pipette, 100μL of a 10% ethanol 90% LGW solution is added to all wells in columns 2-12. Then, 200μL of sample prepared in 10% ethanol 90% LGW is applied to the first column in duplicate rows, and serially diluted across columns 1 through 11 using a 1:2 dilution factor. Column 12 receives no chemical and contains only 100μL of 10% ethanol solution. All wells receive 200μL of the yeast/CPRG solution. Column 12 contains only assay solvent, yeast/CPRG and, therefore, acts as the negative control and a check for cytotoxicity. The plate is then sealed with plate sealing film and allowed to shake in an incubator-shaker for 5 minutes at 30°C and 200rpm. The shaker is turned off and the plate left to incubate at 30°C for 48 hours . On the fifth day of the assay, 100μL of supernatant is transferred from each well of the 96-deep-well plate to a 96-flat-well plate and the absorbance of each well at 540nm (wavelength at which chlorophenol red maximally absorbs) and 620nm (wavelength used as a turbidity measure) is recorded.

A standard operation procedure (SOP) has been established for testing E2 and 4-nonylphenol (4-NP) with the YES assay in the Weinberg laboratory and is described in Nunes (2013). Atrazine (ATZ), triclosan (TCS), and carbamazepine (CBZ) were tested for estrogenic activity up to their solubility limits in water (summarized in Table 1), while caffeine (CAFF) and DEET were evaluated up to the millimolar level and no higher because no indication of an estrogenic response was observed at those concentrations. Stocks of each target chemical were prepared in ethanol and stored in an upright freezer at -20°C. On the third day of the assay, a working solution of each chemical is prepared fresh by performing a 1:10 dilution of the chemical stock such that the percentage of ethanol in the working solution does not exceed 10% (e.g. 100μL of chemical stock prepared in 100% ethanol diluted into 900μL of LGW for a total

volume of 1mL). The structures of the test chemicals are shown in Figure 3 and the working solution concentration for each chemical is summarized in Table 1.

Table 1. Target Chemical Solubility Data

Chemical	Molecular Weight (g/mol)	Water Solubility (mg/L)	Water Solubility (M)	Working Solution Concentration (M)
Triclosan	289.54	[a]12.0	4.14E-05	4.00E-05
4-Nonylphenol	220.35	[b] 6.25	2.84E-05	2.50E-05
17-β-Estradiol	272.38	[c]3.90	1.43E-05	5.00E-08
Atrazine	215.68	[d]33.0	1.61E-04	1.60E-04
Carbamazepine	236.27	[e]18.0	1.11E-01	2.00E-03
Caffeine	194.19	[f]1.60E+04	7.62E-05	7.50E-05
DEET	191.30	[g]2.00E+03	5.85E-02	2.00E-03

[a] European Commission: Scientific Community on Consumer Safety, 2010
[b] California Environmental Protection Agency, 2009
[c] Pubchem, 2017
[d] DeMarini & Hoar Zahm, 1999
[e] United States Environmental Protection Agency, 2004
[f] Sigma Aldrich, 2017
[g] California Environmental Protection Agency, 2000

2.2 Target Chemical Data Analysis

A corrected absorbance is calculated for each well of the concentration dilution series for a given chemical using equation 1:

$$Corrected\ Absorbance = Abs_{540} - (Abs_{620} - Abs_{620\ neg\ control}) \qquad (1)$$

The value obtained when the absorbance of the negative control at 620nm is subtracted from the absorbance of the well at 620nm gives the overall turbidity measure of the sample and is subtracted from the absorbance of that same well at 540nm so that only the absorbance of chlorophenol red is being measured. Cytotoxicity is monitored by comparing the absorbance value of the well at 620nm to the absorbance of the negative control at 620nm. The negative control contains no sample, and, thus, any turbidity present in the negative control can be used as a surrogate for yeast cell density. If the turbidity (measured at 620nm) of any well containing

sample is less than 10% of the negative control, the concentration of that well is said to be cytotoxic and is not included in any data analysis.

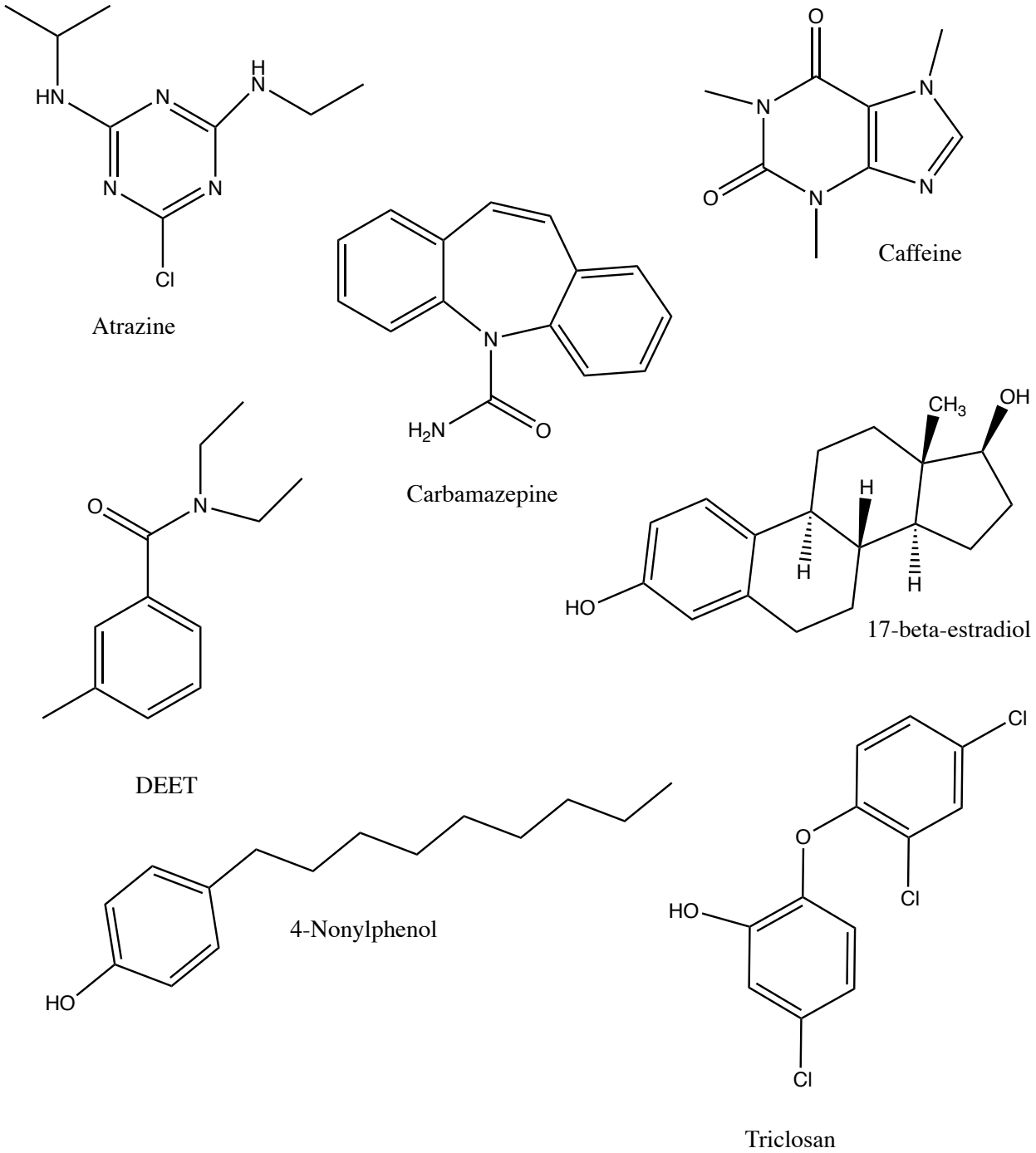

Figure 3. Chemical structures of the seven target contaminants.

Duplicate absorbance measures of each well are plotted against the log transformed molar concentration using GraphPad Prism 4.3 (GraphPad Software; La Jolla, CA), and a sigmoidal dose response curve with variable hillslope is fit to the data. GraphPad Software then calculates an EC50 for each chemical that gives an estrogenic response. An estrogen equivalent factor (EEF), which relates the potency of the EDC to the potency of E2, was calculated for each compound using equation 2 (Murk et. al., 2002, Leusch et. al., 2010):

$$EEF = \frac{EC50(E2)}{EC50(Test\ Chemical)} \quad (2)$$

The calculated EEFs were then used in conjunction with chemical analysis data to calculate predicted EEQs for each sample, as discussed in section 2.8.

2.3 Wastewater Source

The source wastewater used for the column pilot study was collected from the Jordan Lake Business Center (JLBC) located in Apex North Carolina, which uses a subsurface vertical flow sediment filter as the onsite wastewater treatment. Sewage produced by the approximately 60 occupants at the business center is collected in a septic tank and then treated through a series of subsurface sediment filters, beginning with a bed that is aerobic in nature, followed by a bed that is anaerobic, and finishing with a second aerobic sand bed. The volume of source wastewater needed for an entire dose interval study was collected from the storage tank in the field site prior to the start of each study to ensure uniformity of the column influent, which can change over time based on the changes in production of wastewater. Wastewater was stored in 25L polyethylene containers in a cold room set to 4°C for a maximum of three weeks while one dose interval study was being conducted.

2.4 Aerobic and Hypoxic Batch Study

In order to better understand the mechanisms of remediation occurring in the pilot-scale constructed wetland discussed in section 2.5 below, a batch study was conducted as described in Holmes (2017) to analyze the effect of oxygenation on contaminant removal. Briefly, Stalite (discussed in section 2.5 below) was incubated at 25°C with wastewater for two months to establish a biofilm on the Stalite, while oxygen was bubbled into the wastewater to ensure proper bacterial growth. Then, 60g of the Stalite was transferred into six acid washed 1L amber bottles. Wastewater was spiked with the 7 target contaminants to obtain a final concentration of 100µg/L of each chemical (this procedure is described in more detail in section 2.5 below), and 600mL of spiked wastewater was aliquoted into the bottles containing Stalite. The samples were allowed to incubate and shake at 100rpm and 25°C. Prior to incubation, aerobic conditions were developed in three of the bottles by flushing them with air until achieving a dissolved O_2 reading of at least 7mg/L, and the samples were then left uncovered to allow for free exchange of gases. Low oxygen conditions were developed in the other three bottles prior to incubation by flooding them with argon until achieving a dissolved O_2 reading of less than 3mg/L and then capping for incubation. While this is a higher level of oxygen than is typically found for hypoxic conditions (typically defined as 1mg/L of dissolved O_2) there were limitations in the system used by Holmes (2017) that prevented lower oxygen conditions from being obtained (Martinez-Lavanchy et. al., 2015). As a result, a low enough level of oxygen (<3.0mg/L) was obtained that was expected to go hypoxic in the column studies and batch study vessels after sealing and incubating. For each set of hypoxic and aerobic conditions, one sample was sacrificed for chemical and YES analysis after 1 hour, the next after 8 hours, and the final after 24 hours of incubation. Samples were sacrificed by filtering the aqueous portion through 2.7µm and 1.5µm

Whatman (Fisher Scientific; Suwanee, Georgia) glass fibre filters followed by a Sartorius (Fisher Scientific; Suwanee, Georgia) 0.45μm nylon filter. Samples were then processed via SPE, as described in section 2.6.

2.5 Pilot-Scale Constructed Wetland Set Up

The pilot-scale CW was conducted by another student as part of the larger research project and is described in detail in Holmes (2017). The pilot-scale CW utilized large stainless steel vertical columns (10 cm x 100 cm) each containing a column of substrate to reproduce a subsurface vertical flow wetland in the laboratory and a schematic is presented in Figure 4. The columns were packed with Stalite, a slate aggregate produced by rotary kiln processing of volcanic ash sediment naturally occurring in North Carolina (Stalite, 2009). Stalite is characterized by a high porosity and surface area, making it ideal for colonization of bacterial communities. The columns were stored in a cold room set at 4°C (to prevent additional bacterial degradation of column effluent in the collection vessel) and wrapped with heating wire set at 25°C to promote bacterial growth and encourage the development of a biofilm on the Stalite. Prior to any experiments, each column was conditioned by closing the effluent port on the column and filling it with wastewater. The presence of a biofilm was confirmed using a FastDNA Spin Kit for DNA soil extraction (MoBio; Carlsbad, CA) prior to any dosing studies. The columns were run in series, with the first column being aerobic in nature, the second being hypoxic, and the third being aerobic. Aerobic conditions were developed by leaving the aerobic columns uncovered, unlike the hypoxic column which was sealed using a metal cap on the top of the column. Hypoxic conditions were developed by flooding the column with argon, which has a higher density than oxygen, to displace any oxygen that may have been present in the column.

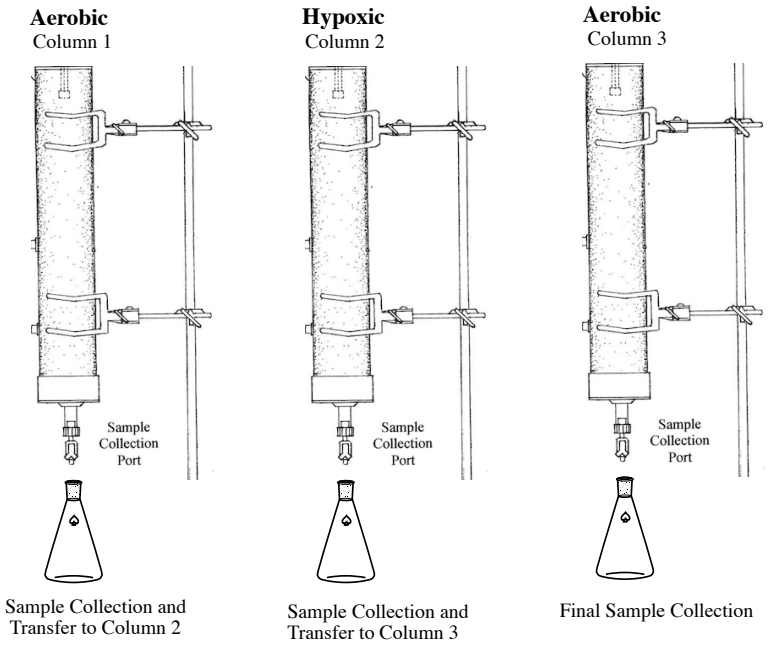

Figure 4. Schematic of the pilot-scale constructed wetland.

Five different dose interval studies – 8, 12, 24, 36 and 8 hour flooding – were tested to determine the relationship between dose interval and target contaminant removal as described in Holmes (2017). Wastewater was spiked with each target chemical (ATZ, DEET, 4-NP, CBZ, CAFF, E2, and TCS) to yield an overall spike level of 100µg/L for each chemical. Chemical stocks, each at 1g/L, were prepared in ACS grade methanol (Honeywell Burdick & Jackson; Muskegon, MI) and stored at 4°C. Due to solubility limitations, a stock of 1g/L of caffeine was prepared in LGW instead of methanol. Each study was conducted for nine days based on data from a previous round of column studies described in Holmes (2017). For each study, a total of 1.61L of spiked wastewater was poured into the top of the columns daily (over the course of five minutes), separated into aliquots based on the dose interval such that the total daily application did not exceed 1.61L (dose scenarios and volumes are summarized in Table 2).

Table 2. Summary of Pilot-Scale CW Optimization Study Parameters

Dose Interval (hours)	Doses Per Day	Total Wastewater Applied Per Dose Interval (L)	Total Wastewater Applied Daily (L)	Hours between Sample Collection
8	3	0.537	1.61	24
12	2	0.805	1.61	24
24	1	1.61	1.61	24
36	0.5	1.61	1.61	36
8 (flooding)	3	0.537	1.61	24

On the first day of each study, a volume of spiked wastewater (summarized in Table 2) was applied only to the first aerobic column and allowed to freely drain into a collection vessel. After the first dose interval had passed, the effluent from the aerobic column was poured into the top of the hypoxic column and allowed to freely drain into a collection vessel, while a second dose was applied to the first aerobic column. After the second interval, the effluent from the hypoxic column was transferred to top of the last aerobic column, and after the appropriate dose interval the process was repeated. For the 8 hour flooding dose interval, instead of freely collecting each column was saturated with wastewater and only allowed to drain at the end of the dose interval. Once the wastewater had passed through all three columns (three days for all studies except the 36 hour study, which took 4.5 days to pass through the final column), samples from column three were collected in 1L amber bottles and stored at 4°C until extraction. On the ninth and final day of the study, samples were collected from each column such that the sample collected from column 1 had only passed through the first aerobic column, the sample from column 2 had passed through the first aerobic and hypoxic columns, and the sample from column three had passed through all three columns. Prior to solid phase extraction of wastewater samples, 60mL aliquots of wastewater samples were set aside for routine water quality parameter testing, as discussed in detail in Holmes (2017).

2.6 Sample Processing

All materials and chemicals used for SPE are summarized in Appendix B. At the end of each study, all samples were filtered using stacked 2.7µm and 1.5µm Whatman glass fibre filters, followed by a Sartorius 0.45µm nylon filter, which was replaced as needed due to clogging of the filter. 250mL aliquots of each sample were transferred to acid-washed amber bottles using a graduated cylinder, and spiked with 12.5µL of a stock of atenolol at 1g/L (prepared in methanol) as the surrogate standard for quality control. For each dose interval study, duplicate samples of the source wastewater were prepared for characterization of the column influent prior to spiking. Four aliquots of the source wastewater prepared with the target chemicals spiked in at 5, 10, 50, and 100µg/L were used to build a standard addition calibration curve from which quantification of the target chemicals in all samples was achieved. The full SPE procedure was originally described by Stanford and Weinberg (2007) with a few modifications by Holmes (2017). Samples that were intended only for chemical analysis were eluted from the SPE cartridges into conical vials, blown down using a gentle stream of high purity nitrogen and low heat (30°C), and reconstituted in 2mL of methanol, yielding an overall concentration factor of 125. Samples that were intended for both chemical and YES analysis were processed and eluted from the cartridges using the same method with slight modifications to the reconstitution procedure as discussed in the next section.

2.7 Preparation of Samples for YES Analysis

From the samples processed and eluted as described in section 2.5, unspiked wastewater influent, spiked wastewater influent, an intermediate time point in the study, and final day samples collected from columns 1,2, and 3, were selected for each dose interval study for analysis on the YES assay in order to be able to monitor estrogenic activity removal trends over

time. The eluted extracts selected for both chemical and YES analysis were split into two aliquots of equal volumes, transferred to conical vials, and blown down to dryness using a gentle stream of high purity nitrogen and low heat (30°C). For each eluted sample that was split and blown down, one aliquot was reconstituted in 1mL of methanol for chemical analysis while the second was reconstituted in 1mL of molecular biology grade ethanol for analysis using the YES assay. Separate aliquots are required because ethanol is compatible with the YES assay but not chemical analysis.

All extracts have undergone the same overall concentration factor of 125 from wastewater sample to extract. However, samples that have undergone different levels of estrogenic activity removal needed to be differentially diluted in order to see a full dose response curve on the YES assay. As a result, the processing of each SPE extract for application on the YES assay is different and must be accounted for in data analysis. The unspiked source wastewater used for each study had low levels of estrogenic activity to begin with and, therefore, was not diluted prior to application on the YES assay. As a result, the unspiked sample had to be blown down a second time and reconstituted 1:1 in assay solvent (e.g. 1mL of extract blown down and reconstituted in 1mL of 10% ethanol solution). Similarly, for samples that required dilution factors less than 1:10 (e.g. 1:4, 1:2), an aliquot was transferred into a conical vial, blown down to dryness, and reconstituted in a 10% ethanol to ensure that all working dilutions of the extracts were prepared such that the final percentage of ethanol did not exceed 10%. Tables 3 through 7 summarize the sample identity and required dilution factor for all samples analyzed on the YES assay presented in this thesis.

Table 3. 8 hour Dose Interval Samples and Preparation for YES

Sample Name	Sample Identity	Degree of Treatment	Dilution Factor for Reconstituted Extract
T0 Unspiked	Source WW	Untreated	[a]No dilution
T0 Spiked	WW + 100µg/L spike of	Untreated	1:100
T3C3*	First WW sample collected	All 3 columns	1:4
T8C1	Final WW sample collected	Column 1 only	1:20
T8C2	Final WW sample collected	Columns 1 and 2	1:4
T8C3	Final WW sample collected	All 3 columns	1:2

*T#C$ where: T= Time point
#= Sample collection number (i.e. 3 means it was the third sample collected)
C= Column
$= Column the sample was collected from

[a]No dilution indicates a 1:1 reconstitution into 10% ethanol as described in section 2.6

Table 4. 8 hour Flooding Dose Interval Samples and Preparation for YES

Sample Name	Sample Identity	Degree of Treatment	Dilution Factor for Reconstituted Extract
T0 Unspiked	Source WW	Untreated	No dilution
T0 Spiked	WW + 100µg/L spike of	Untreated	1:80
T3C3	Fourth WW sample collected	All 3 columns	1:2
T8C1	Final WW sample collected	Column 1 only	1:50
T8C2	Final WW sample collected	Columns 1 and 2	No dilution
T8C3	Final WW sample collected	All 3 columns	1:2

Table 5. 12 hour Dose Interval Samples and Preparation for YES

Sample Name	Sample Identity	Degree of Treatment	Dilution Factor for Reconstituted Extract
T0 Unspiked	Source WW	Untreated	No dilution
T0 Spiked	WW + 100µg/L spike of	Untreated	1:100
T3C3	Third WW sample collected	All 3 columns	No dilution
T7C1	Final WW sample collected	Column 1 only	1:80
T7C2	Final WW sample collected	Columns 1 and 2	1:20
T7C3	Final WW sample collected	All 3 columns	1:4

Table 6. 24 hour Dose Interval Samples and Preparation for YES

Sample Name	Sample Identity	Degree of Treatment	Dilution Factor of Reconstituted Extract
T0 Unspiked	Source WW	Untreated	No dilution
T0 Spiked	WW + 100μg/L spike of	Untreated	1:100
T5C3	Second WW sample collected	All 3 columns	1:10
T9C1	Final WW sample collected	Column 1 only	1:80
T9C2	Final WW sample collected	Columns 1 and 2	1:20
T9C3	Final WW sample collected	All 3 columns	1:10

Table 7. 36 hour Dose Interval Samples and Preparation for YES

Sample Name	Sample Identity	Degree of Treatment	Dilution Factor of Reconstituted Extract
T0 Unspiked	Source WW	Untreated	No dilution
T0 Spiked	WW + 100μg/L spike of	Untreated	1:100
T4C1	Final WW sample collected	Column 1 only	1:100
T4C2	Final WW sample collected	Column 1 and 2	1:20
T4C3	Final WW sample collected	All 3 columns	1:10

2.8 Mixture Analysis of Seven Target Compounds

A standard mix of the contaminants was prepared at the concentration that would be expected in the SPE extract, which undergoes a 125-fold concentration factor, assuming 100% recovery of target analytes (i.e. 12.5mg/L). First, a 12.5mg/L solution was prepared in methanol for all contaminants except caffeine, which was prepared in LGW due to solubility limitations. The 12.5mg/L mixture was prepared by transferring 125μL of each individual 1g/L stock (in methanol) of E2, 4-NP, ATZ, CBZ, DEET, and TCS to a 10mL volumetric flask and filling to volume with methanol. A 12.5mg/L solution of caffeine was prepared by transferring 125μL of a 1g/L CAFF stock (prepared in LGW) to a 10mL volumetric flask and filling to volume with

LGW. A working solution was prepared by diluting this concentrated standard in the same manner as the extracts for application on the YES assay. This was done by transferring 13µL of the 12.5mg/L dilution of CAFF and 13µL of the 12.5mg/L mix of E2, 4-NP, ATZ, CBZ, DEET, and TCS to a test tube, adding 162µL ethanol, and 1320µL LGW and vortexing to mix. This mixture was then analyzed in the same manner as the SPE extracts described in section 2.9 to calculate the EEQs of the mixture, which was then compared to the sum of the individual chemical EEQs.

2.9 Data Analysis for SPE Extracts

YES data for the SPE extracts were processed identically to the data for individual chemicals, with two exceptions. First, the corrected absorbance was plotted against the volume of undiluted SPE extract present in each well as expressed in L/well equivalents (Leusch et. al., 2010). Doing so allows for the sample dilution factor to be included in the dose response curve and ensures all dose response curve analysis is in terms of the pure extract and not the working solution. For typical analysis of an individual chemical on the YES assay, an EC50 is calculated from a plot of absorbance versus chemical concentration, but the same cannot be done for an environmental extract of unknown concentration. Instead the absorbance of each well is plotted against the volume of undiluted extract in each well and an effective volume 50% (EV50) (i.e. the volume of extract producing 50% of the maximal response) is calculated (Leusch et.al., 2010). EEQ can then be determined using equation 3, which relates the concentration of E2 that would produce the half maximal response to the volume of extract that would produce the same half maximal response (Leusch et. al., 2010):

$$EEQ = \frac{EC50(E2)}{EV50(Sample)} \quad (3)$$

The EC50 of E2 is reported as the ng/well concentration that corresponds to the half maximal response, and the EV50 of the sample is reported as the L/well concentration of the undiluted extract, resulting in an EEQ in units of ng/L. This allows for a calculation of total estrogenic activity without requiring knowledge of the mixture components. Each extract is tested three times by preparing a fresh dilution of an aliquot of the extract on the third day of the YES assay. This allows for calculation of an average EEQ and its corresponding standard deviation.

To determine if any of the observed differences in estrogenic activity removal efficiencies for batch study samples were statistically significant a Student's t-test for two sample means with unequal variance was conducted in Microsoft Excel at the alpha=0.10 significance level. As percent reductions are not binary in nature and can take on any value between 0% and 100%, a t-test is the most appropriate statistical test (as compared to a z-test which is used for binomial variables and proportions). Differences in removal efficiencies were determined to be statistically significant when the associated p-value was less than α=0.10.

2.10. Calculation of Predicted EEQ

Sample extracts were analyzed for the 7 target chemicals using reverse phase high pressure liquid chromatography with detection by tandem mass spectrophotometry (LCMS) in electrospray ionization mode as described in Holmes (2017). Individual recoveries of each target compound were calculated using a method of standard addition, as described by Holmes (2017), and were used to adjust the concentration of the estrogenic targets in predicted EEQ calculations. Chemical concentrations are reported as the concentration present in the extract, and not the sample wastewater.

Total predicted EEQs for samples with different concentrations of estrogenic compounds required first calculating the EEF for each estrogenic compound using equation 1 and then using equation 4 (where n represents a given estrogenic target compound):

$$Predicted\ EEQ = \sum_{i=1}^{n} EEF_n \cdot [Concentration]_n \quad (4)$$

Only species that were determined to be estrogenic on the YES assay during analysis of the individual target compounds in the early part of the study are used when calculating a predicted EEQ for the extract.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Individual Target Compound Estrogenic Activity

Two of the 7 target compounds, E2 and 4-NP, are known estrogenic chemicals, and their estrogenic activity, which is summarized in Table 8, was confirmed by the YES assay. The mean and standard deviation EC50 for E2, which is also the positive control for the YES assay, over the entire 18 month study was found to be 0.539 ± 0.190 nM (n=59) and an example dose response curve is shown in Figure 5.

Table 8. Summary of Estrogenic Target Chemical Activity

	E2 (n=59)	4-NP (n=4)	ATZ (n=6)
Average EC50 (M)	5.39E-10	6.98E-07	3.99E-05
Standard Deviation (M)	1.90E-10	1.03E-07	1.68E-05
EEF	1.00	0.000771	0.0000135

The values in Table 8 are on the same order of magnitude as those reported in the literature for the YES assay, which range from 0.2nM to 0.25nM for E2 (Arnold, Robinson, Notides, Guillette, & McLachlan, 1996; Folmar et.al., 2002; Svobodová, Plačková, Novotná, & Cajthaml, 2009).

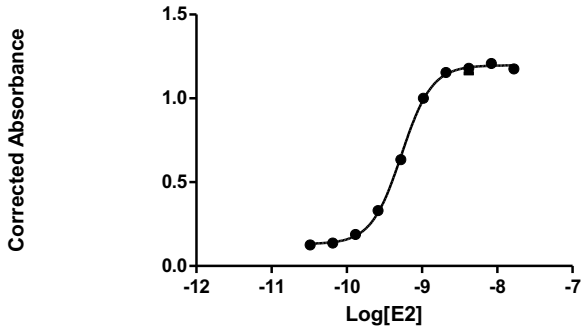

Figure 5. Example YES assay dose response curve for serial dilutions (1.67E-08M to 3.25E-11M) of the assay positive control, 17-β-estradiol in 10% ethanol (EC50 = 5.336E-10M).

The average EC50 for 4-NP was found to be 0.698 ± 0.103 μM (n=4), a value that falls in the range reported in the literature for the YES assay, which is 0.35-210μM (Folmar et. al., 2002; Svobodová, Plačková, Novotná, & Cajthaml, 2009). Atrazine was also found to be estrogenic with an average EC50 of 39.9 ± 16.8 μM (n=6). Example dose response curves for 4-NP and atrazine are shown in Figures 6 and 7, respectively.

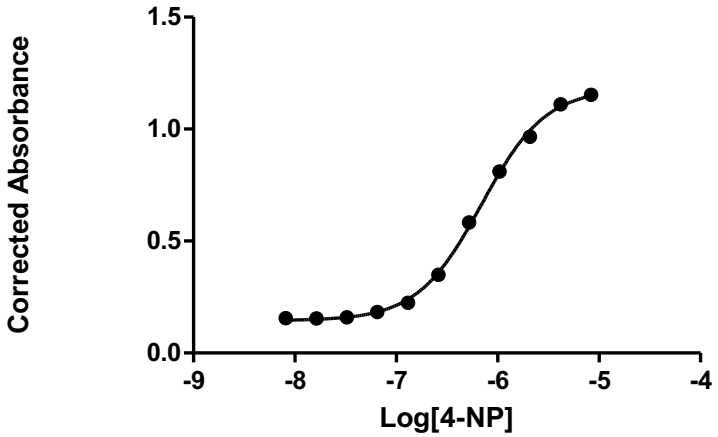

Figure 6. Example YES assay dose response curve for serial dilutions of 4-nonylphenol (8.29E-06M to 1.62E-08M) in 10% ethanol (EC50= 6.924E-07).

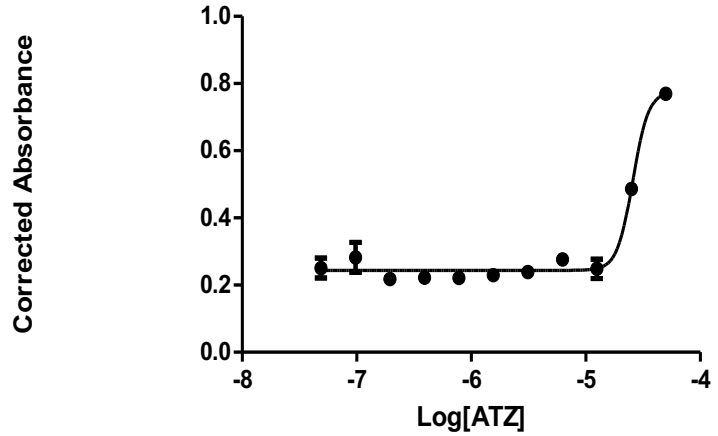

Figure 7. Example YES assay dose response curve for serial dilutions of atrazine (5.07E-05M to 4.95E-08M) in 10% ethanol (EC50= 2.57E-05).

There is conflicting information in the literature regarding the estrogenic activity of atrazine and its ability to bind to the human estrogen receptor. Previous research has not found atrazine to activate β-galactosidase transcription in the YES assay; however, it was shown to inhibit the binding of E2 in a human ERα competitive binding assays with a corresponding inhibitory concentration 50% value of 358μM (Bistan, Podgorelec, Marinšek Logar, & Tišler, 2012; Scippo et al., 2004). This indicates that atrazine is capable of binding to the ER and thus has the potential to activate the β-galactosidase pathway, as confirmed in this current study. Table 8 summarizes the average EC50 values of the three estrogenic chemicals, along with their resultant EEFs which are calculated as described in equation 2 of Chapter 2. While the EC50 of E2 is higher in this set of results than other reported research, the resultant EEF of 4-NP (0.000771) is still in good agreement with reported literature value of 0.00063, indicating the resultant EEFs can still be used to calculate predicted EEQs from chemical analysis data (Zhao et. al., 2011).

Carbamazepine and Triclosan were tested (n=3) up to their limit of solubility in water (solubilities given in Table 1 of Chapter 2), and were not found to be estrogenic on the YES assay, as shown by the lack of dose response in Figures 1 and 2 of Appendix C. Caffeine and DEET were tested (n=3) up to millimolar levels, nearly two orders of magnitude higher than environmental occurrence levels, and showed no induction of β-galactosidase, as seen in Figures 3 and 4 of Appendix C. Carbamazepine and caffeine have been previously tested on the YES assay and there was no detected induction of β-galactosidase activity in those experiments (Fent, Escher, & Caminada, 2006). Triclosan has been shown to be antiestrogenic in work involving recombinant ovarian cancer reporter gene assays (ER CALUX assays), and, therefore, was not able to induce reporter gene activation at any concentration tested (Ahn et. al., 2008). Triclosan

was also tested *in vivo* in recombinant gene assays conducted in nine different fish species, and showed little transactivation of any of the nine fish ERα (Miyagawa et. al., 2014). DEET is the only compound among the 7 examined in this study here that has not previously been tested for its estrogenic activity in a reporter gene system in the literature, and, as such, cannot be compared to any biologically relevant results.

3.2 EEQ for the Mixture of the Seven Target Compounds

A standard mixture of the 7 target compounds in solvent, prepared at the extract concentration assuming 100% recovery of target analytes (i.e. 12.5mg/L), had an average experimental EEQ of 10.5 ± 1.79 mg/L and the method comparison results are summarized in Table 9. An example dose response curve for the mixture is presented in Figure 8.

Table 9. Average EEQ for Mix of the Seven Targets Spiked in Solvent at 12.5mg/L Each

Replicate EEQ (mg/L)	Replicate EEQ (mg/L)	Replicate EEQ (mg/L)	Average EEQ and Standard Deviation (mg/L)	Predicted EEQ (mg/L)	Percent Difference	p-value
12.16	10.6	8.59	10.5 ± 1.79	12.5	17.8%	0.0929

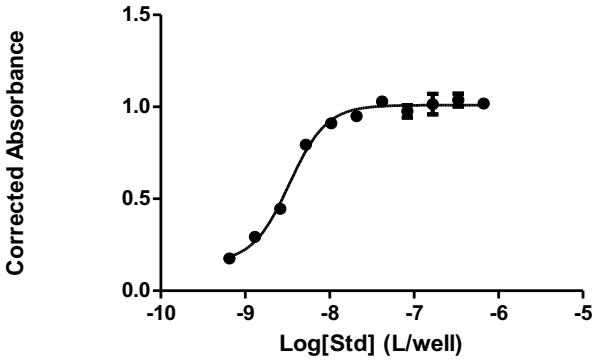

Figure 8. Example YES assay dose response curve for serial dilutions (6.67E-07 L/well to 6.51E-10 L/well) of the standard extract mix in 10% ethanol, EV50=3.59E-09 L/well.

The mixture was tested to determine whether the 7 targets exhibited additivity, antagonism, or potential synergy when combined in a single sample. A predicted EEQ for the mixture is calculated for the sample using equation 4 from Chapter 2, and an example of this equation is shown below using the quantified EEFs presented in Table 8 for the three major estrogenic components of the mixture (E2, 4-NP, ATZ).

$$\sum EEQ = 1.00 \cdot [E2] + 0.000771 \cdot [4-NP] + 0.0000135 \cdot [ATZ] \quad (5)$$

It is clear from the predicted EEQ (12.51mg/L) that the majority of the estrogenic activity in the mixture is caused by the presence of the endogenous hormone, E2. The percent difference in EEQ between the predicted (12.5mg/L) and the YES derived EEQ (10.5 $\pm$ 1.79 mg/L) is approximately 17.8%. A student's t test for two sample means assuming unequal variances was conducted to determine if the predicted and experimentally derived EEQ are significantly different. This was done by comparing the three experimental replicate EEQ calculated for the mixture (from Table 9) against the predicted value of 12.51 mg/L for each replicate (n=3, degrees of freedom= 2) at the α=0.10 significance level. The difference was found to be statistically significant ($p < 0.10$). Since the predicted value is consistently higher than the observed value, this indicates antagonism might be occurring in the mixture, which would cause the observed activity to be reduced compared to the predicted activity. Antagonism can partially be explained by the presence of a known ER antagonist, triclosan, in the mixture, which has shown to be antiestrogenic in several estrogen receptor-based bioassays (Ahn et. al., 2008). In addition, the 4-NP used in this study is a technical grade mix of isomers, some of which have been shown to be more antiestrogenic than estrogenic in MVLN cell lines utilizing the human ER (Preuss, Gurer-Orhan, Meerman, & Ratte, 2010). The ability of 4-NP isomers to act as both

an ER agonist and antagonist could also account for the lower than predicted activity in the mixture.

3.3 Aerobic and Hypoxic Batch Study Results

The same source wastewater was used for the aerobic and hypoxic batch studies and, as such, the average values of the EEQs for the unspiked and spiked wastewater extracts are identical for the two sets of conditions. The results for samples sacrificed for SPE after 1, 8, and 24 hours of incubation are presented in Tables 10 and 11, respectively. The unspiked wastewater extract had an average EEQ of 9.45 ± 1.98 μg/L, which was elevated to 10.1 ± 1.86 mg/L upon spiking with 100 μg/L of each of the 7 target chemicals.

Table 10. Aerobic Batch Study YES Results for SPE Extracts

Sample	Replicate EEQ	Replicate EEQ	Replicate EEQ	Average EEQ ± Standard deviation
T0 Unspiked	7.55 μg/L	9.31 μg/L	11.5 μg/L	9.45 ± 1.98 μg/L
T0 + 100 μg/L	11.7 mg/L	10.7 mg/L	8.02 mg/L	10.1 ± 1.86 mg/L
1hr	5.28 mg/L	5.78 mg/L	7.45 mg/L	6.17 ± 1.14 mg/L
8hr	5.71 mg/L	6.63 mg/L	8.71 mg/L	7.01 ± 1.54 mg/L
24hr	3.03 mg/L	3.65 mg/L	3.30 mg/L	3.33 ± 0.307 mg/L

Table 11. Hypoxic Batch Study YES Results for SPE Extracts

Sample	Replicate EEQ	Replicate EEQ	Replicate EEQ	Average EEQ ± Standard deviation
T0 Unspiked	7.54 μg/L	9.31 μg/L	11.5 μg/L	9.45 ± 1.98 μg/L
T0 + 100 μg/L	11.7 mg/L	10.7 mg/L	8.02 mg/L	10.1 ± 1.86 mg/L
1hr	8.06 mg/L	6.38 mg/L	6.31 mg/L	6.92 ± 0.993 mg/L
8hr	6.27 mg/L	5.07 mg/L	6.07 mg/L	5.80 ± 0.641 mg/L
24hr	3.24 mg/L	2.50 mg/L	2.45 mg/L	2.73 ± 0.441 mg/L

The removal efficiencies for each batch study time point are calculated from the YES derived EEQ and are presented in Table 12. Overall, hypoxic conditions were associated with the highest rate of removal (75.6%) of estrogenic activity from a starting EEQ of 10.1 $\pm$ 1.86 mg/L to a final EEQ of 2.73 $\pm$ 0.441 mg/L over the course of 24 hours of incubation time.

Table 12. Batch Study Removal Efficiencies

Incubation Time	Aerobic Removal (%)	Hypoxic Removal (%)	p-value[a]
1hr	44.8	38.1	0.218
8hr	37.2	48.1	0.0858
24hr	70.2	75.6	0.0628

[a]: p-value for a student t test for difference of two samples (aerobic and hypoxic) assuming unequal variance at the α=0.10 significance level.

In order to perform the t test, each replicate EEQ for the aerobic and hypoxic conditions was used to calculate three corresponding percent removals (as seen in Table D.1 in Appendix D) for each incubation time (1, 8, 24 hours). Then a t-test for two sample means was conducted using the replicate percent removals to determine whether the mean removals for the two conditions (aerobic and hypoxic) were truly different for each incubation time (n=3, degrees of freedom= 2). Hypoxic conditions had higher rates of removal after 8 and 24 hours of incubation (48.1% and 75.6%), which are statistically significant ($p < 0.10$). Hypoxic conditions yielded a lower rate of removal after 1 hour (38.1%) than aerobic conditions (44.8%) but this difference was not found to be statistically significant at any alpha level. As expected, the removal efficiency increased with increased incubation time for both conditions, except the aerobic 8 hour extract, which had a reduced removal efficiency (37.2%) compared to the 1 hour extract. Differences in removal efficiencies between the batch studies are a result of the difference microbial communities that grow under each set of conditions. Aerobic bacteria dominate under aerobic conditions, while hypoxic conditions increase the portion of the biofilm that is anaerobic

in nature. Certain compounds are degraded preferentially under aerobic and anaerobic conditions, and, thus, changing the level of oxygenation changes the degradation of the target compounds by the biofilm.

The predicted (calculated from the sum of the analyte concentrations from LCMS analysis) and bioassay EEQ for each extract are summarized in Table 13. The analyte concentrations for each extract were adjusted using the individual chemical method recovery data (recovery data is summarized in Table D.2 of Appendix D) to calculate the actual analyte concentration in the SPE extract prior to calculating predicted EEQ. Nonylphenol was the only target compound detected (LCMS results are summarized in Table D.3 in Appendix D) in the unspiked wastewater extract and its presence accounts for 39.3% of the observed estrogenic activity based on these calculations. Samples that spend the longest time incubated with the Stalite (i.e. 24 hour aerobic and 24 hour hypoxic samples) had very low percentages (0.165% and 0.207 % respectively) of observed activity that could be predicted from the target analytes. Overall, the hypoxic conditions had the lowest percentage of activity that could be predicted from the estrogenic compounds targeted in this study for all time points. For all samples, the observed activity using the YES assay was much higher than could be predicted from chemical analysis, indicating the possible formation of estrogenic metabolites that are not captured in the LCMS analysis. This indicates that the YES assay provides additional insight into the estrogenic activity of treated wastewater effluent, activity which would be greatly underestimated if samples are analyzed only by chemical analysis. Taken together, this indicates that the YES assay can be used to track the removal of estrogenic activity through treatment processes, and can account for the transformation of estrogenic species (or metabolites) occurring in wastewater treatment.

Table 13 Comparison of EEQs for SPE Extracts for the Batch Study from LCMS and YES Data

	Aerobic			Hypoxic		
Time point	Predicted EEQ	Average Observed EEQ	Activity Explained by Target Estrogenic Analytes (%)	Predicted EEQ	Average Observed EEQ	Activity Explained by Target Estrogenic Analytes (%)
T0 Unspiked	3.71 μg/L	9.45 μg/L	39.3	3.71 μg/L	9.45 μg/L	39.3
T0 + 100 μg/L	1.26 mg/L	10.1 mg/L	12.4	1.26 mg/L	10.1 mg/L	12.4
1 hour	2.44 mg/L	6.17 mg/L	39.5	94.4 μg/L	6.92 mg/L	1.37
8 hours	1.42 mg/L	7.01 mg/L	20.3	4.17 μg/L	5.80 mg/L	0.0719
24 hours	5.48 μg/L	3.33 mg/L	0.165	5.65 μg/L	2.73 mg/L	0.207

3.4. Estrogenic Activity of Column Study Samples

Activity of Source Wastewater

The estrogenic activity of the wastewater prior to being spiked for the column study remained fairly consistent throughout the duration of the study. The wastewater used in the 8 and 36 hour dosing studies was collected from the JLBC CW field site at the same time, which is why the EEQ for the unspiked water extracts are identical for those two studies. Overall, the EEQ of the unspiked extract was the lowest for the 8hr flooding dose interval (EEQ= 0.861 $\pm$ 0.139 μg/L) and highest for the 8 and 36 hour dosing studies (EEQ= 2.63 $\pm$ 0.437 μg/L), with the others falling somewhere in between.

Activity of the Spiked Extracted Wastewater

A predicted EEQ was calculated for the extracted spiked wastewater (i .e. wastewater spiked with 100 μg/L of each chemical and processed through SPE prior to any application on the pilot scale CW) used for each dosing study. A predicted EEQ was calculated from the concentration of three estrogenic chemicals (E2, 4-NP, and ATZ) as determined by LCMS, using

equation 5. As with the batch study, the actual concentration of analyte in each SPE extract was calculated by adjusting for the individual chemical method recovery data (recovery data is summarized in Table D.2 of Appendix D). A comparison of the predicted EEQ with the experimental bioassay results is presented in Table 14. Three of the extracted spiked wastewater samples (8 hour, 24 hour, and 36 hour flooding) showed average EEQ much higher than what is predicted from chemical analysis at 6.20 $\pm$ 1.92 mg/L for the 8 and 36 hour and 6.90 $\pm$ 2.72 mg/L for the 24 hour dose interval. The remaining two spiked extracts (8 hour flooding, and 12 hour) had YES assay-derived EEQ one order of magnitude and 50% lower than the predicted value, respectively. YES assay-derived EEQs are a measure of net estrogenic activity (i.e. sum of estrogenic activity minus the sum of antiestrogenic activity) of a mixture. As a result, lower activity observed using the YES assay indicates that there are likely antiestrogenic compounds that are present in the wastewater background that are lowering the observed activity, but are not targeted in the LCMS method. Higher activity that is seen in the extracted spiked wastewater for the 8, 24, and 36 hour extracts could be explained by the presence of compounds in the background wastewater that influence how the target chemicals behave in a mixture. While the activity in these samples is higher than predicted from chemical analysis, it is still lower in all samples than the activity observed in standard mixture prepared in solvent (as discussed in section 3.2), further supporting the hypothesis that the increased observed activity is the result of interference of chemicals in the wastewater matrix and not from interactions occurring between target analytes.

Table 14. Comparison of EEQ Calculated from LCMS* and YES# Data for Spiked Wastewater

Dose Interval	EEQ# of Extracted Wastewater Spiked at 100 μg/L	Predicted EEQ*
8 hour	6.20 ± 1.92 mg/L	
8 hour flooding	128± 14.9 μg/L	
12 hour	693 ± 86.6 μg/L	1.26 mg/L
24 hour	6.90 ± 2.72 mg/L	
36 hour	6.20 ± 1.92 mg/L	

*: Indicates predicted EEQs calculated from the chemical concentrations in the extracts as determine by LCMS.
#: Indicates EEQs calculated from YES assay response to the extract.

8 Hour Dosing Study

The unspiked wastewater for the 8 hour study had an EEQ of 2.63 ± 0.437 μg/L, which was elevated to 6.20 ± 1.92 mg/L upon spiking of each target compound at 100 μg/L. The bioassay derived EEQ values for each SPE extract are summarized in Table 15. Samples indicated as T8 are those collected on the final day of the study and the sample denoted as T3 represents the third column 3 sample collected for this dosing study.

Table 15. 8 Hour Dose Interval Study YES Results for Solid Phase Extracts

Sample	Replicate EEQ	Replicate EEQ	Replicate EEQ	Average EEQ ± Standard deviation
T0 Unspiked	3.12 μg/L	2.29 μg/L	2.49 μg/L	2.63 ± 0.437 μg/L
T0 +100μg/L	8.21 mg/L	5.99 mg/L	4.40 mg/L	6.20 ± 1.92 mg/L
T8C1	2.70 mg/L	1.90 mg/L	2.63 mg/L	2.40 ± 0.443 mg/L
T8C2	7.19 μg/L	4.76 μg/L	9.44 μg/L	7.13 ± 2.34 μg/L
T8C3	1.82 μg/L	8.93 μg/L	1.82 μg/L	4.19 ± 4.10 μg/L
T3C3*	4.85 μg/L	1.19 μg/L	4.60 μg/L	7.12 ± 4.14 μg/L

*T#C$ where: T= Timepoint
#= Sample collection number (i.e. 3 means it was the third sample collected)
C= Column
$= Column number the sample was collected from

The combined removal of estrogenic activity from the spiked influent applied to column 1 after passing through all three columns (i.e. samples T3C3 and T8C3) was 99.9%. Samples

T3C3 and T8C3 showed the same overall removal of estrogenic activity, indicating that the removal efficiency remained consistent throughout the length of the study. While it is clear that a large reduction (61.1%) in EEQ (from an average of 6.20 mg/L to 2.40 mg/L) occurs in the first aerobic column, the combined sequence of columns 1, 2, and 3 leads to an overall reduction of 99.9%. Looking at the relative reductions for each column, it becomes clear that the hypoxic column (column 2) facilities the highest reduction in EEQ at 99.7%. The reduction in estrogenic activity between column 2 and column 3 is much lower at 41.3%. Table 16 summarizes overall and relative removal between columns in this dosing study.

Table 16. 8 Hour Dose Interval Study Removal Efficiencies

Sample	Removal (%)	Relative Removal (%)
T8C1	61.1	61.1
T8C2	99.9	99.7
T8C3	99.9	41.3
T3C3	99.9	N/A

N/A indicates the relative removal cannot be calculated because the column 2 effluent (i.e. column 3 influent) was not characterized for estrogenic activity. Only final day samples characterized all three column effluents for their activity.

The predicted EEQ along with the observed bioassay EEQ for each time point are compared in Table 17. None of the estrogenic activity that was observed in the unspiked extract could be predicted from the LCMS data as all three estrogenic targets were below detection limit (results that are summarized in Table D.4 in Appendix D). For all extracts that had passed through at least one column of the CW except T3C3, the percent of activity that could be explained from chemical analysis was small, ranging from 0.966% to 9.02%. This underestimation of activity is likely caused by transformation of the parent compounds into their metabolites, some of which, such as estrone and estriol (metabolites of E2), are known to be estrogenic but are not captured by the targeted analysis used in this study. T3C3 was the

exception, and 75.1% of its activity could be predicted from the 4-NP that remained in the sample. The difference seen between T3C3 and T8C3 could be explained by an accumulation of weakly estrogenic metabolites in column 3 over the course of the study, which would not show up in chemical analysis, but could explain why the majority of the activity in T3C3 can be traced back to the presence of the target compound, 4-NP, but the activity in T8C3 cannot.

Table 17. Comparison of EEQ for SPE Extracts of 8 Hour Study from LCMS* and YES Data[#]

Sample	Predicted EEQ*	Average EEQ[#]	Percent of Observed Activity Explained by the Target Estrogenic Analytes
T0 Unspiked	<0.004 μg/L	2.63 μg/L	<0.002
T0 +100μg/L	1.26 mg/L	6.20 mg/L	20.3
T8C1	217 μg/L	2.41 mg/L	9.02
T8C2	0.0689 μg/L	7.13 μg/L	0.966
T8C3	0.0696 μg/L	4.19 μg/L	1.66
T3C3	5.35 μg/L	7.12 μg/L	75.1

*: Indicates predicted EEQs calculated from the chemical concentrations in the extracts as determine by LCMS.
#: Indicates EEQs calculated from YES assay response to the extract.

8 Hour Flooding Dosing Interval

The unspiked wastewater extract for the 8hr flooding dose interval was the least potent of all wastewater used for the dosing studies with an average EEQ of 0.861 ± 0.139 μg/L, which was increased to 128 ± 14.9 μg/L upon spiking with each of the target chemicals at 100μg/L. The EEQ for each extract are summarized in Table 18. Comparing the EEQ for the extracted unspiked wastewater from the different studies indicates that there is variability within the background activity of the wastewater, which can explain some of the differences in observed EEQ across samples. As discussed previously, the low EEQ of the spiked wastewater extract

used in the 8 hour study is likely caused by the presence of antiestrogenic compounds in the wastewater matrix that will reduce the activity observed in the spiked wastewater extract.

Table 18. 8 Hour Flooding Dose Interval YES Results for Solid Phase Extracts

Sample	EEQ	EEQ	EEQ	Average EEQ ± Standard deviation
T0 Unspiked	0.763 μg/L	0.960 μg/L	N/A*	0.861 μg/L
T0 +100μg/L	141 μg/L	132 μg/L	112 μg/L	128± 14.9 μg/L
T8C1	1.10 mg/L	2.00 mg/L	1.90 mg/L	1.67 ± 0.494 mg/L
T8C2	8.45 μg/L	2.56 μg/L	3.46 μg/L	4.82 ± 3.17 μg/L
T8C3	2.76 μg/L	2.07 μg/L	2.36 μg/L	2.40 ± 0.344 μg/L
T3C3	11.4 μg/L	14.1 μg/L	14.2 μg/L	13.2 ± 1.56 μg/L

*A third replicate EEQ is not available for the T0 unspiked extract because there was not enough sample. As a result a standard deviation cannot be calculated for this sample.

The removal efficiency for extracted samples that had passed through all three columns increased slightly for the duration of the study, with the removal in T3C3 being 89.7% compared to T8C3 which was 98.1%. The negative removal seen in column 1 (-1200%) could partially be explained by selective removal of antiestrogenic compounds or the conversion of antiestrogenic compounds into estrogenic compounds in the first column. If antiestrogenic compounds are being preferentially removed in column 1 (or converted into species that are estrogenic), it would result in an increase of the net activity seen in the YES assay results. Therefore, the majority of the removal of estrogenic activity is actually occurring in column 2, which agrees with the removal of estrogenic activity observed for column 2 (96.2%) and the high relative removal occurring in column 2 (99.7%). Moreover, it becomes clear that the majority of the activity is removed in the second column because, while column 3 shows a moderate relative reduction of 50.3%, column 3 only contributes an additional 1.9% to the overall removal. This data is summarized in Table 19.

Table 19. 8 Hour Flooding Dose Interval Study Removal Efficiencies

Sample	Removal (%)	Relative Removal (%)
T8C1	-1200	-1200
T8C2	96.2	99.7
T8C3	98.1	50.3
T3C3	89.7	N/A

N/A indicates the relative removal cannot be calculated because the column 2 effluent (i.e. column 3 influent) was not characterized for estrogenic activity. Only final day samples characterized all three column effluents for their activity.

The predicted EEQs from the LCMS data in Table D.5. in Appendix D along with the bioassay EEQs for the 8hr flooding study are summarized in Table 20. Atrazine and nonylphenol were detected in the unspiked wastewater extract and their presence explains 48.2% of the activity that was observed using the YES assay (results are summarized in Table D.4 in Appendix D). Samples that had passed through column 3 (T3C3 and T8C3) had some of the lowest percentages of activity that could be predicted based on the target estrogenic analytes (1.70% and 6.08%, respectively). This same phenomenon is seen in the sample from column 2 (T8C2), which only has 11.0% of its estrogenic activity explained by the predicted EEQs. This underestimation of activity is likely caused by the removal of the target chemicals via bacterial metabolism into estrogenic metabolites which are not captured by the LCMS analysis used in this study. For column 1, however, the percent of activity that could be predicted from chemical analysis was quite high at 78.7%, which indicates that there is very little conversion of the target compounds into estrogenic metabolites. If column 1 was exhibiting low removal of the estrogenic target compounds because it was preferentially removing the antiestrogenic compounds in the background wastewater matrix, it would explain both the negative removal in activity seen in column 1 and the high percentage of activity that can be explained by the presence of the estrogenic parent compounds. Such mechanistic evaluation (i.e. antagonism,

agonism) would not be able to be attained from LCMS analysis and, as a result, information regarding the selectivity of the columns and the conversion of the parent compounds to their metabolites would not be attained without evaluation on the YES assay.

Table 20. Comparison of EEQs for SPE Extracts of 8hr Flooding from LCMS* and YES# Data

Sample	Predicted EEQ*	Average EEQ#	Percent of Observed Activity Explained by the Target Estrogenic Analytes
T0 Unspiked	0.336 μg/L	0.861 μg/L	39.0
T0 +100μg/L	1.26 mg/L	128 μg/L	980
T8C1	1.31 mg/L	1.67 mg/L	78.7
T8C2	0.529 μg/L	4.82 μg/L	11.0
T8C3	0.146 μg/L	2.40 μg/L	6.08
T3C3	0.224 μg/L	13.2 μg/L	1.70

*: Indicates predicted EEQs calculated from the chemical concentrations in the extracts as determine by LCMS.
#: Indicates EEQs calculated from YES assay response to the extract.

12 Hour Dosing Study

The average measured EEQ of the unspiked extract was 1.75 ± 0.90 μg/L, which was elevated to 693 ± 86.6 μg/L upon spiking of the 7 targets. As was seen in the 8hr flooding study, the observed EEQs for the spiked extract are an order of magnitude lower than what is predicted from the chemical analysis (1.26 mg/L). As discussed previously, the low EEQ of the spiked wastewater extract used in this study is likely caused by the presence of antiestrogenic compounds in the wastewater matrix that will reduce the activity observed in the spiked wastewater extract. The EEQ calculated for each sample in the 12 hour study is presented in Table 21. Samples coded T7 were the final samples collected for the 12 hour study.

Table 21. 12 Hour Dose Interval YES Results for SPE Extracts

Sample	Replicate EEQ	Replicate EEQ	Replicate EEQ	Average EEQ ± Standard deviation
T0 Unspiked	1.12 μg/L	2.39 μg/L	2.46 μg/L	1.99 ± 0.755 μg/L
T0 +100μg/L	609 μg/L	689 μg/L	782 μg/L	693 ± 86.6 μg/L
T7C1	6.90 mg/L	2.29 mg/L	2.81 mg/L	3.99 ± 2.53 mg/L
T7C3	13.2 μg/L	10.4 μg/L	15.4 μg/L	13.0 ± 2.52 μg/L
T3C3	0.425 μg/L	0.758 μg/L	0.873 μg/L	0.684 ± 0.234 μg/L

The combined removal of estrogenic activity from the spiked influent applied to column 1 after passing through all three columns appears to decrease slightly through the duration of the study. Sample T3C3 had an overall removal of 99.9% while T7C3 showed a slightly lower removal at 96.5%. A t test for two sample means assuming unequal variance was run using replicate percent differences for the two samples, which are summarized in Table D.6 in Appendix D) (n=3, degrees of freedom=2). The difference in percent removal between T3C3 and T7C3 were found to be statistically significant at both the α=0.10 and α=0.05 significant level ($p < 0.05$). This result indicates that there may be an accumulation of weakly estrogenic metabolites in the third column over the course of the study, or that some of the adsorbed compounds are sloughing off of the Stalite and returning to the aqueous phase.

The negative removal seen in column 1 (-476%) could partially be explained by selective removal of antiestrogenic compounds or the conversion of antiestrogenic compounds into estrogenic compounds in the first column, similar to what was seen for the 8 hour flooding study. If antiestrogenic compounds are being preferentially removed in column 1 (or converted into species that are estrogenic), it would result in an increase of the net activity seen in the YES assay results for column 1. Similar to the results of the 8 hour flooding study, the majority of the removal of estrogenic species is actually occurring in column 2, which agrees with the high net

and relative removals in EEQ observed for column 2 (96.5% and 99.4%, respectively). As was the case with the 8 hour study, the majority of the activity is removed in the second column because, while column 3 shows a moderate relative reduction of 46.6%, column 3 only contributes an addition 1.6% to the overall removal, data that is summarized in Table 22.

Table 22. 12 Hour Dose Interval Study Removal Efficiencies

Sample	Removal (%)	Relative Removal (%)
T7C1	-476	-476
T7C2	96.5	99.4
T7C3	98.1	46.6
T3C3	99.9	N/A

N/A indicates the relative removal cannot be calculated because the column 2 effluent (i.e. column 3 influent) was not characterized for estrogenic activity. Only final day samples characterized all three column effluents for their activity.

The EEQ predicted from chemical analysis along with the bioassay EEQs for the 12hr study are summarized in Table 23. Nonylphenol was the only estrogenic target detected in the unspiked wastewater extract but its presence explains only 3.43% of the activity that was observed using the YES assay (results that are summarized in Table D.7 of Appendix D). As with the previous two studies, samples that had passed through all three columns (T3C3 and T7C3) had a low percent of activity that could be predicted based on the target estrogenic analytes (13.8% and 0.838%, respectively), likely due to metabolism of target compounds into weakly estrogenic metabolites.

Table 23. Comparison of EEQs for SPE Extracts of 12 Hour Study from LCMS* and YES Data[#]

Sample	Predicted EEQ*	Average EEQ#	Percent of Observed Activity Explained by the Target Estrogenic Analytes
T0 Unspiked	0.683 μg/L	1.99 μg/L	3.43
T0 +100μg/L	1.26 mg/L	693 μg/L	181
T7C1	319 μg/L	4.00 mg/L	7.99
T7C2	0.0832 μg/L	24.3μg/L	0.342
T7C3	0.0109 μg/L	13.0 μg/L	0.838
T3C3	0.0941 μg/L	0.684 μg/L	13.8

*: Indicates predicted EEQs calculated from the chemical concentrations in the extracts as determine by LCMS.
#: Indicates EEQs calculated from YES assay response to the extract.

24 Hour Dosing Study

The unspiked wastewater extract had an EEQ measured by the YES assay of 2.16 ± 0.801 μg/L, which was elevated to 6.901 ± 2.72 mg/L after it was spiked with 100 μg/L of each of the 7 target chemicals. The removal efficiency of the 24 hour study (Table 25) stayed consistent during the duration of the study with the removal for T5C3 and T9C3 being 97.7% and 98.0%, respectively. The EEQs for each sample are summarized in Table 24 and the removal efficiencies are presented in Table 24. Both the overall removals and the relative removals increased for each successive column, with the final column having the highest overall relative removal efficiency at 85.2%. Unlike previous studies, column 3 facilitated an appreciable increase in removal, increasing the net removal of the CW by 11.6%. This same trend is not seen in the previous dosing intervals, indicating the removal efficiency of each column may depend on the dosing interval employed.

Table 24. 24 Hour Dose Interval Study YES Results for Solid Phase Extracts

Sample	Replicate EEQ	Replicate EEQ	Replicate EEQ	Average EEQ ± Standard deviation
T0 Unspiked	2.73 μg/L	1.24 μg/L	2.50 μg/L	2.16 ± 0.801 μg/L
T0 +100μg/L	3.82 mg/L	12.6 mg/L	7.95 mg/L	6.90 ± 2.72 mg/L
T9C1	3.16 mg/L	3.86 mg/L	3.75 mg/L	3.53 ± 0.374 mg/L
T9C2	970 μg/L	1320 μg/L	1080 μg/L	1.12 ± 0.181 mg/L
T9C3	135 μg/L	336 μg/L	160 μg/L	210 ± 110 μg/L
T5C3	270 μg/L	98.0 μg/L	188 μg/L	185 ± 86.1 μg/L

Table 25. 24 Hour Dose Interval Study Removal Efficiencies

Sample	Removal (%)	Relative Removal (%)
T9C1	52.9	52.9
T9C2	86.4	71.2
T9C3	98.0	85.2
T5C3	97.7	N/A

N/A indicates the relative removal cannot be calculated because the column 2 effluent (i.e. column 3 influent) was not characterized for estrogenic activity. Only final day samples characterized all three column effluents for their activity.

The predicted and bioassay EEQs for the 24 hour study are compared in Table 26. As with the 12 hour dosing interval, nonylphenol was the only estrogenic target chemical detected in the unspiked wastewater extract and its presence explains only 10.6% of the activity that was observed using the YES assay (results that are summarized in Table D.8 of Appendix D). As with other dosing intervals, the percentage of activity that could be predicted from chemical analysis was lowest for samples that were treated through all three columns (T5C3, T9C3) and were still quite low for column 1 and 2 samples. The consistent underestimation of activity by chemical analysis across all studies indicates that microbial metabolism, and not just sorption, is facilitating the reduction in EEQ. The consistent underestimation of estrogenic activity predicted from chemical analysis in all dosing studies underscores the value that a biological screening tool such as the YES assay can add to water quality monitoring. Overall, the results indicate that

chemical analysis is not sufficient to capture the toxicological risk of an environmental sample, and points to the need to evaluate samples with a widely available bioassay, such as the YES.

Table 26. Comparison of EEQs for SPE Extracts of 24 Hour Study from LCMS* and YES Data#

Sample	Predicted EEQ	Average EEQ	Percent of Observed Activity Explained by the Target Estrogenic Analytes
T0 Unspiked	0.185 μg/L	2.16 μg/L	8.60
T0 +100μg/L	1.26 mg/L	8.10 mg/L	33.2
T9C1	430 μg/L	3.59 mg/L	11.9
T9C2	693 μg/L	1.12 mg/L	6.17
T9C3	12.1 μg/L	210 μg/L	5.75
T5C3	10.6 μg/L	185 μg/L	5.73

*: Indicates predicted EEQs calculated from the chemical concentrations in the extracts as determine by LCMS.
#: Indicates EEQs calculated from YES assay response to the extract.

36 Hour Dose Interval

As mentioned previously, the wastewater used for the 36 hour study was collected at the same time as the 8hr study and so the average EEQ for the unspiked and spiked wastewater samples are identical at 2.63 ± 0.437 μg/L and 6.20 ± 1.92 mg/L, respectively. The EEQ for each sample is summarized in Table 27. Samples coded as T4 were the final samples collected for the 36 hour study. The 36 hour dose interval had the lowest overall removal of estrogenic activity at 96.2%. The same trend that was observed in the 24 hour dosing interval, which saw the net removals increase steadily with treatment by each successive column, was seen in this study with the column 2 and 3 increasing the net removal by 49.8% and 11.6%, respectively. This phenomenon is not seen in the shorter dose intervals. However, as with all studies, column 2 showed highest relative removal at 76.4%.

Table 27. 36 Hour Dose Interval Study YES Results for Solid Phase Extracts

Sample	Replicate EEQ	Replicate EEQ	Replicate EEQ	Average EEQ ± Standard deviation
T0 Unspiked	3.12 μg/L	2.29 μg/L	2.49 μg/L	2.63 ± 0.437 μg/L
T0 +100μg/L	8.21 mg/L	7.17 mg/L	4.89 mg/L	6.20 ± 1.92 mg/L
T4C1	5.02 mg/L	5.98 mg/L	4.86 mg/L	5.29 ± 0.608 mg/L
T4C2	1.02 mg/L	1.17 mg/L	1.56 mg/L	1.25 ± 0.283 mg/L
T4C3	0.191 mg/L	0.376 mg/L	0.360 mg/L	0.309 ± 0.102 mg/L

Table 28. 36 Hour Dose Interval Study Removal Efficiencies

Sample	Removal (%)	Relative Removal (%)
T4C1	34.8	34.8
T4C2	84.6	76.4
T4C3	96.2	75.3

The predicted and measured bioassay EEQs for the 36 hour study are compared in Table 29. Concentrations used to calculate predictions are presented in Table D.9 in Appendix D. Atrazine was the only estrogenic target detected in the unspiked wastewater extract and its presence explains only 0.01% of the activity that was observed using the YES assay. As with the previous dosing studies, the presence of target analytes explains very little of the observed activity in samples that passed through column 2 and column 3 (T4C2 and T4C3). Column 1 had a higher percent of activity (12.8%) that could be explained by chemical analysis as compared to the other columns, likely due to the reduced rate of removal of estrogenic activity observed in the first column as compared to columns 2 and 3.

Table 29. Comparison of EEQs for SPE Extracts of 36 Hour Study from LCMS* and YES# Data

Sample	Predicted EEQ	Average EEQ	Percent of Observed Activity Explained by the Target Estrogenic Analytes
T0 Unspiked	0.00155 μg/L	2.63 μg/L	0.059
T0 +100μg/L	1.26 mg/L	6.20 mg/L	20.3
T4C1	6.79 mg/L	5.29 mg/L	12.8
T4C2	0.0437 μg/L	1.25 mg/L	0.003
T4C3	0.0347 μg/L	309 μg/L	0.01

*: Indicates predicted EEQs calculated from the chemical concentrations in the extracts as determine by LCMS.
#: Indicates EEQs calculated from YES assay response to the extract.

3.5 Discussion

The results of the five dosing studies indicate that the removal of estrogenic activity decreases as the length of the dosing interval increases, as can be seen in Figure 9. Overall, the 8 hour and 8 hour flooding dose intervals have the best overall removal at 99.9% and 98.5% respectively, but even the worst performing 36 hour dose interval still had high removal at 96.2%. However, small changes in removal efficiency can lead to significant differences in effluent activity with the best performing dose intervals having final estrogenic activities two orders of magnitude lower than that of the worst performing intervals: 4.19 ± 4.10 μg/L and 2.40 ± 0.344 μg/L in SPE for the 8 hour studies as compared to 210 ± 110 μg/L 309 ± 102 μg/L for the 24 hour and 36 hour studies respectively.

Table 30 summarizes the chemical removal for each column as calculated from the LCMS analysis (Holmes, 2017). The 24 and 36 hour studies showed the worst removal of E2 (66% and 48%, respectively) which explains the low removal of EEQ observed in column 1 samples for those dosing studies (52.9% and 34.8%, respectively).

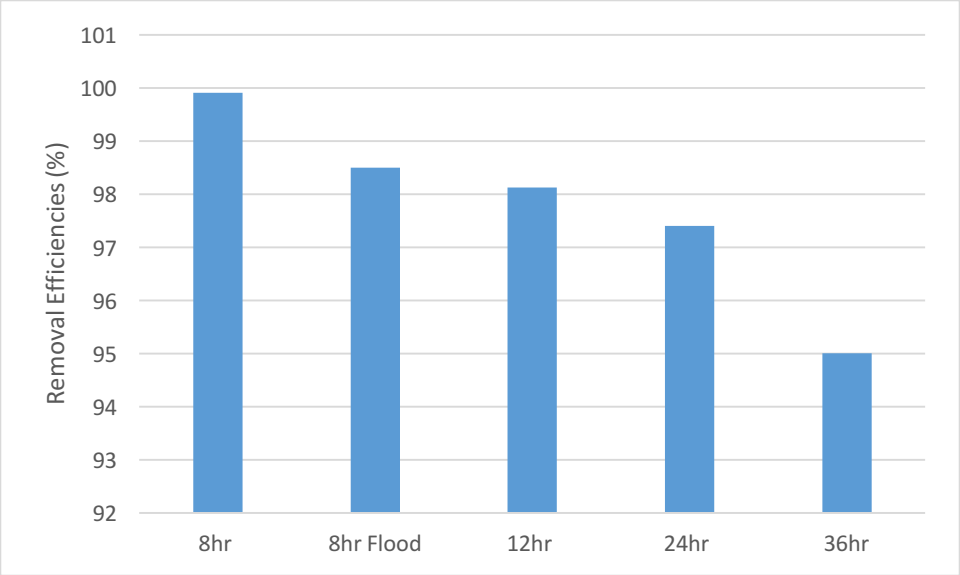

Figure 9. Overall estrogenic activity removals for the dosing intervals as calculated using YES assay-derived EEQ values.

The removal in EEQ for the best performing 8 hour dose interval in column 1 (61.1%) also tracked with the high removal of E2 in the first column (88.4%). However, column 1 from the 8 hour flooding and 12 hour showed negative removals of EEQ (i.e. an increase in estrogenic activity) despite having high rates of removal of E2 (≥ 95% and 73% respectively). This phenomenon could be caused by a preferential removal of antiestrogenic activity in column 1, which is not captured in the LCMS analysis, in conjunction with the conversion of E2 into its less potent estrogenic metabolites. The presence of antiestrogenic compounds in the source water would cause the EEQ of the extracted spiked source water to be depressed relative to the column 1 extracts, causing a negative removal of EEQ. The removal of such compounds in conjunction with the conversion of E2 into its metabolites would cause the net activity of column 1, as measured by the YES assay, to increase relative to the spiked extract.

Table 30. Removal Efficiencies of Estrogenic Analytes Calculated from LCMS Data

Dose Interval (h)	Column	ATZ (%)	E2 (%)	NP (%)
8 Flooding	1	38	≥ 95	≥ 95
	2	-1.6	≥ 99.9	92
	3	-2.4	≥ 99.9	≥ 95
	3 (Avg) n=16	3.3 ± 9.5	≥ 99.9	≥ 95
8	1	20	84	≥ 95
	2	27	≥ 99.9	≥ 95
	3	27	≥ 99.9	≥ 95
	3 (Avg) n=16	27 ± 6.0	≥ 99.9	89 ± 13
12	1	4.1	73	≥ 95
	2	13	≥ 99.9	≥ 95
	3	5	≥ 99.9	≥ 95
	3 (Avg) n=14	10 ± 5.4	≥ 99.9	≥ 95
24	1	11	66	74
	2	24	95	94
	3	56	≥ 98	≥ 98
	3 (Avg) n=12	46 ± 18	≥ 98	95 ± 3.8
36	1	-14	48	94
	2	19	≥ 95	≥ 95
	3	18	≥ 95	≥ 95
	3 (Avg) n=8	30 ± 10	≥ 95	≥ 95

Nonylphenol had high rates of removal in extracts from all 3 columns at ≥ 95% except for some of 24 hour study extracts, while atrazine had low removal in all of the column 1 samples, ranging from 4.1% to 38%. However, atrazine is nearly five orders of magnitude less potent than E2, and its presence in column 1 extracts only accounts for a small fraction of the observed EEQ in all column 1 extracts. This indicates that in addition to the low removal of atrazine, conversion of target analytes into estrogenic compounds of lower potency must be occurring.

For the dosing intervals that yielded the best overall reduction in EEQ after treatment by all three columns (8 hour, 8 hour flooding, and 12 hour), E2 could not be detected in any of the column 2 or column 3 samples. This was not due to failure of the method, as E2 was detected in all of the extracted spiked wastewater standards, and such standards yielded a linear response

between peak area and concentration. As a result, removals of E2 are estimated to be ≥ 99.9% for those samples, and the high removal for E2 is reflected in the high reduction of EEQ. The removal of E2, 4-NP, and ATZ for the less efficient dose intervals (24 and 36 hour) increased steadily between columns 1, 2, and 3, which tracks with the increased removal efficiency of each column as seen in the YES assay. While the removal of 4-NP was equally high, ATZ actually had higher rates of removal for all columns for the 24 and 36 hour dose intervals. However, the removal of ATZ has less of an impact on the observed EEQ than 4-NP or E2 due to its lower potency. Moreover, while removal of E2 was still high (≥ 98% and ≥ 95% for the column 3 averages of the 24 and 36 hour studies, respectively), E2 was still detected in all three column extracts for those studies. Thus, it is likely the reduced removal of E2 that is causing the column 3 EEQs of the 24 and 36 hour studies to be two orders of magnitude higher than the best performing studies, in which E2 could not be detected.

For all dosing studies, the highest net removal in activity was achieved in the second column, as seen in Figure 10. Column 1 for the 8 hour flooding and 12 hour dosing intervals had negative reductions in estrogenic activity, which is likely caused by selective removal of antiestrogenic compounds in columns 1, followed by selective removal of estrogenic compounds in column 2. Looking solely at the net reductions observed in each column does not tell the whole story, however, and it becomes necessary to look at the relative removal efficiency of each column, which is presented in Figure 11.

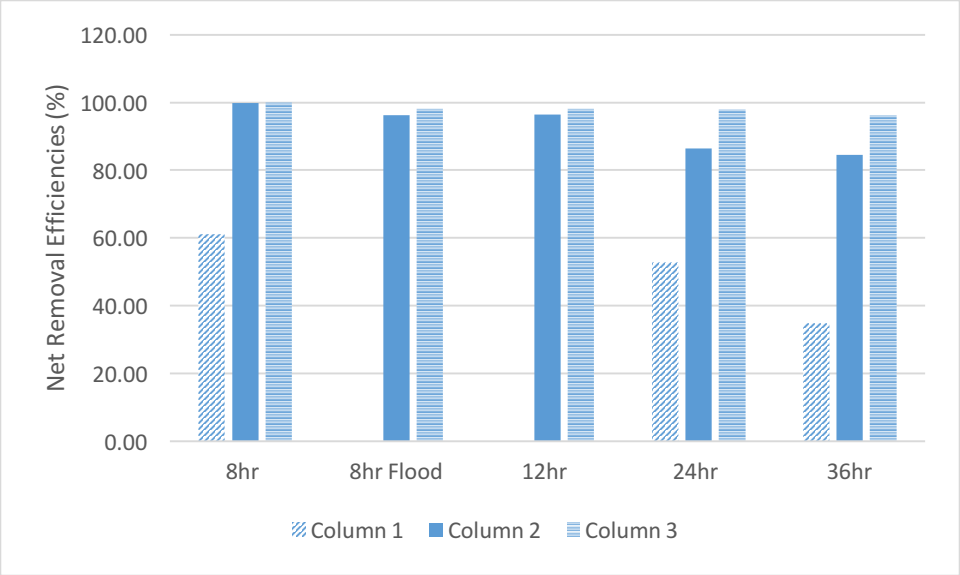

Figure 10. Cumulative removal efficiencies as calculated using YES assay-derived EEQ values, by column. Note, column 1 data from the 8hr flooding and 12 hour dose interval has been excluded due to the negative removals observed.

In terms of column efficiency, two patterns arise regarding chemical degradation and estrogenic activity removal, which appear to be dependent on the length of the dose interval employed. For the shorter 8 hour, 8 hour flooding and 12 hour studies, the hypoxic column had the highest overall reduction of estrogenic activity relative to the its column influent. The longer dosing intervals do not obey this same pattern. In the 24 hour and 36 hour studies, the relative removals steadily increased in each column. Taken together, this information indicates that even when there is a large reduction in the first column, the second and third columns continue to reduce the estrogenic activity of the wastewater and contribute to the overall reduction in activity. In either case, higher net removals (99.9% in the best performing 8 hour study) are achieved by utilizing the columns in series. For the 24 hour dosing study, the net removal increased 33.5% from column 1 (52.9%) to column 2 (86.4%), and 11.6% from column 2 to column 3 (98%). The need to use the columns in series becomes even more apparent when

looking at the 36 hour study, in which the net removal increased 49.8% from column 1 (34.8%) to column 2 (84.6%) and 11.6% in column 3.

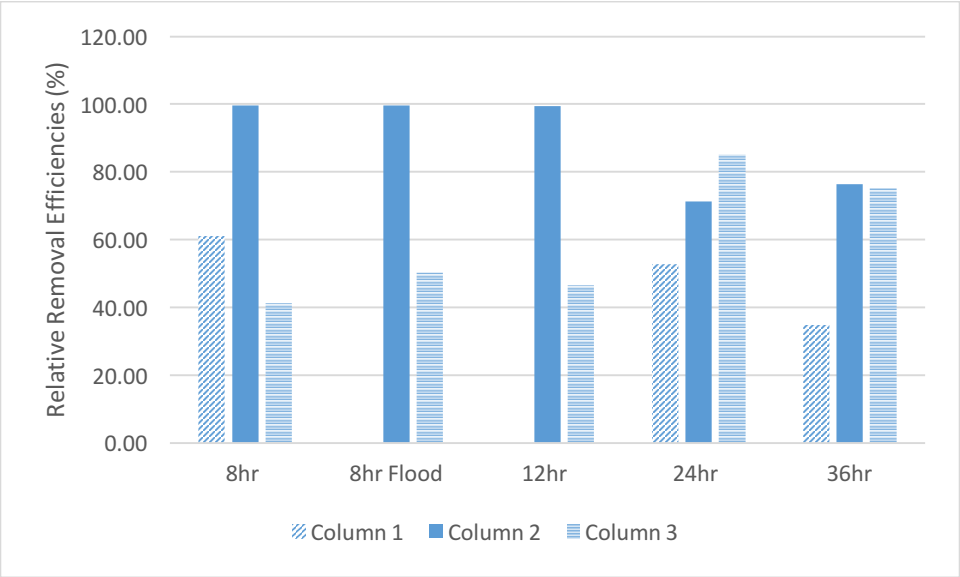

Figure 11. Relative removal efficiencies of each column for five pilot-scale CW dosing intervals as calculated using YES assay derived EEQ values

The results from the batch study also indicate that the efficiency of the column conditions, aerobic or hypoxic, will depend on the length of contact time between the wastewater and the biofilm. As seen in Figure 12, aerobic conditions yield a slightly higher reduction in estrogenic activity during shorter periods of incubation (1 hour) than do hypoxic conditions, although these differences are not statistically significant at the α=0.10 significance level. However, hypoxic conditions yield higher rates of removal after 8 and 24 hours of incubation, and these results were found to be statistically significant ($p<0.10$). Therefore, it appears that the efficiency of each set of conditions are dependent on the dosing interval employed, and the need to have two aerobic or two hypoxic stages of the CW may depend on the dosing parameters that being employed.

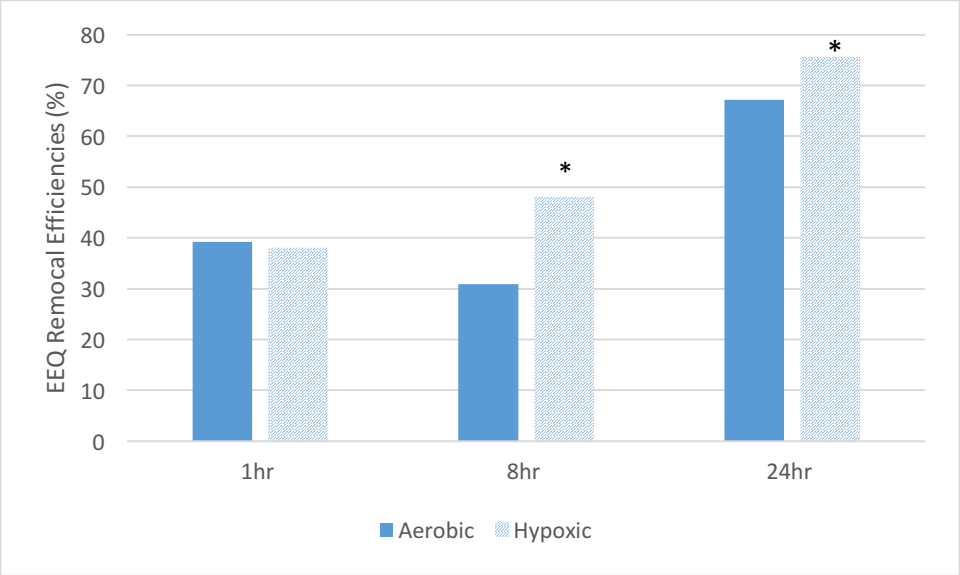

Figure 12. Comparison of removal efficiencies for the aerobic and hypoxic batch studies, as calculated using the YES assay. * indicates statistically significant difference in removal (α=0.10).

When comparing predicted and YES assay-derived EEQs across all samples, targeted chemical analysis appears insufficient for predicting the activity of complex environmental samples. Of the 37 SPE extracts tested on the YES assay, 35 of them had observed activity that was much higher than could be predicted from the concentrations of the three estrogenic target compounds, E2, 4-NP, and ATZ. This indicates that the majority of the activity of the sample was from other chemicals not targeted in this analysis. The remaining two samples had predicted activities that were higher than the activity observed on the YES assay, which can be caused by the presence of the triclosan, a known antiestrogenic species, in addition to other antiestrogenic compounds present in the background wastewater. The consistent underestimation of activity, confirms that chemical analysis alone is an insufficient method to monitor estrogenic activity of complex environmental samples. Moreover, it confirms the benefit of using a widely available bioassay, such as the YES, to screen environmental samples and track pollutant removal efficiencies of different treatment processes.

CHAPTER 4: CONLCUSIONS AND FUTURE WORK

4.1 Conclusions

The primary objective of this research was to demonstrate how the YES assay could be used to estimate the estrogenic activity of influent wastewater and track the removal of that activity as the wastewater was treated through the various stages of the pilot-scale CW. While there have been efforts to look at the ability of CW to remove emerging contaminants, and thus remediate polluted wastewater, this study attempts to correlate the removal of the contaminants with a biologically relevant endpoint. The second major objective was to be able to predict the estrogenic activity from chemical analysis and compare it to the YES assay-derived activity. Doing so would further validate the utility of using a biological assay, in conjunction with traditional approaches, to better estimate the activity of wastewater effluent, monitor the effectiveness of different treatments and reduce downstream exposure.

The yeast estrogen screen was able to confirm the estrogenic activity of three (E2, ATZ, 4-NP) of the 7 emerging contaminants used in this pilot study. Evaluation of the mixture of all 7 target compounds in solvent indicated that there was possible antagonism occurring in the mixture, which is likely caused, but not confirmed, by the presence of the known ER against, triclosan.

Wastewater collected over the course of several months from the constructed wetland at the JLBC in Apex, NC showed levels of estrogenic activity with EEQ ranging from 0.861 μg/L to 9.45 μg/L. Taking into account the concentration factor (125) allows for a rough approximation of the EEQ this would represent in wastewater effluent from the site, which

would be in the range of 6.89 ng/L to 75.6 ng/L. However, it is important to note that these values only represent an approximation of the wastewater's activity because chemical recovery through the SPE method can only be calculated for the target compounds, and thus might such a calculation may underestimate the activity in the source wastewater. The EEQ represent the equivalent concentration of E2 that would be required to produce the same overall activity that is observed for the wastewater samples. In a study looking at estrogenic activity of sewage treatment in Australia and New Zealand, the EEQ of raw sewage samples ranged from 15 ng/L to 185 ng/L and conventionally treated effluent had EEQ ranging from 6.0 ng/L to 143 ng/L (Leusch et. al., 2006), indicating the JLBC is achieving similar rates of removal as municipal wastewater treatment.

Adding 100 μg/L of each of 7 chemicals to this wastewater prior to the laboratory studies elevated the EEQ by two to three orders of magnitude and allowed for careful monitoring of the effect of treatment on their remediation by both the YES assay and LCMS analysis. Treatment of the wastewater through sequential columns containing the same solid substrate as used at JLBC with either hypoxic or aerobic conditions was able to reduce the EEQ to near background levels in all but the 24 and 36 hour dosing intervals. The effectiveness of the columns was highest for the 8 hour dosing interval and decreased as the length of the dosing interval increased. Two studies, the 8 hour flooding and 12 hour, indicated that column 1 might have been preferentially removing antiestrogenic activity, causing the calculated percent removals to be negative. This could be confirmed by further investigation with the YES assay. The YES assay could be used to evaluate antiestrogenic activity, as well as estrogenic activity, by co-exposing the yeast to a constant concentration of E2 while the concentration of the sample is varied to create a dose

dilution curve. This would allow for the calculation of an inhibitory concentration 50%, much like what is calculated for the competitive binding receptor assays.

For all studies except the 8 hour dose interval, hypoxic conditions were associated with better removal efficiencies and contributed to significant reductions in EEQ. This phenomenon was confirmed in isothermal batch studies where hypoxic conditions showed higher rates of removal after 8 and 24 hours of incubation, and these differences were found to be statistically significant ($p < 0.10$).

The removal of estrogenic activity from the spiked wastewater tracked most closely with that of the most potent estrogenic species, E2, whose removal was highest in the 8 hour, 8 hour flooding, and 12 hour dosing studies. Since E2 is so much more potent than the EDCs tested in this study (and most other EDCs), dose intervals that maximize the removal of E2 should be used in operation of the CW if the overall goal is to optimize such a system for estrogenic activity removal. Such operations would, therefore, need to utilize shorter dosing intervals, such as the 8 hour dosing interval, to achieve maximum removal of E2. However, estrogenic activity still persisted at low levels even when E2 could not be detected in final effluent samples, which is likely the result of conversion of E2 into its less potent but still estrogenic metabolites. Background levels of estrogenic activity persisted even after most of the parent compound was removed, and may require the addition of a second hypoxic stage to the CW to further reduce the activity in the effluent. Overall, the results of the study indicate that a restructuring of the current order of the treatment system to allow for two phases of hypoxic conditions in the full scale constructed wetland could result in higher rates of estrogenic activity removal.

The presence of the target analytes detected by LCMS could only explain low percentages of the total observed activity. In 35 out of 37 of the samples analyzed, chemical analysis consistently underestimated the activity of the sample, resulting in predicted EEQ often orders of magnitude lower than were observed using the YES. On the other hand, two samples overestimated the activity in the extracts, likely due to the presence of antiestrogenic species, whose presence would reduce the observed net activity. Taken together, this information shows that chemical analysis alone is insufficient to monitor a toxicological endpoint, such as estrogenic activity. This further suggests that bioassays can act as useful screening tools, especially when conducting studies to optimize water treatment for hazard reduction.

4.2 Future Work

Future expansions of this work should include evaluating and quantifying both the estrogenic and antiestrogenic activity of target compounds being spiked into the wastewater. If the ultimate goal is to be able to predict the activity of environmental samples from chemical analysis, this work could be expanded to quantify the potency of these major estrogenic metabolites and include these estrogenic metabolites in future chemical analysis. However, there is a limit to the number of compounds that it is feasible to target in chemical analysis, which is why bioassay analysis can prove useful. In addition, column extracts could also be evaluated for their antiestrogenic activity using the YES assay, which would provide further insight into potential preferential removal of activity (antiestrogenic or estrogenic) occurring in the different stages of the CW.

As mentioned previously, hypoxic conditions showed better removals of estrogenic activity, indicating that a change in the order of the columns employed in the pilot-scale CW

could facilitate increased removal of estrogenic activity. Future work could explore the removal efficiency of a CW utilizing a hypoxic column, followed by an aerobic column, followed by a second hypoxic column and compare the removal efficiencies seen in the best performing dosing intervals. Given the sensitivity of many aquatic species to potent estrogens, even small increases in the removal of estrogenic activity by a treatment method can have a protective effect on wildlife in receiving waters by helping to reduce their exposure to estrogenic activity.

A limitation of this study was testing the effectiveness of the CW on removal of target compounds and estrogenic activity from a single wastewater matrix. The wastewater used in the present study had already been treated by the JLBC CW and, as a result, is cleaner and has a reduced organic carbon load than other water types. Therefore, it would be interesting to see if this system would continue to achieve high rates of removal of estrogenic activity for a range of influent waters. This would provide a stronger case for using CWs to treat a wide variety of water types. In addition, this study only examined one relevant toxicological endpoint (estrogenic activity). Future work could expand to include other forms of endocrine activity, including antiestrogenic activity, and (anti)-androgenic activity, which can also pose a threat to human and wildlife health.

APPENDIX A: YEAST ESTROGEN SCREEN (YES) ASSAY PROTOCOL

Part 1: Materials needed

UV-VIS 96-well plate reader (Molecular Devices, EMAX; Sunnyvale, California)

Shaking Incubator (Shel Lab; Cornelius, Oregon)

Weighing scale (Sartorius; Goettingen, Germany)

Disposable sterile vacuum filter/storage system (Corning Incorporated; Corning, New York)

Disposable (100 x 15 mm) sterile petri dishes (Fisher Scientific; Suwanee, Georgia)

Disposable 96-flat well bottom microplates (Fisher Scientific; Suwanee, Georgia)

Disposable 96-deep well 1 mL plates (Fisher Scientific; Suwanee, Georgia)

Disposable Reagent Reservoirs (VWR; Radnor, Pennsylvania)

50mL Falcon tubes (Fisher Scientific; Suwanee, Georgia)

Test tubes (Fisher Scientific; Suwanee, Georgia)

Plate Sealing Film (Corning Incorporated; Corning, New York)

Vortex mixer (Barnstead/Thermolyne; Dubuque, Iowa)

Original potable pipette aid (Drummond Scientific; Broomall, Pennsylvania)

50 – 300 µL 8 multichannel pipettor (Thermo Labsystems; Vantaa, Finland)

100 – 1000 µL pipettor (Fisher Scientific; Dubuque, Iowa)

Disposable (1 – 250 µL) pipette tips (Fisher Scientific; Suwanee, Georgia)

20 -200 µL pipettor (Pipetman; Middleton, Wisconsin)

10 and 25 mL plastic disposable pipettes (Fisher Scientific; Raleigh, North Carolina)

Part 2: Chemicals Needed

Chemical	CAS #	Brand/Source used by Weinberg lab
[a]KH_2PO_4	7778-77-0	Fisher Scientific; Fair Lawn, NJ
Ammonium sulfate	7783-20-2	Mallinckrodt; Paris, KY
[b]KOH	71769-53-4	Fisher Scientific; Fair Lawn, NJ
Casamino acids	65072-00-6	Fisher Scientific; Fair Lawn, NJ
Adenine	73-24-5	Alfa Aesar; Ward Hill, MA
Bacto agar	2014-03-31	BD; Sparks, MD
[c]$Fe_2(SO_4)_3$	10028-22-5	MP Biomedicals; Santa Ana, CA
D (+) Glucose	50-99-7	Fluka; St. Louis, MO
L-aspartic acid	56-84-8	MP Biomedicals; Santa Ana, CA
L-threonine	72-19-5	Acros Organics; New Jersey, NJ
[d]$MgSO_4 * 7H_2O$	10034-99-8	Acros Organics; New Jersey, NJ
[e]$CuSO_4 * 5H_2O$	7758-98-7	Sigma Aldrich; St. Louis, MO
[f]CPRG	99792-79-7	Sigma Aldrich; St. Louis, MO
Thiamine	59-43-8	Sigma Aldrich; St. Louis, MO
Pyridoxine	65-23-6	Sigma Aldrich; St. Louis, MO
D-pantothenic acid hemicalcium salt	137-08-6	Sigma Aldrich; St. Louis, MO
Inositol	87-89-8	Acros Organics; New Jersey, NJ
Biotin	58-85-5	Kodak; Rochester, NY
[g]Difco yeast nitrogen base	2014-11-30	BD; Sparks, MD
Adenine Sulfate	321-30-2	Acros Organics; New Jersey, NJ
Dextrose Anhydrous	50-99-7	Fisher Scientific; Fair Lawn, NJ
17-β-Estradiol (98.1%)	50-28-2	Sigma Life Sciences; St. Louis, MO
4-Nonylphenol (technical grade)	84852-15-3	Aldrich Chemistry; St. Louis, MO
Atrazine (99.1%)	613-068-00-7	Fluka Sigma Aldrich; St. Louis, MO
Caffeine Anhydrous	58-08-2	Fluka Sigma Aldrich; St. Louis, MO
Carbamazepine (99%)	298-46-4	Acros Organics; Geel, Belgium
[h]DEET (98.1%)	134-62-3	Fluka Sigma Aldrich; St. Louis, MO
Triclosan	3380-34-5	Sigma Aldrich; St. Louis, MO
Ethanol (Molecular Biology Grade)	64-17-5	Fisher Bioreagents; Geel, Belgium

[a]KH_2PO_4: Potassium dihydrogen phosphate
[b]KOH pellets: Potassium hydroxide
[c]$Fe_2(SO_4)_3$: Iron (III) sulfate
[d]$MgSO_4 * 7H_2O$: Magnesium sulfate septahydrate
[e]$CuSO_4 * 5H_2O$: Cupric sulfate pentahydrate
[f]CPRG: chlorophenol red-β-D-galactopyranoside
[g]Difco yeast nitrogen base is free of amino acids and ammonium sulfate
[h]DEET: *N,N*-Diethyl-*m*-toluamide

Part 3: Preparation of solutions and buffers

Minimal Medium (1L) + L-aspartic acid + L-threonine

- Dissolve the following components in 1L LGW
- 13.61 g KH_2PO_4
- 1.98 g $(NH_4)_2SO_4$
- 4.2 g KOH pellets
- 0.2 g $MgSO_4 * 7H_2O$
- 6.72 g casamino acids
- Add 1 mL of 10 mg/L $Fe_2(SO_4)_3$ (prepared just before making the media), 27.4 mL L-aspartic acid solution and 8.7 mL L-threonine solution
- Filter sterilize using a disposable filter/storage sterilization system and store the solution at room temperature

L-aspartic acid solution (4 mg/mL) for the assay medium

- Dissolve 0.120 g L-aspartic acid in 30 mL of LGW
- Filter sterilize using a disposable filter/storage sterilization system and store at 4°C

L-threonine solution (24 mg/mL) for the assay medium

- Dissolve 0.240 g L-threonine in 10 mL of LGW
- Filter sterilize using a disposable filter/storage sterilization system and store at 4°C

D (+) Glucose (20% solution) for the preparation of the growth media

- Dissolve 40.00 g glucose in 200 mL LGW
- Filter sterilize using a disposable filter/storage sterilization system and store at room temperature

Copper (II) sulfate (20mM) for the preparation of the growth media

- Dissolve 0.128 g $CuSO_4$ * $5H_2O$ in 40 mL LGW
- Filter sterilize using a disposable filter/storage sterilization system and store at room temperature

Vitamin solution for the preparation of the growth media

- Using a microbalance, weigh out the following:
- 8 mg thiamine
- 8 mg pyridoxine
- 8 mg D-pantothenic acid hemicalcium salt
- 40 mg inositol
- Dissolve in 180 mL of LGW
- Add 20 mL biotin (2 mg/100 mL LGW)
- Filter sterilize using a filter/storage sterilization system and store at 4°C

Chlorophenol red-β-D-galactopyranoside (CPRG)

- Dissolve 0.01 g CPRG in 1 mL of LGW and store in an amber vial at 4°C

Growth media for yeast culture

- Prepare growth media fresh just before use
- Add 23.8 mL assay medium (minimal media + L-aspartic acid + L-threonine), 2.5 mL of the 20% D (+)-glucose solution, 0.25 mL of the vitamin solution and 63 µL of the 20mM $CuSO_4$ solution to a 50 mL falcon tube and vortex.

Ura-Trp Solid media for yeast cell propagation (500 mL):

- 3.35 g Yeast Nitrogen Base without amino acids and ammonium sulfate
- 2.5 g ammonium sulfate
- 2.5 mL of 4 mg/mL adenine sulfate solution
- 10 g anhydrous dextrose
- 2.5 g casamino acids
- Dissolve in 500 mL LGW
- Filter sterilize using a disposable filter/storage sterilization system
- Add 10 g of bacto agar to 500 mL liquid Ura-Trp media in sterile storage vessel
- Shake well and transfer to a 1L Erlenmeyer flask
- Autoclave using the liquid cycle and limit sterilization time to 15 minutes because a longer one will degrade dextrose in the media
- Place autoclaved container under hood and allow to cool until it can be handled without difficulty. Do not shake the contents of the autoclaved bottle at this point because this would cause bubbles to be present in the media
- Pour 20-25 mL of media into disposable sterile petri dishes
- Let stand at room temperature to harden
- Place petri plates in dated ziploc bags and store at 4°C.

17-β-estradiol (E2) solutions (in 100% ethanol):

- 1E-2M E2 stock: dissolve 27.38 mg E2 in a 10 mL volumetric flask with ethanol
- 1E-4M E2: add 100 µL of 1E-2M E2 stock solution to a 10 mL volumetric flask with ethanol
- 1E-6M E2: add 100 µL of 1E-4M E2 solution to a 10 mL volumetric flask with ethanol
- All solutions should be stored in amber vials at -20°C

5E-8M E2 working solution (in 10% ethanol):

- Add 9 mL LGW to an amber vial
- Add 0.5 mL ethanol and 0.5 mL 1E-6M E2 solution to the vial
- Mix well. Store at 4°C
- Make working solution fresh on day of assay for best results

10% Ethanol solution:

- Add 9mL LGW to a 50mL falcon tube
- Add 1mL ethanol
- Vortex to mix
- Prepare 10mL of 10% ethanol solution per 96-deep well plate being tested

Part 4: Yeast Cell Propagation and Assay Procedure:

1. Yeast cell propagation

I. Cell growth on Ura-Trp plates: A petri dish containing viable yeast colonies was provided by North Carolina State University and this dish was subsequently used for streaking the next yeast generations.

1) Using a sterile wand (i.e. metal wand dipped in ethanol and sterilized by a flame), streak a single colony of yeast from a previous plate onto an Ura-Trp solid media plate. Seal the plate with parafilm and incubate at 30°C. After 60 - 72 hours (when individual colonies have reached 1-2 mm in diameter), the plates should be removed from the incubator and stored at 4°C.

NOTE: Plates containing yeast colonies can be stored at 4°C for 2 months; however, it is a good practice to streak fresh plates at least every month in order to maintain the yeast colonies.

2. Assay Procedure

DAY 1

II. Grow yeast cells in growth media:

1) Aliquot 7 mL of growth media into a 50 mL sterile falcon tube.

2) Using a sterile wand, pick up one independent colony from a solid agar plate and transfer it to the liquid media in the falcon tube.

3) Incubate the yeast cells at 30°C in an incubator and shaker at ~200 rpm for 48 hours.

DAY 3

III. Determination of yeast cell density and its dilution using growth media:

1) Vortex the falcon tube containing the 48 hour yeast suspension culture by using a vortex mixer. Ensure that the 96 well plate reader is turned on at least 10 minutes prior to use.

2) Plate 100 µL (in triplicate) of the yeast suspension on 96-well flat bottom microplate. Plate 100 µL (in triplicate) of the growth media on the same plate. Read the plate at an absorbance of 620 nm in a plate reader.

3) Calculate the total volume of yeast cell solution that is used for each set of experiments:

   a) Subtract $Abs_{620 growth\ media}$ from $Abs_{620 Yeast}$

   b) Solve for x: $(Abs_{620 Yeast} - Abs_{620 growth\ media})(x\ \mu L) = (0.15)(100\ \mu L)$

4) Solve for y: $(x\ \mu L)/(100 \mu L) = (y\ mL)/25\ mL)$

5) y mL of yeast + (25-y) mL of growth media = dilution of the yeast suspension needed for the assay.

6) Prepare the yeast dilution calculated in 5). In order to ensure that there is enough yeast solution, make 25 mL of diluted yeast suspension per plate being tested.

7) Check the yeast dilution to make sure that the Abs_{620} falls in the range of 0.15 and 0.20 (Once again, subtract $Abs_{620 growth\ media}$ to account for background).

8) Add 10 µL of CPRG solution per 1 mL of diluted yeast solution and vortex to mix.

IV. Prepare assay plates:

1) Using a multichannel pipettor, add 100 µL of 10% ethanol to each of the wells in columns 12-2 of a 96 well flat bottom plate.

2) Add 200 µL of the positive control (E2) working solution to the first column of rows

A and B (Wells A1 and B1) as shown in Figure A.1. Add 200 µL of sample prepared in 10% ethanol, in duplicate, to the first wells in rows C-H.

3) Using a multichannel pipettor, dilute each column serially in 1:2 dilutions. (Transfer 100 µL of sample from column 1 to column 2; mix thoroughly by aspirating, then transfer 100 µL from column 2 to column 3). Continue the serial dilution across entire plate until column 11. After mixing the contents of column 11, withdraw 100 µL and discard as waste so that the column 12 wells contain only 10% ethanol, yeast and CPRG to act as the negative control for the assay, as shown in Figure A.1.

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td colspan="11" rowspan="2">E2</td><td rowspan="8">Negative control (10% ethanol)</td></tr>
<tr><td>B</td></tr>
<tr><td>C</td><td colspan="11" rowspan="2">Sample 1 in rows C and D</td></tr>
<tr><td>D</td></tr>
<tr><td>E</td><td colspan="11" rowspan="2">Sample 2 in rows E and F</td></tr>
<tr><td>F</td></tr>
<tr><td>G</td><td colspan="11" rowspan="2">Sample 3 in rows G and H</td></tr>
<tr><td>H</td></tr>
</table>

Figure A.1 YES Assay Template

V. Exposure of yeast cell to samples:

1) Add 200 µL of the yeast/CPRG solution to each well of the 96 deep well plate containing the samples, E2 and negative control, starting in column 12, which contains the negative control, and ending in column 1 to avoid cross contamination of wells . Ensure that each pipette has the same level of diluted yeast solution and no bubbles prior to placing in wells.

2) Cover plate with a plate sealing film and incubate at 30°C while shaking at ~200 rpm for 3 minutes. Remove the plate from the shaker and leave in the incubator for 48 hours. Avoid opening and closing the incubator until incubation period has elapsed.

DAY 5

VI. Measurement of optical densities (Endpoint):

1) Measure the absorbance of the plate at 540 and 620 nm. Calculate the corrected absorbance as the difference between the chemical absorbance at 540 nm and the value obtained when the blank absorbance at 620 nm is subtracted from the chemical absorbance at 620 nm. Dose-response curves are obtained by plotting the corrected absorbance against the sample concentrations.

2) Compare the sample's absorbance at 620 nm to that of the negative control's average. Values that are 10% less than that of the negative control's average are taken as

indication of cytotoxicity to the yeast cells due to the presence of the sample. Such cytotoxic concentrations are excluded from the data set used to plot the dose response curves for estrogenic activity.

APPENDIX B: MATERIALS FOR SOLID PHASE EXTRACTION PROCEDURE

Part 1: Materials needed

Supleco Vacuum Port Manifold (Sigma Aldrich; St. Louis, MO)

Strata-X 20mg/6mL Cartridges (Phenomenex; Torrance, CA)

Strata SAX 500mg/3mL Strong Anion Exchange Cartridges (Phenomenex; Torrance, CA)

Strata Si1 Silica Cartridges 300mg/3mL (Phenomenex; Torrance, CA)

Whatman GF/D 2.7μm glass fibre filters (Fisher Scientific; Suwanee, Georgia)

Whatman 934-AH 1.5μm glass fibre filters (Fisher Scientific; Suwanee, Georgia)

Sartorius Nylon 0.45μm nylon filter (Fisher Scientific; Suwanee, Georgia)

Part 2: Chemicals Needed

Chemical	CAS #	Brand/Source used by Weinberg lab
Methanol (ACS Grade)	67-56-1	Honeywell Burdick & Jackson; Muskegon, MI
Methyl-tert-butyl Ether (≥99%)	1634-04-4	Sigma Aldrich; St. Louis, MO
Ammonium Hydroxide (ACS)	1336-21-6	Fisher Chemicals; Geel, Belgium
Citric Acid Monohydrate	5949-29-1	Sigma Aldrich; St. Louis, MO
Atenolol	190017	MP Biomedicals; Santa Ana, CA
Ethanol (Molecular Biology Grade)	64-17-5	Fisher Bioreagents; Geel, Belgium

APPENDIX C: SUPPLEMENTAL DOSE RESPONSE CURVES

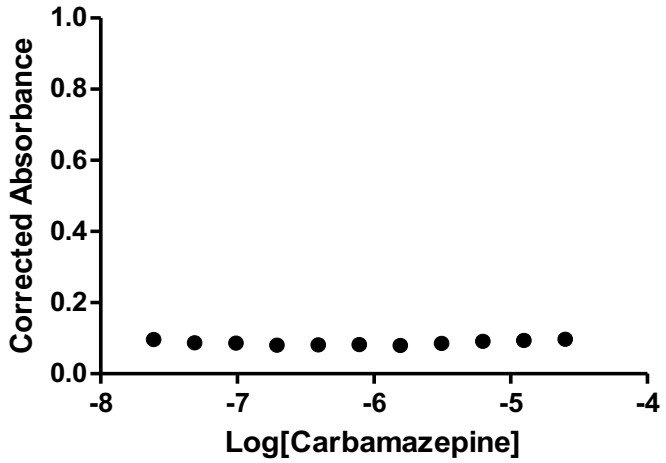

Figure C.1. Example YES assay dose response curve for serial dilutions of carbamazepine (2.53E-05M to 2.47E-08).

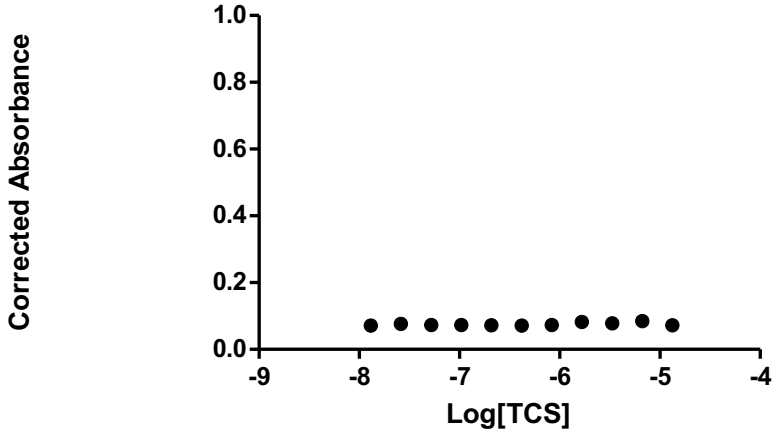

Figure C.2. Example YES assay dose response curve for serial dilutions of triclosan (1.33E-05M to 1.302E-08M).

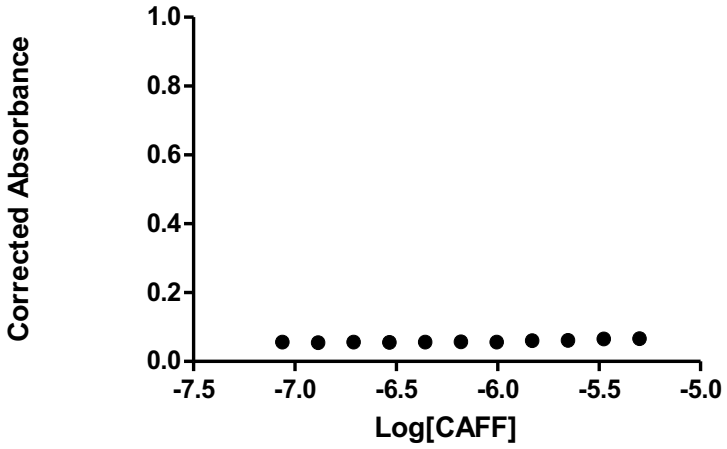

Figure C.3. Example YES assay dose response curve for serial dilutions of caffeine (1.25E-04M to 2.17E-06M).

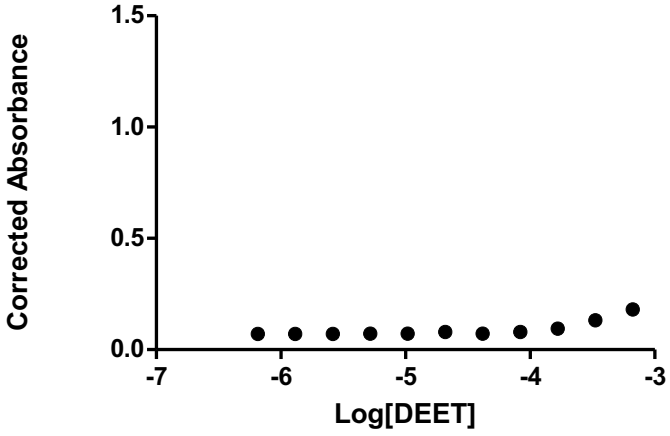

Figure C.4. Example YES assay dose response curve for serial dilutions of DEET (6.67E-04M to 6.51E-07).

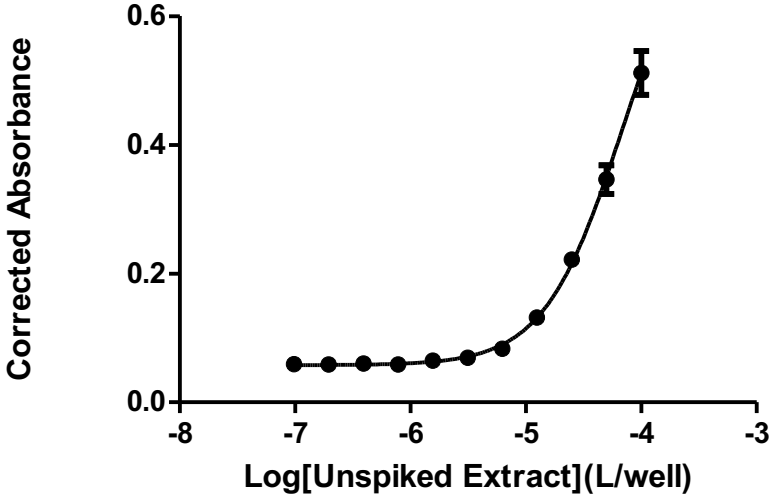

Figure C.5. Example dose response curve for the source wastewater (unspiked extract) used in the 8hr dosing study, EV50 = 5.734E-05 (L/well).

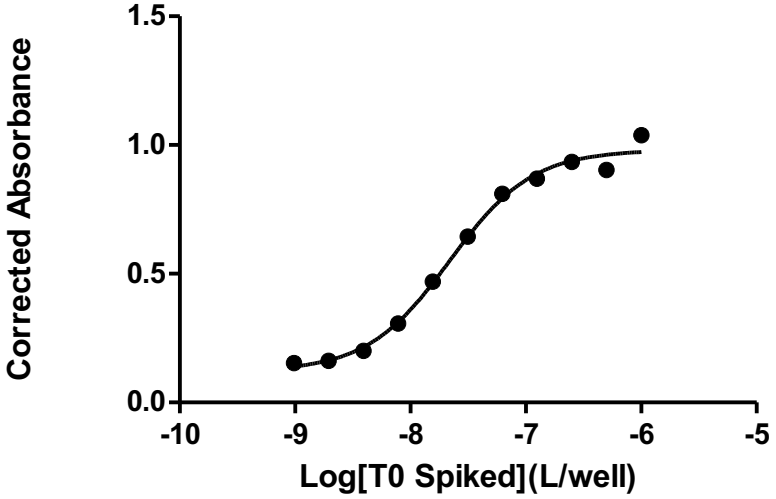

Figure C.6. Example dose response curve for the wastewater spiked with the 7 target compounds at 100 μg/L (T0 spiked) used in the 8 hour study, EV50 = 2.188E-08 L/well.

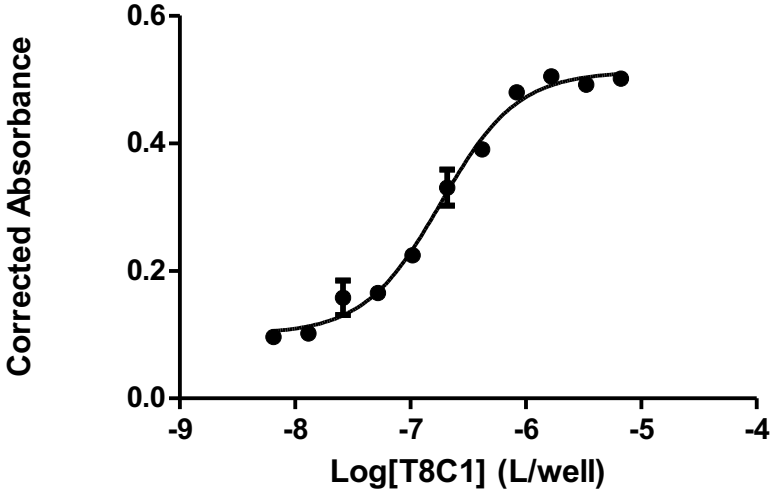

Figure C.7. Example dose response curve for the extract treated only by the first aerobic column (T8C1) in the 8 hour dosing study, EV50 = 1.835E-07 L/well.

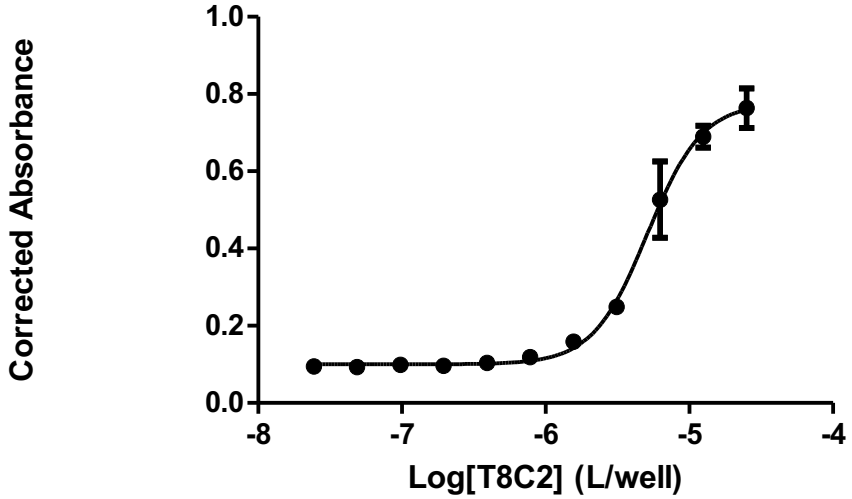

Figure C.8. Example dose response curve for the extract treated by the first aerobic and second hypoxic columns (T8C2) for the 8 hour dosing study, EV50 = 5.117E-06 L/well.

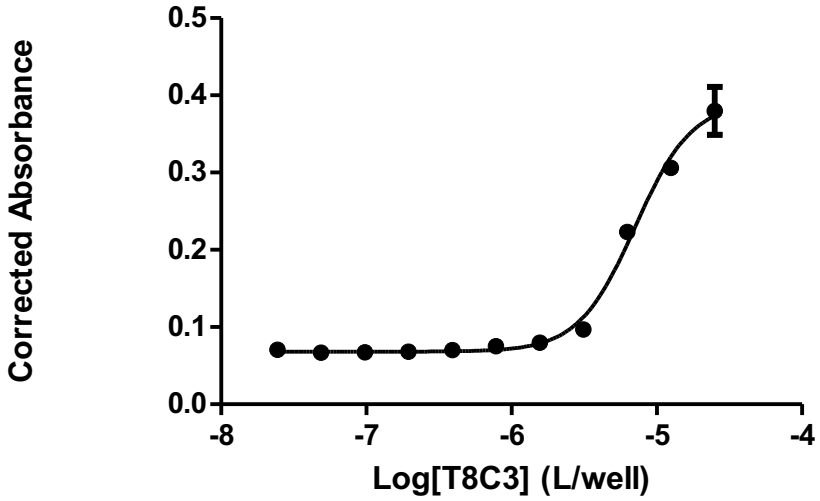

Figure C.9. Example dose response curve for the extract treated by all three columns (T8C3) in the 8 hour study, EV50 = 7.125E-06 L/well.

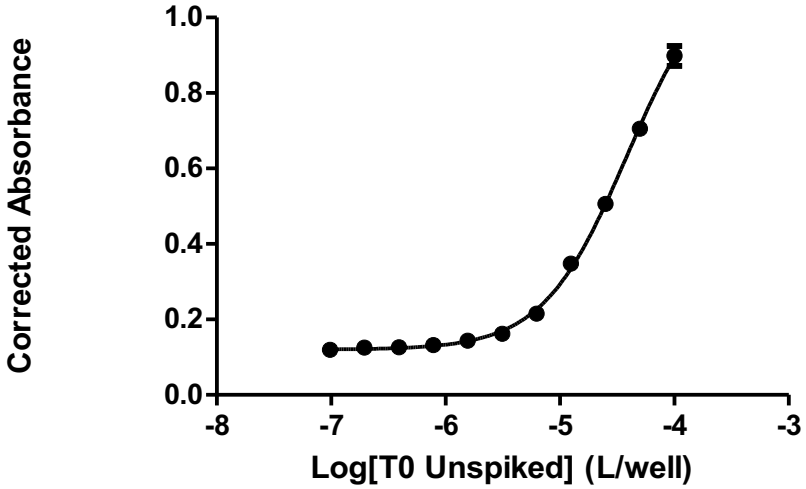

Figure C.10. Example dose response curve for the source wastewater (T0 unspiked) used in the 8 hour flooding dosing study, EV50 = 3.839E-05 L/well.

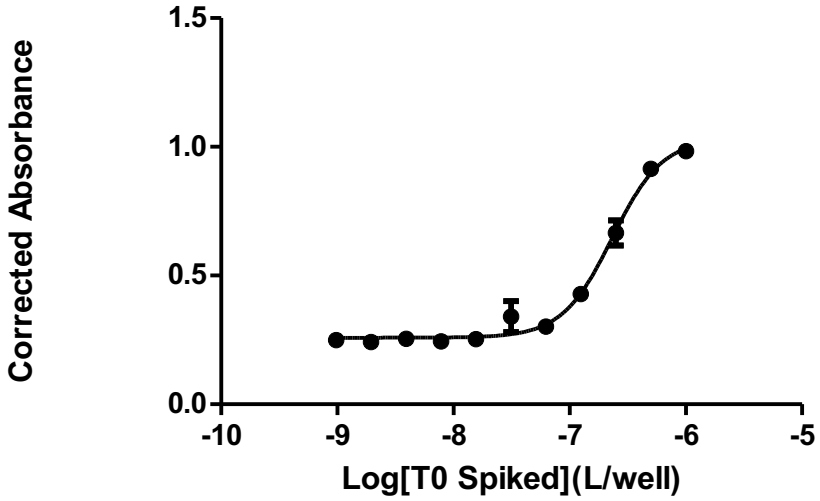

Figure C.11. Example dose response curve for the wastewater spiked with the 7 target compounds at 100 μg/L (T0 spiked) used in the 8 hour flooding study, EV50 = 2.327E-07 L/well.

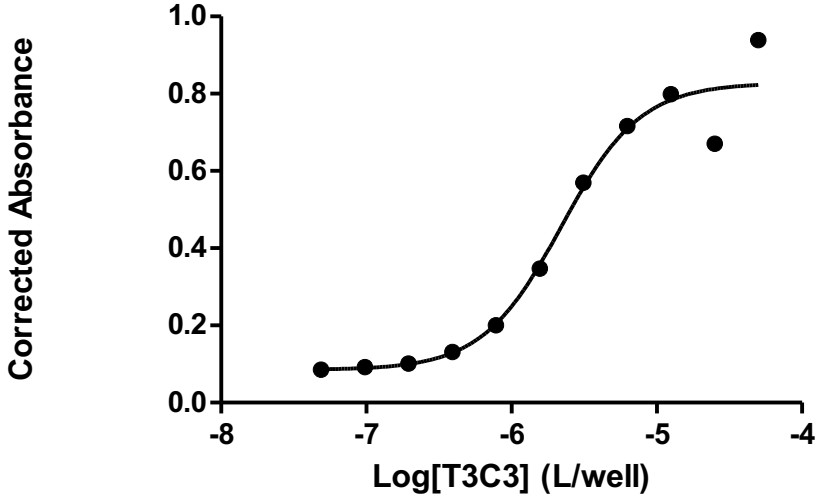

Figure C.12. Example dose response curve for the extract treated by all three columns (T3C3) in the 8 hour flooding study, EV50 = 2.203E-06 L/well.

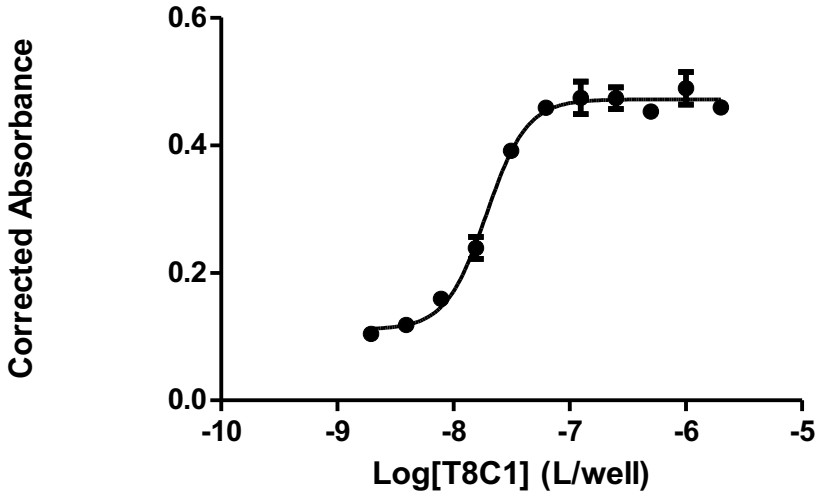

Figure C.13. Example dose response curve for the extract treated by the first aerobic column (T8C1) in the 8 hour flooding dosing study, EV50 = 1.918E-08 L/well.

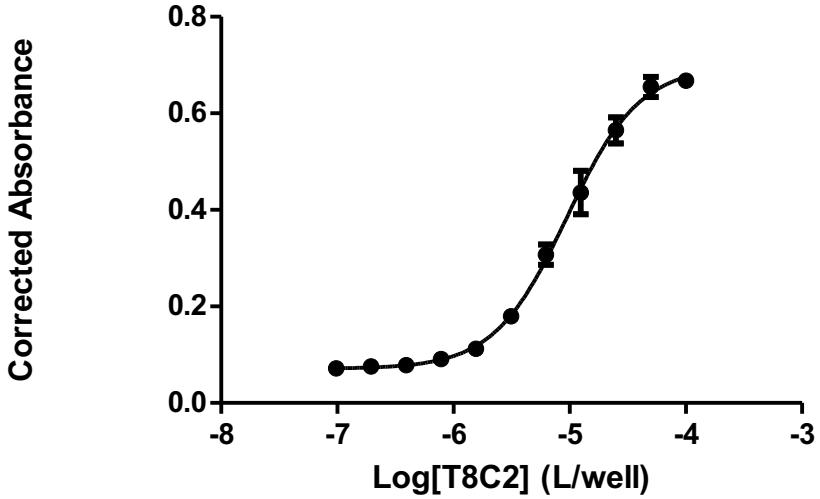

Figure C.14. Example dose response curve for the extract treated by the first aerobic and second hypoxic columns (T8C2) for the 8 hour flooding study, EV50 = 9.468E-06 L/well.

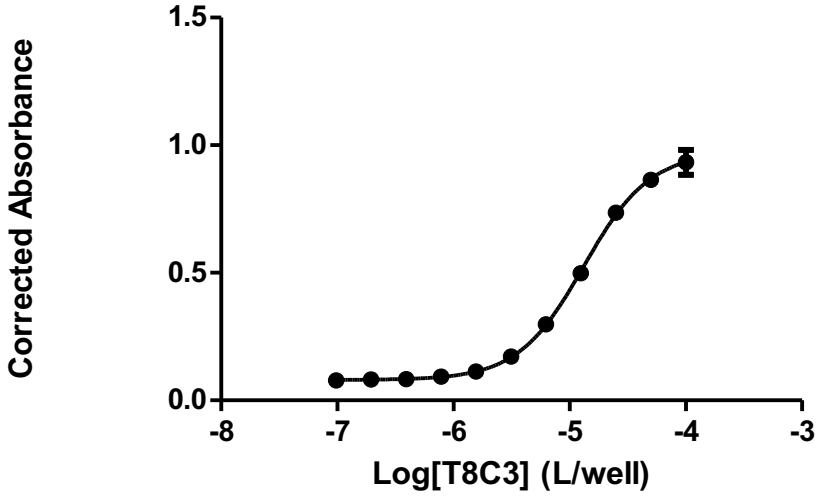

Figure C.15. Example dose response curve extract treated by all three columns (T8C3) in the 8 hour flooding study, EV50 = 1.325E-05 L/well.

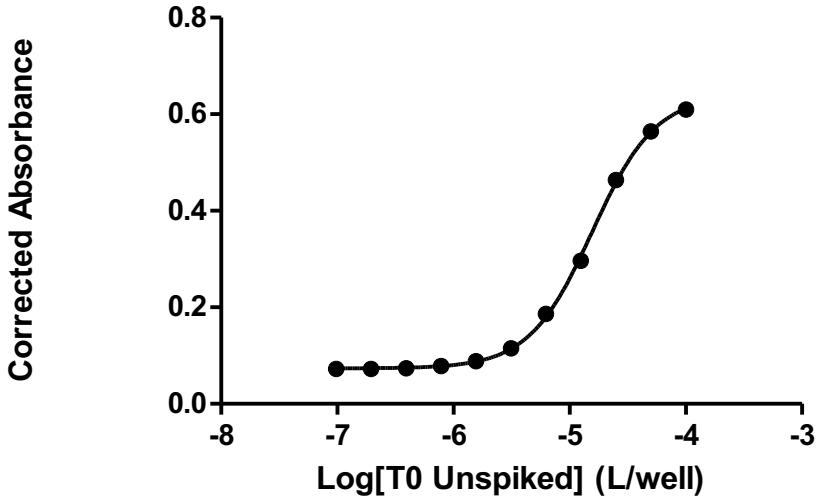

Figure C.16. Example dose response curve for source wastewater (T0 unspiked) used in the 12 hour dosing study, EV50 = 1.565 E-05 L/well.

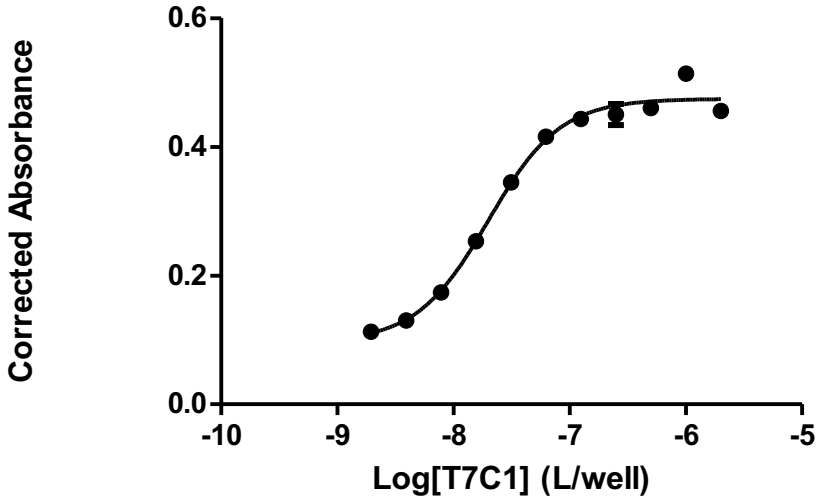

Figure C.17. Example dose response curve for the extract treated only by the first aerobic column (T7C1) in 12 hour dosing study, EV50 = 1.997E-08 L/well.

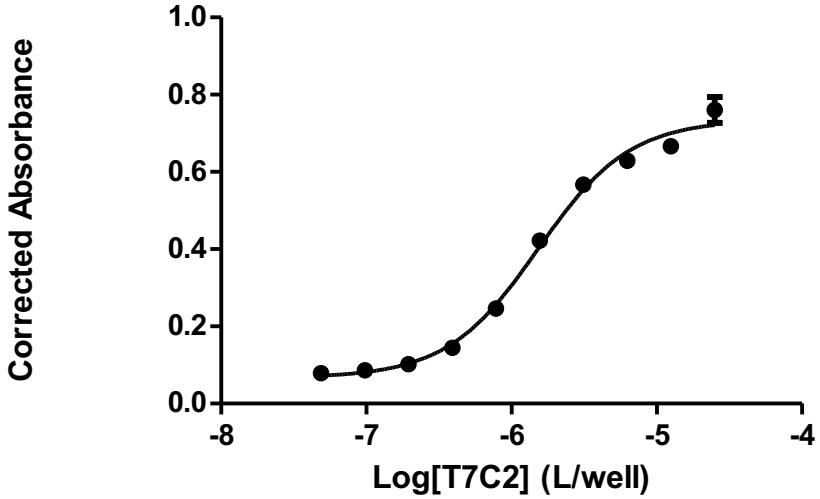

Figure C.18. Example dose response curve for extract treated by the first aerobic and second hypoxic columns (T7C2) for the 12 hour dosing study, EV50 = 1.526-06 L/well.

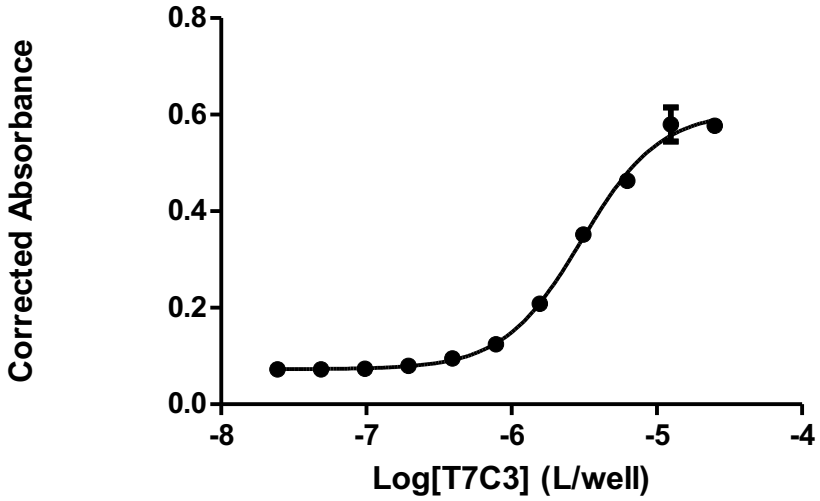

Figure C.19. Example dose response curve for extract treated by all three columns (T7C3) in the 12 hour dosing study, EV50 = 3.036E-06 L/well.

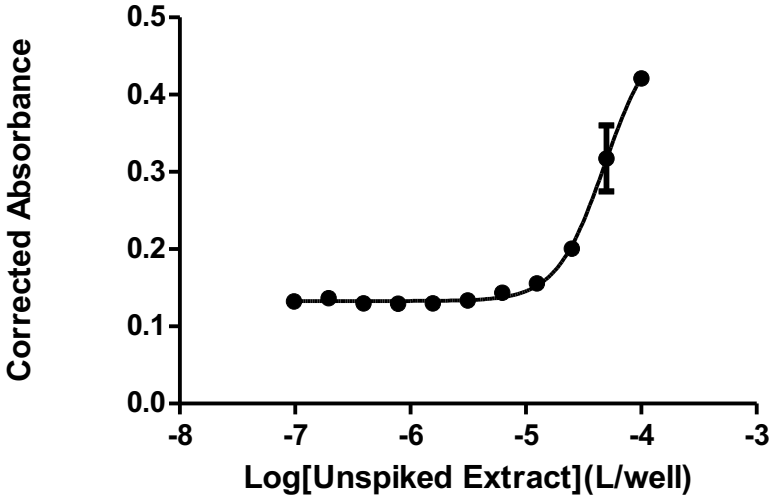

Figure C.20 Example dose response curve for the source wastewater (Unspiked Extract) used in the 24 hour dosing study, EV50 = 4.799E-05 L/well.

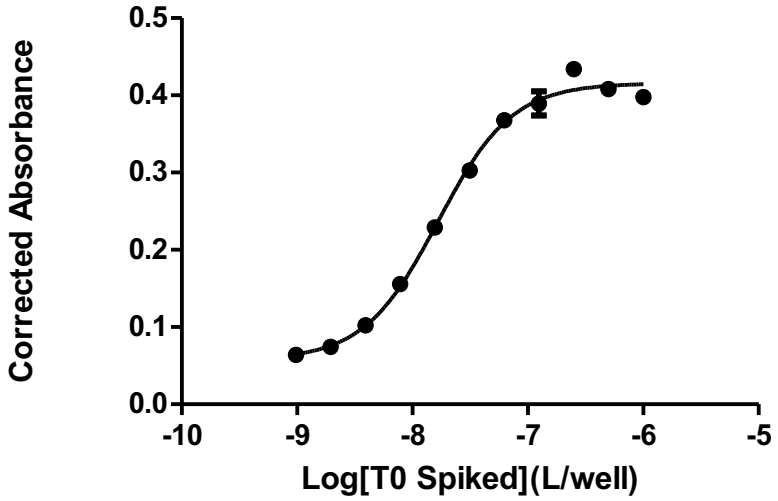

Figure C.21. Example dose response curve for the wastewater spiked with the 7 target compounds at 100 μg/L (T0 Spiked) used in the 24 hour study, EV50 = 1.663E-08 L/well.

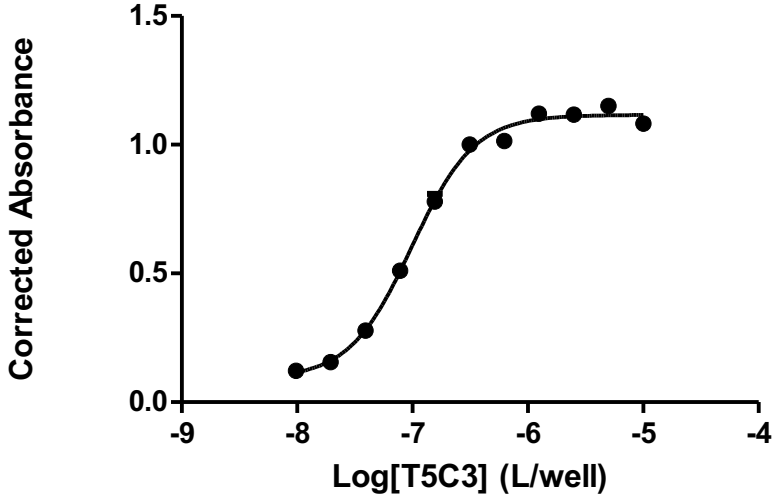

Figure C.22. Example dose response curve for extract treated by all three columns (T5C3) in the 24 hour study, EV50 = 9.874E-08 L/well.

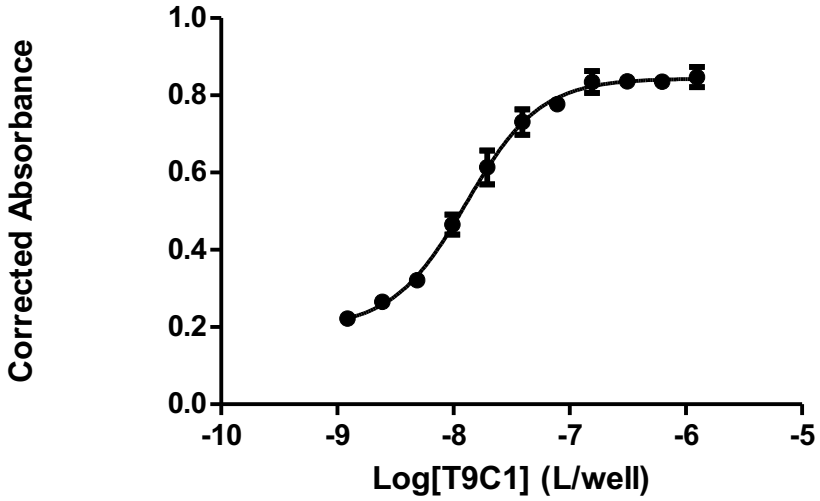

Figure C.23. Example dose response curve for the extract treated by the first aerobic column (T9C1) in the 24 hour dosing study, EV50 = 1.289E-08 L/well.

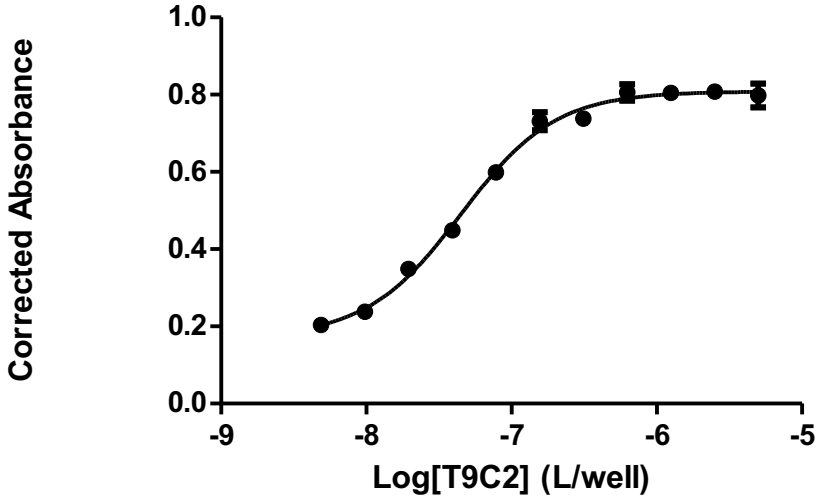

Figure C.24. Example dose response curve for the extract treated by the first aerobic and second hypoxic columns (T9C2) in the 24 hour study, EV50 = 4.478E-08 L/well.

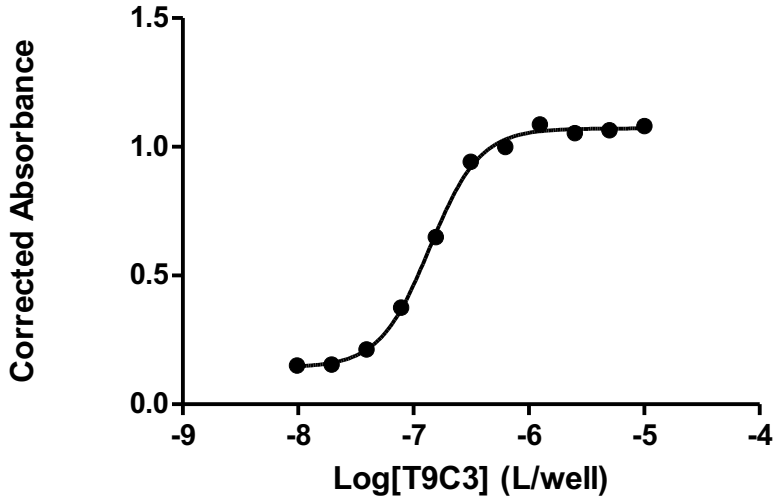

Figure C.25. Example dose response curve for extract treated by all three columns (T9C3) for the 24 hour dosing study, EV50 = 1.382E-07 L/well.

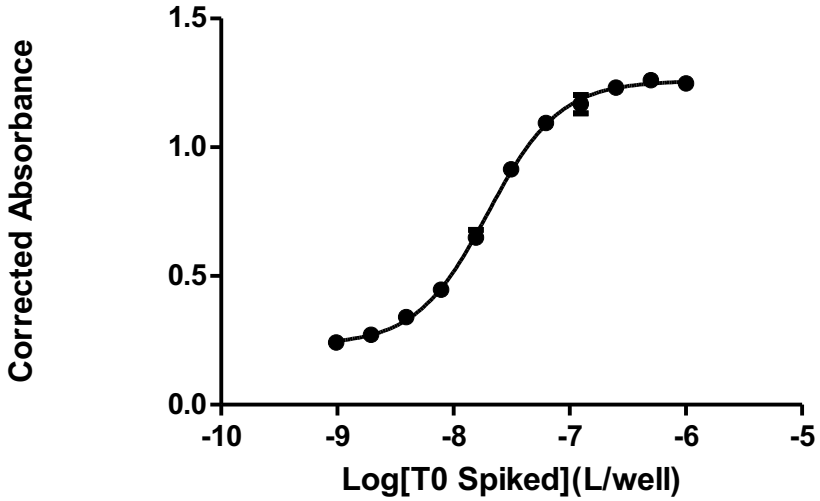

Figure C.26. Example dose response curve for the wastewater spiked with the 7 target compounds at 100 μg/L (T0 spiked) used in the 36 hour study, EV50 = 1.984E-08 L/well.

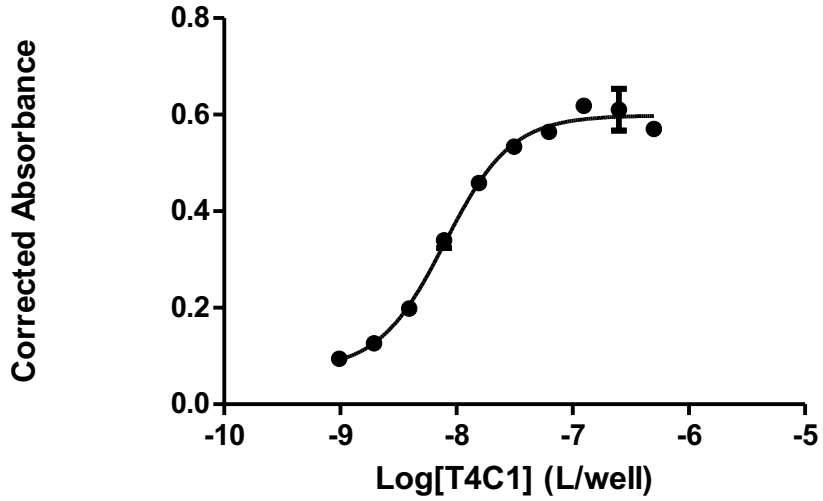

Figure C.27. Example dose response curve for the extract treated by the first aerobic column (T4C1) in the 36 hour study, EV50 = 7.939E-09 L/well.

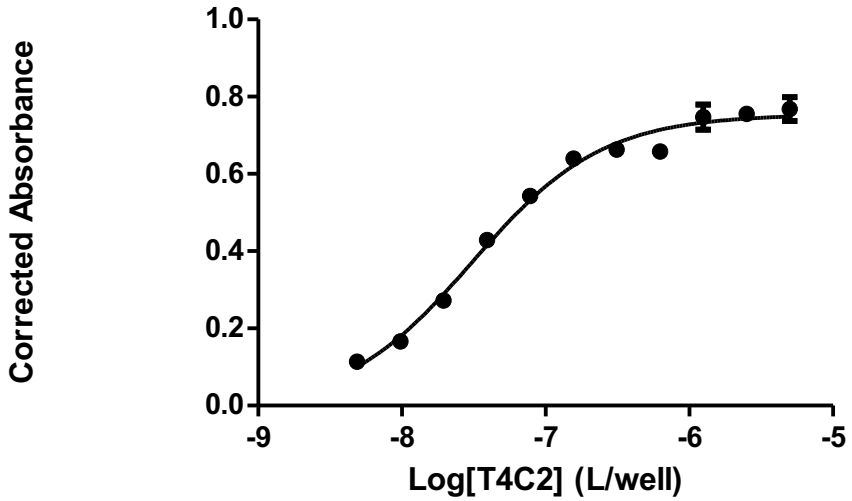

Figure C.28. Example dose response curve for the extract treated by the first aerobic and second hypoxic columns (T4C2) in the 36 hour study, EV50 = 3.089E-08 L/well.

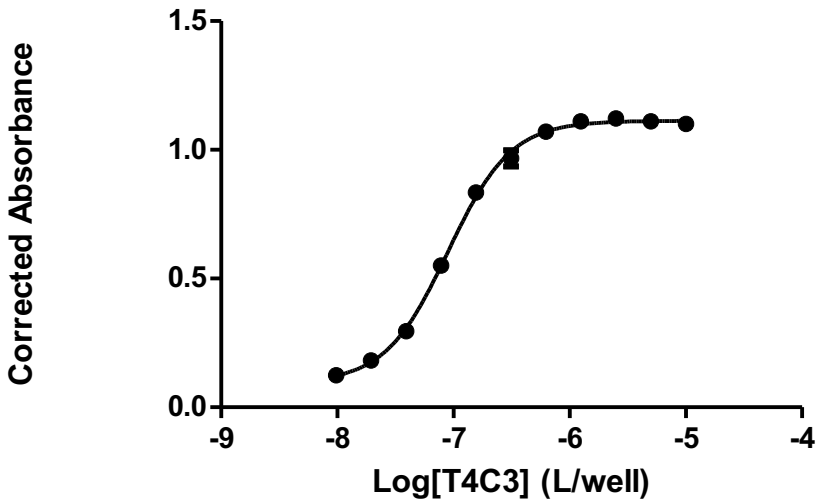

Figure C.29. Example dose response curve for extract treated by all three columns (T4C3) in the 36 hour study, EV50 = 8.958E-08 L/well.

APPENDIX D: SUPPLEMENTAL TABLES

Chemical concentrations presented in this appendix comes from LCMS analysis conducted by and described in detail by Holmes (2017). Chemical concentrations are given as the concentration of the analyte in the extract and not the source wastewater, and have all been adjusted to account for the different recoveries listed in Table D.1. The limit of detection for this method is 5 μg/L. Non detect indicates no peak was observed on the LCMS for that chemical.

Table D.1. Batch Study Replicate Percent Removals

	Aerobic			Hypoxic		
Sample	Replicate Removal (%)	Replicate Removal (%)	Replicate Removal (%)	Replicate Removal (%)	Replicate Removal (%)	Replicate Removal (%)
1 Hour	47.9	43.0	26.6	20.5	37.1	37.8
8 Hours	43.7	34.6	14.1	38.2	50.0	40.2
24 Hours	70.1	64.0	67.4	68.1	75.3	75.9

Table D.2. Calculated SPE Method Recovery for each Target Analyte

	ATZ	CAF	CBZ	DEET	E2	NP
Percent Recovery	53%	49%	77%	30%	10%	81%

Table D.3. Estrogenic Analyte Concentrations Detected by LCMS for Batch Study SPE Extracts

Sample	ATZ	E2	4-NP
T0 Unspiked	<5 μg/L	<5 μg/L	4.81 mg/L
T0 + 100 μg/L[b]	6.63 mg/L	1.25 mg/L	10.1 mg/L
1hr Aerobic	344 μg/L	2.43 mg/L	6.27mg/L
8hr Aerobic	399 μg/L	1.42 mg/L	8.43 mg/L
24hr Aerobic	303 μg/L	<5 μg/L	7.10 mg/L
1hr Hypoxic	364 μg/L	89.5 μg/L	6.30 mg/L
8hr Hypoxic	356 μg/L	<5 μg/L	5.40 mg/L
24hr Hypoxic	340 μg/L	<5 μg/L	7.32 mg/L

Table D.4. Estrogenic Analyte Concentrations Detected by LCMS for 8hr Study SPE Extracts

Sample	ATZ	E2	4-NP
T0 Unspiked	<5 μg/L	Non Detect	<5 μg/L
T0 + 100 μg/L	6.63 mg/L	1.25 mg/L	10.1 mg/L
T3C3	5.78 mg/L	Non Detect	6.83 mg/L
T8C1	6.06 mg/L	2.17 mg/L	<5 μg/L
T8C2	5.11 mg/L	Non Detect	<5 μg/L
T8C3	5.16 mg/L	Non Detect	<5 μg/L

Table D.5. Estrogenic Analyte Concentrations Detected by LCMS for 8hr Flooding SPE Extracts

Sample	ATZ	E2	4-NP
T0 Unspiked	61.2 μg/L	Non Detect	434 μg/L
T0 + 100 μg/L	6.63 mg/L	1.25 mg/L	10.1 mg/L
T3C3	5.44 mg/L	Non Detect	196 μg/L
T8C1	6.60 mg/L	1.31 mg/L	690 μg/L
T8C2	7.12 mg/L	Non Detect	561 μg/L
T8C3	6.13 mg/L	Non Detect	81.7 μg/L

Table D.6. 12 Hour Column 3 Extract Replicate Removals

	Replicate Removal (%)	Replicate Removal (%)	Replicate Removal (%)
T3C3	99.9	99.9	99.9
T7C3	98.1	98.5	97.8

Table D.7. Estrogenic Analyte Concentrations Detected by LCMS for 12hr Study SPE Extracts

Sample	ATZ	E2	4-NP
T0 Unspiked	<5 μg/L	Non Detect	88.5 μg/L
T0 + 100 μg/L	6.63 mg/L	1.25 mg/L	10.1 mg/L
T3C3	6.98 mg/L	Non Detect	<5 μg/L
T7C1	8.67 mg/L	319 μg/L	1.06 mg/L
T7C2	6.12 mg/L	Non Detect	<5 μg/L
T7C3	8.07 mg/L	Non Detect	<5 μg/L

Table D.8. Estrogenic Analyte Concentrations Detected by LCMS for 24hr Study SPE Extracts

Sample	ATZ	E2	4-NP
T0 Unspiked	<5 μg/L	Non Detect	240 μg/L
T0 + 100 μg/L	6.63 mg/L	1.25 mg/L	10.1 mg/L
T5C3	4.05 mg/L	10.3 μg/L	265 μg/L
T9C1	7.87 mg/L	428 μg/L	2.02 mg/L
T9C2	7.61 mg/L	68.8 μg/L	593 μg/L
T9C3	2.68 mg/L	11.9 μg/L	193 μg/L

Table D.9. Estrogenic Analyte Concentrations Detected by LCMS for 36hr Study SPE Extracts

Sample	ATZ	E2	4-NP
T0 Unspiked	115 μg/L	Non Detect	<5 μg/L
T0 + 100 μg/L	6.63 mg/L	1.25 mg/L	10.1 mg/L
T4C1	6.59 mg/L	679 μg/L	383 μg/L
T4C2	3.24 mg/L	Non Detect	<5 μg/L
T4C3	2.57 mg/L	Non Detect	<5 μg/L

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