OCCURRENCE AND CONTROL OF ESTROGENIC AND ANDROGENIC ACTIVITY IN WATER

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ABSTRACT

Breanne Elizabeth Holmes: Occurrence and Control of Estrogenic and Androgenic Activity in Water
(Under the direction of Howard S. Weinberg)

Endocrine disrupting compounds (EDCs), including pesticides, plasticizers, and personal care products that accumulate in wastewater, are exogenous chemicals that can alter hormonal regulation and gene transcription, in part by mimicking natural hormones and binding to receptors. Environmental EDC exposures can cause profound effects for humans, ranging from breast, prostate, and lung cancer to obesity and infertility. Some epidemiological studies indicate disinfection by-products (DBPs), formed during drinking water treatment, could cause similar adverse health effects. A more complete assessment of endocrine active DBP structural classes and the remediation of known EDCs from wastewater are required. This dissertation identifies EDCs of concern in drinking water and evaluates the removal of estrogenic activity from wastewater using a combination of chemical and biochemical techniques.

Using receptor-binding assays and *in silico* molecular docking, the binding affinity of 21 DBPs were tested individually and in binary mixtures at concentrations ranging from 0.1 nM to 2 mM for their affinity to the human estrogen alpha and chimp androgen receptor. 14 DBPs were found to bind to the androgen receptor, at IC_{50} values in the range of 1.86 mM for 2,3-dichloropropionamide to 13.5 μM for 3,4,5,6-tetrachloro-benzoquinone, and were predicted to adopt the antagonist conformation. For the estrogen receptor, 9 of the 21 DBPs were able to bind weakly, with affinities ranging from IC_{50} values of 1.44 mM for dibromoacetonitrile to 148 μM

for bromopicrin, which was contrary to *in silico* predictions that showed a low probability of binding. In binary mixtures, the DBPs followed concentration addition for both receptors.

The yeast estrogen screen (YES) was then used to evaluate remediation of estrogenic activity in a simulated vertical flow filtration on-site wastewater treatment system. A laboratory-scale column study of dose loading intervals of 8, 12, 24, 36, and 48h free flowing and 8h saturated conditions showed higher removal of target anthropogenic chemicals and estrogenic activity with decreasing dose intervals. Moreover, higher levels of activity in the treated wastewater were measured by the YES assay than explained by the chemical concentrations. These results highlight the utility of complementary chemical and biological analyses for studying the effects of treatment on wastewater quality.

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LIST OF ABBREVIATIONS

AR	Androgen receptor
ARBA	Androgen receptor binding assay
ATZ	Atrazine
BAM	2-bromoacetamide
BDCM	Bromodichloromethane
BOD	Biological oxygen demand
3-BPN	3-bromopropionitrile
CA	Concentration addition
CH	Chloral hydrate
CAF	Caffeine
chAR	Chimp androgen receptor
CBZ	Carbamazepine
CFU	Colony forming units
CSA	Chlorosuccinic acid
2-CP	2-chlorophenol
CPRG	Chlorophenol red-β-D-galactopyranoside
CW	Constructed Wetland
CWA	Clean Water Act
DBAA	Dibromoacetic acid
DBAN	Dibromoacetonitrile
DBP	Disinfection by-product
DBPN	2,3-dibromopropionitrile

DCAN	Dichloroacetonitrile
DCBQ	2,6-dichloro-1,4-benzoquinone
DCP	2,3-dichloropropionamide
DDT	Dichlorodiphenyltrichloroethane
DHT	5α-dihydrotestosterone
DIAA	Diiodoacetic acid
DN	Dissolved nitrogen
DO	Dissolved oxygen
DOC	Dissolved organic carbon
DTT	DL-dithiothreitol
E2	17β-estradiol
EC_{50}	Effect concentration (50%)
EDCs	Endocrine disrupting compounds
EDSP	Endocrine Disruptor Screening Program
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EEF	Estradiol effect factor
EEQ	Estradiol equivalents
ER	Estrogen receptor
ERBA	Estrogen receptor binding assay
FWSCWs	Free water surface constructed wetlands
GR	Glucocoriticoid receptor
HAA	Haloacetic acids
hER	Human estrogen receptor

HFCW	Horizontal flow constructed wetland
HPLC	High performance liquid chromatography
[^{3}H]-R188h	[17 α-methyl-^{3}H]-methyltrienolone
HRT	Hydraulic retention time
IAA	Iodoacetic acid
IC_{50}	Inhibition concentration (50%)
JLBC	Jordan lake business center
LBD	Ligand-binding domain
LGW	Lab grade water
MCA	Mucochloric acid
MPN	Most probable number
MX	3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone
NB	Nonbinder
NDMA	*N*-nitrosodimethylamine
4-NP	4-*n*-nonylphenol
NPDES	National Pollutant Discharge Elimination System
NR	Nuclear receptor
PCR	Polymerase Chain Reaction
PAO	Phosphorus accumulating organisms
PR	Progesterone Receptor
R1881	Methyltrienolone
RBA	Relative binding affinity
SB	Slight binder

SFCWs	Subsurface flow constructed wetlands
SPE	Solid phase extraction
TCS	Triclosan
TR	Thyroid receptor
TBNM	Tribromonitromethane
TCAN	Trichloroacetonitrile
TCBQ	3,4,5,6-tetrachloro-1,2-benzoquinone
TCP	2,4,6- trichlorophenol
THMs	Trihalomethanes
TN	Total nitrogen
TOC	Total organic carbon
UPLC-ESI-MS	Ultra performance liquid chromatography- electrospray ionization- mass spectrometry
U.S. EPA	United States Environmental Protection Agency
VFCWs	Vertical flow constructed wetlands
VFSF	Vertical flow sand filter
YAS	Yeast androgen screen
YES	Yeast estrogen screen

CHAPTER 1: INTRODUCTION TO ENDOCRINE ACTIVITY IN WATER

1.1. Research Motivations

The human right to water and sanitation was officially recognized by the United Nations General Assembly in July 2010 through Resolution 64/292 (1). While the use of chlorine to disinfect drinking waters first emerged in 1850 and became widely used in the early 1900's, access to clean drinking water globally has yet to be realized. Even in countries where drinking water treatment is commonplace, chemical contaminants in source waters or those produced during treatment, such as disinfection by-products (DBPs), undermine what is considered to be a safe and clean drinking water. Sanitation, in the form of wastewater treatment, has its own host of contaminant concerns including relatively high levels of pharmaceuticals, personal care products, DBPs and antibiotic-resistant bacteria, many of which can reach downstream drinking water sources (2-6). The confluence of these concerns is that the treatment of our water for consumption or our wastewater for release into the environment could still contribute to issues of public health.

In the U.S. providing and maintaining safe drinking water, including preventing waterborne outbreaks, is mandated under the Safe Drinking Water Act (SDWA). This act regulates over 80 contaminants and requires monitoring hundreds of water quality parameters to help provide safe drinking water to nearly 300 million people (7). Disinfectants, such as chlorine or chloramine, are used as part of water treatment to meet the SDWA guidelines because they are efficient at inactivating disease-causing components often found in source water, including *Giardia*, *Campylobacter*, and norovirus. As strong oxidants, these disinfectants are also capable

of reacting with natural organic matter from the decomposition of organic material present in decaying leaves and other plants, thus forming DBPs (8). Furthermore, because disinfection is used in both drinking water and wastewater treatment, these low-level contaminants are found in potable water or are released in treated effluent into the environment. These compounds, often containing halogens, are comprised of a large variety of structural classes and have generally unknown toxicities.

Despite there being over 800 identified DBPs (9, 10), current United States Environmental Protection Agency (US EPA) regulations only address a small subset of them, including the sum of four trihalomethanes (THMs) and five haloacetic acids (HAAs), chlorite, and bromate (11). Regulations in the European Union and World Health Organization expand that list further to include chloral hydrate, dichloroacetonitrile, dibromoacetonitrile, trichloroacetonitrile, cyanogen chloride, formaldehyde, and 2,4,6-trichlorophenol (11). Epidemiological and laboratory study evidence links the presence of DBPs to a number of disease states, including bladder cancer, colorectal cancer, and adverse reproductive outcomes, emphasizing the need for a more refined understanding of some of the biological pathways at risk (12-16). Of particular concern is whether the DBPs that result from conventional water treatment through chlorine or chloramine disinfection can be implicated in endocrine disruption, a target system proposed as a potential target of DBPs for these compounds but for which no such evaluation exists. Epidemiological studies have suggested that DBPs present in drinking water could be the cause of spontaneous abortion (17), as well as cardiovascular defects, low birth weight, and neural tube defects (18). Although these chemicals are typically only in the μg/L to ng/L range in finished drinking waters (9, 12), their potency has not been measured, which would indicate what risk they pose to endocrine disruption. Higher potency chemicals can

still be threats at lower concentrations and similarly, mixtures of a large number of low potency chemicals even at low concentrations could have a higher overall effect due to the additive effect of the mixture (19, 20). In order to understand the risk posed by DBP exposure it is necessary to evaluate the interaction of DBPs with endocrine signaling pathways and receptor proteins. An amendment to the SDWA in 1996 helped to establish the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) through the U.S. EPA (21). The result, the Endocrine Disruptor Screening Program (EDSP), is a two-tiered approach to screening pesticides and other environmental chemical contaminants for their activity in the estrogen, androgen, or thyroid hormone systems.

While the EDSP requires investigation into whether DBPs are endocrine active, there are other common contaminants in waste streams that show activity and present health risks (21). These compounds are of concern further in the life cycle of water as they enter the environment from wastewater discharges without removal during treatment (22, 23) and then pose a risk to aquatic life as well as surface waters (24, 25). A number of common wastewater chemical contaminants are known endocrine disrupting compounds (EDCs) and include some pesticides, herbicides, and hormonal supplements (15). While conventional wastewater treatment plants treat the influent water to remove aggregate indicators of pollution (such as biochemical oxygen demand), they are unable to fully remove EDCs (23, 26). Rural populations which usually treat their wastewater onsite are, likewise, a source of endocrine activity in the environment because of their even more limited approach to EDC degradation, for example through septic systems (27). However, alternatives to such systems requiring minimal resources and low costs include constructed wetlands (CWs) and these are increasingly common in developing countries where large-scale plants are not economically feasible (28-30). In the U.S. CWs are of becoming more

commonly considered where wastewater can be reused for landscaping, toilet flushing, and other means (31). The use of a CW system to improve wastewater quality before reuse or release into the environment allows for passive remediation of anthropogenic contaminants with resulting decreased risk to the receiving water body and wildlife (32, 33). Numerous examples have been published in the literature on studies examining the use of CWs for various aspects of wastewater treatment, including vertical and horizontal flow CWs and biofilters (34) for the removal of organic matter, nitrogen content (35, 36), and pharmaceutical contaminants (37). However, there has been limited attention paid to the use of CWs for the optimization of the removal of EDCs.

The U.S. EPA regulates wastewater discharge under the Clean Water Act (CWA) and National Pollutant Discharge Elimination System (NPDES), regulations which are designed to establish limits on pollution of the nation's waterways and set water quality standards for surface waters. While they help to manage surface water pollution levels in the U.S., they do not address the issue of groundwater pollution through the transport of chemicals from decentralized wastewater treatment systems, landfill leachates, surface runoff from biosolid applications or infiltration from land applications. The water from these sources often contains a diverse group of personal care products, hormones, pharmaceuticals, and other chemicals present in households which could be transported through the soil to reach groundwater, thus impacting drinking water (3, 6, 27). Due to increasing water scarcity, wastewater reuse for non-potable applications is employed often without knowing the fate of the chemical contaminants in the water. In one such format, constructed wetlands (CWs) are a clear and promising form of green engineering for water reuse that are being refined for use in decentralized and developing locations (28, 38, 39). These systems not only allow for the treatment of wastewater to generate improved effluent

quality, but can also result in treatment of waste streams with lower energy input than conventional systems making them a viable alternative treatment technique.

This dissertation focuses on water quality as it relates to human health, as well as its potential impact on the total environment. Through the fundamental study of the endocrine activity of DBPs, together with the fate and removal of persistent anthropogenic compounds and their endocrine activity during CW treatment of wastewater, an understanding of the endocrine activity of drinking water and the removal of activity in wastewater is achieved.

1.2. Knowledge Gap

Signaling for a number of biological functions in the human body is controlled through the master regulatory network known as the endocrine system. This pathway is comprised of a group of glands including the adrenal, hypothalamus, ovaries, pancreas, parathyroid, pineal, pituitary, testes, thymus, and thyroid, which release hormones to transport signals throughout the body. These hormones are used to control gene transcription for a variety of biological functions including: sexual development, reproductive health, metabolism, stress levels, and sleep (40). The regulation of gene transcription occurs through two main types of hormones: protein hormones including peptides and amino acids, and steroid hormones that are produced from cholesterol. Targets of steroid hormones include hormone receptors known within the family of nuclear receptors (NRs), which are a group of structurally similar transcription factors of which 48 have been identified in mammals (41, 42). Within NRs, the specific hormone receptors include the estrogen receptor (ER), androgen receptor (AR), progesterone receptor (PR), glucocorticoid receptor (GR), thyroid receptor (TR), and mineralocorticoid receptor (40). When exogenous hormones or hormone analogues are able to interfere with the homeostatic activity of these receptors they are known as EDCs.

The activity of EDCs as a class, which include interference with every cellular hormonal pathway, is an ecological and human health concern because of their ability to inhibit or activate the normal gene transcription levels that are controlled by all steroid receptors. The results from human exposure to these compounds can vary widely but include biological endpoints such as infertility, obesity, altered sexual development, neuroendocrinology, cardiovascular endocrinology, and cancer (43, 44). The mechanisms behind these endpoints are not entirely understood but there are a few known ways in which EDCs act to interfere with NR signaling. Normally, when the endogenous ligand binds to its target receptor it causes a conformational change that allows for hetero- or homo-dimerization with another receptor and recruitment of coactivators, to allow for binding at DNA promoter sites and the initiation of gene transcription (40, 45, 46). For EDCs, however, mechanisms expand to include activation through binding of agonists to the NR and initiating recruitment of coactivators that bind to allow for targeted gene transcription to begin, or binding of antagonists that prevent coactivator binding and instead recruit corepressors to inhibit binding to and transcription of gene targets. Examples of the structural changes associated with agonism and antagonism for the androgen receptor, which occur in a similar manner for the other nuclear receptors, are shown in Figure 1.1. Alternatively, EDCs can interfere by binding to one receptor and initiating cross-talk, in which the bound receptor competes for coactivators or for DNA-binding sites with other nuclear receptors (41). There can also be disturbances to signaling when the NRs bind to neighboring promoter sequences that can cause synergistic or inhibitory activity. Finally, the activation of one NR could result in proteasome activation and degradation of the other NR required for dimerization (47, 48). An EDC is considered to be any exogenous compound that is capable of acting through

one of these means to alter normal signaling, and research to date has shown such chemicals are from a number of compound classes.

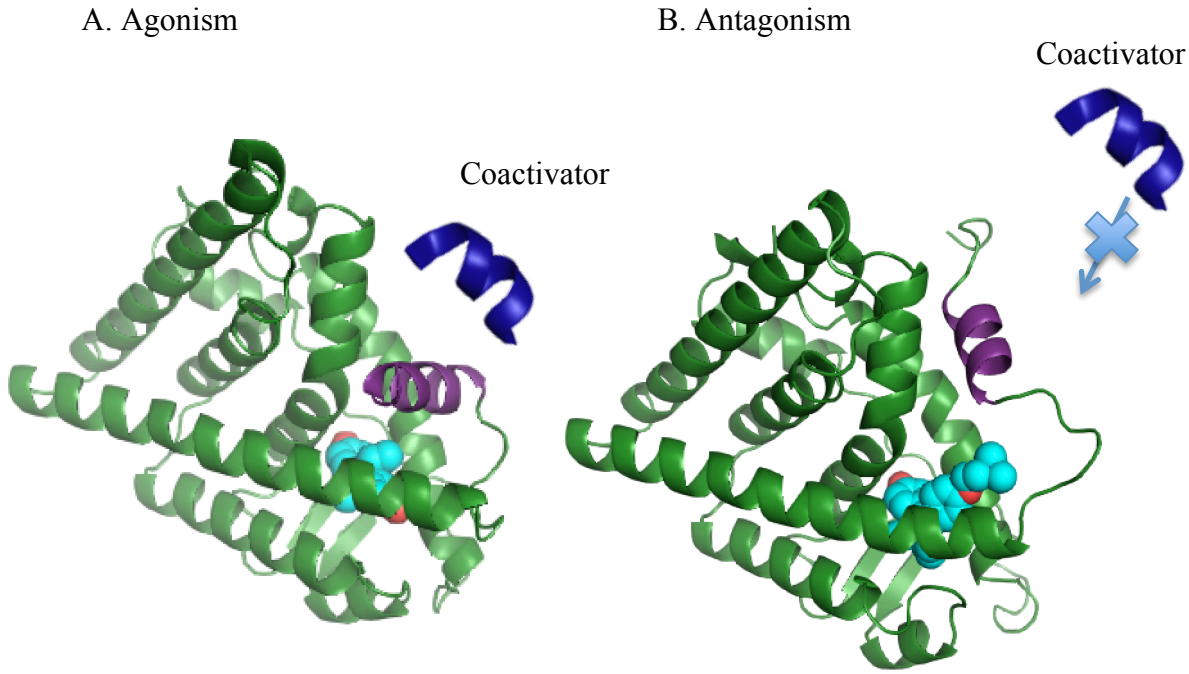

Figure 1.1. A) Agonist binding of the EDC (blue and red) is shown with a conformational change allowing for the recruitment and association with the coactivator. B) Antagonist binding is shown with the EDC (blue and red) preventing the conformational change necessary for the coactivator to interact.

One of the most commonly studied EDCs is bisphenol A, but a variety of other environmental contaminants have been shown to have endocrine disruption potential in humans and other species, including herbicides and pesticides like atrazine, lindane and dichlorodiphenyltrichloroethane (DDT), dioxins, organotin compounds from polyvinyl chloride manufacturing, polychlorinated biphenyls, alkylphenols including nonylphenol, and phthalate plasticizers (15, 48-50). The nature of endocrine disruption from a particular chemical, however, is species-specific which makes them of concern as a health threat for humans and for the total environment (51). Besides humans, there have been endocrine effects studied showing weight changes in birds, intersex and infertility in fish and reptiles, and cancer in mammals, all of which

indicate the importance of understanding the potential of endocrine activity (49). To understand the variety of endocrine related diseases impacting various species, a number of assays have been developed that range from the cellular level to full organism.

Assessing endocrine activity in drinking water and wastewater has been approached through a battery of receptor, cell, and whole organism tests. Examples include the yeast estrogen and androgen screen (YES and YAS, respectively), the *E-screen* assay with MCF-7 BUS breast cancer cells, the chemical activated luciferase expression *in vitro* bioassays (CALUX), screening with the 21-day Medaka fish assays, as well as screening using rat models (13, 52-55). Each of these assays is useful under different test scenarios, but also have their limitations. For example, *in vitro* assays ignore the potential impact of the target chemicals on other cell types that contain different receptor subtypes or membrane proteins, such as cells that contain the ERα subtype compared to the ERβ subtype and have different affinities for the same EDC (56). Conversely, whole organism experiments do not address the species-specific effects that have been observed (42, 54).

Of the many endocrine pathways mentioned, the primary focus for endocrine disruption research has centered on estrogen, androgen, and thyroid activity because of the observed biological outcomes associated with EDC exposures and as a result of the EPA screening program. Estrogen and androgen receptor binding assays, which were used for the research reported in this dissertation, focus on the ability of a chemical to bind at the receptor itself, which is one mechanism for endocrine disruption. While these receptor-based assays do not determine whether binding translates to altered increased or decreased gene transcription, they do help to determine whether a compound has the potential to be endocrine active and if certain chemical classes are of greater concern than others. Furthermore, the endocrine activity of environmental

pollutants, specifically within the estrogen and androgen pathways, is an active area of interest because of their potential to impact a wide variety of species and the range of their biological effects in humans (56, 57). Exposure to and disruption from EDCs has been measured in various water sources as a number of the chemicals previously listed are released in wastewater effluent, while DBPs are formed in both drinking and wastewater (7, 58, 59). For example, herbicides, surfactants, antimicrobials, and steroids are among the common wastewater contaminants that are of interest in this work that have also been shown to be endocrine active (27, 60, 61). Some DBPs have also been implicated as potential EDCs but a large number of them have unknown toxicity and endocrine activity (62). The very nature of both wastewater and drinking water matrices is that of a complex mixture of low-level chemicals, so an understanding of the effect that these mixtures pose on health is of clear need, with some mixtures having been shown to be either additive or synergistic (63). Furthermore, the chronic exposure of wildlife and humans to EDCs demonstrates an important consideration for public health and environmental chemistry. The endpoints tested in most toxicity assays are at high levels but exposure effects have been observed at lower environmentally relevant concentrations, which might be due to prolonged low-level chemical influences (47, 64). Removal of emerging wastewater contaminants and EDCs from wastewater before their introduction into the environment, therefore, is necessary to prevent continued exposure to and accumulation of these compounds.

Since EDCs are persistent contaminants in wastewater and among the so-called "emerging contaminants" in drinking water, understanding their implications for human health when utilizing water reuse and minimizing the risk of exposure are imperative. Access to freshwater is becoming increasingly scarce, with an estimated 700 million people worldwide currently affected and 1.8 billion people expected to be impacted by 2030 (65). Furthermore, the

majority of the impacted population will be in resource-poor settings. Even within the U.S., approximately 80 percent of consumed water is currently being used for agriculture (66). If, instead, safe and effective wastewater treatment could produce effluent to be reused for crop irrigation, this would help to relieve some of the burden from agricultural use on the drinking water supply (67). Utilizing a low-energy engineered design could also allow it to be employed in resource-poor settings, as water scarcity becomes a greater issue (28, 38, 68). Understanding the level of and removal capability of the system to common anthropogenic contaminants, including EDCs, is essential to protecting the overall health of the environment in which the treated water is used and to consumers that would be exposed.

As the hydraulic retention time (HRT) and biodegradation have been shown to be important parameters for the removal of EDCs and other pollutants during wastewater treatment, optimizing a vertical flow sand filter (VFSF) or constructed wetland are logical approaches for an effective low-energy engineering approach (38, 69-72). In particular, one requisite area of investigation is determining which batch-loading interval for a VFSF would best remove common wastewater contaminants and endocrine active compounds. Examples of common wetland designs including surface flow, horizontal subsurface flow, and vertical subsurface flow, as well as a vertical flow sand filter are shown in Figure 1.2 (38, 73). These designs take advantage of different contact times with the substratum and microbial bed, as well as different aeration levels due to their flow patterns. Vertical flow filters are particularly useful because they require less space and can include pulsed flows as might occur in household use, which alternate between aerobic and hypoxic periods, and have been shown to have better removal of contaminants.

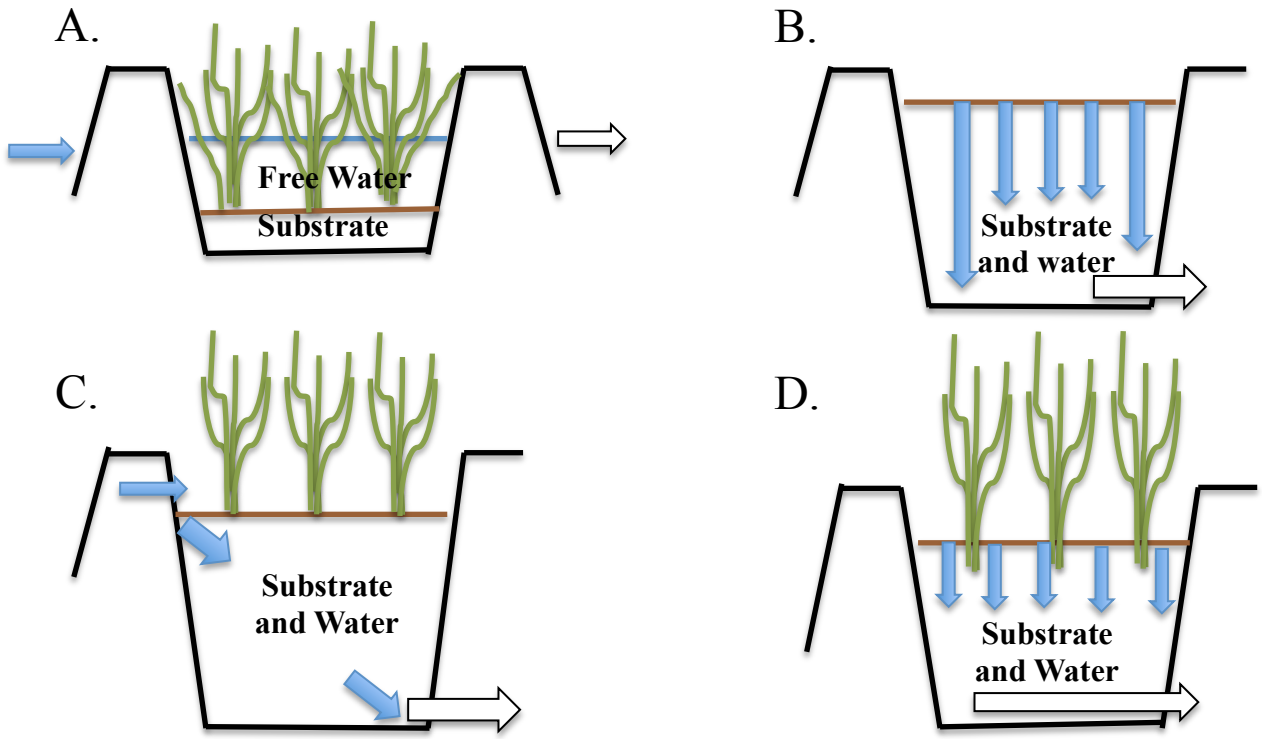

Figure 1.2. Examples of A) a surface flow-constructed wetland, B) a vertical flow sand filter, C) a subsurface flow-constructed wetland, and D) a vertical subsurface flow-constructed wetland.

While sand filters can take a number of forms, they generally function through the periodic release of wastewater from perforated pipes along the top of the sand bed. These primarily work as aerobic, fixed-film bioreactors that can treat wastewater but have also been used for drinking water practices (74). Prior to the release of the wastewater onto the vegetated sand bed, a settling tank, such as a septic tank, is used to remove large settleable solids. The supernatant liquid then moves to pipes that are spread over the surface of the sand bed and are only dosed periodically, which allows for the maintenance of an aerobic and nitrifying biofilm and helps to prevent clogging. This biofilm contains heterotrophs that metabolize the carbon content of the water as a food source with oxygen, and organic nitrogen that has been hydrolyzed to ammonia that is then metabolized by autotrophic bacteria to NO_2^-. The activity of this biofilm

is measured as its biological oxygen demand (BOD) (35, 75). Sand filter designs are efficient because they allow for physical separation, both from the settling tank and the pores of the sand material, as well as chemically through adsorption of dissolved chemicals and pollutants, and biological degradation through the aerobic biofilm. Phosphorus removal in sand filters is accomplished largely through adsorption to sites on the porous media surface, which means its efficiency is limited and media would sometimes need to be changed to accomplish continued removal once these sites are saturated (69, 76, 77). Phosphorus accumulating organisms (PAOs), however, could also contribute to removal (78). Biologically, the biofilm does well to manage organic carbon and nitrogen removal. The high surface area of sand also allows for colonization of microorganisms, which are largely responsible for the water treatment as they use common water pollutants, such as nitrate, BOD, NH_4^+ and organic compounds as nutrients (79). The filter bed is lined to prevent leaching while pipes at the bottom of the filter bed exist to collect the filtered water, which can then be used for landscaping or other purposes.

Chemical degradation in sand filters occurs primarily as a result of microorganisms through respiration or fermentation, with respiration occurring in oxidized environments with a high redox potential (37, 80, 81). It is assumed that most degradation, therefore, occurs in the aerobic zone with the chemical contaminants as a carbon source for heterotrophic bacteria, but cycling from anaerobic to aerobic to anaerobic stages allows for a higher overall nitrogen removal through nitrification and denitrification using a combination of bacteria. Nitrogen removal is important as high nitrogen content in environmental waters can lead to harmful algae blooms and can be toxic to aquatic life. Different functional classes of microbes are responsible for a specific chemical's degradation and these can be enhanced through augmented operational parameters of the sand filter such as adjusting the batch-loading interval to allow for longer

periods of unsaturation. Other important factors for activity include pH, salinity, and the availability of organic carbon (34). The impact of varying the batch-loading interval on the removal of common domestic wastewater contaminants and EDCs in particular, remains an open question.

1.3. Specific Aims

The research reported in this dissertation focused on the use of endocrine activity assays for evaluating water quality both in drinking water and wastewater. Prior to this study little was known about that activity for the chemical by-products resulting from disinfection, and studies measuring the effectiveness of treatment on wastewater effluent quality typically were evaluated by the levels of chemicals and pathogenic microbes, not toxicity.

The known association between contaminants in drinking water and wastewater and negative health endpoints has been well established, but the pathways involved specifically within the endocrine system are as yet not understood. Furthermore, epidemiological evidence suggests an association between consumption of disinfected drinking water and endocrine-controlled health outcomes, such as premature birth and infertility. The need for a fundamental understanding of the structural classes and trends of DBPs that could be the cause of this link is necessary in order to address this issue. Specifically, the activity of structurally different DBPs with the estrogen or androgen receptors is an important and, as yet, unanswered question for the field of environmental toxicology. It was hypothesized that by studying a diverse group of DBPs for their ability to bind to these receptors, an understanding of the chemical underpinnings involved in the development of these negative health outcomes might be gained.

In environmental samples, understanding the health risks of exposures must logically move towards understanding the behavior of a complex mixture and not just sole chemicals. As

environmental samples contain a number of low-level contaminants that might be undetectable individually, it is often more logical to evaluate their overall activity. For example, the behavior of persistent environmental contaminants including pesticides, herbicides, hormones and surfactants need to be understood in relation to how these compounds behave in wastewater and how well these compound classes can be removed in rural settings. While there is considerable work in the literature to understand specific compound class removal using both constructed wetlands and vertical flow filters, there remains a gap in knowledge for how to address overall endocrine activity from a diverse set of compounds. The poor removal of endocrine activity in large-scale wastewater treatment and the observed estrogenic activity in domestic and rural wastewater emphasize this need. By using a bioassay that measures overall estrogenic activity through the expression of a reporter gene, it is possible to determine the change in total activity and account for chemical transformations that could still contribute even at low levels. In this dissertation research, it was anticipated that a vertical flow filter could be optimized for the removal of target compounds from major persistent chemical classes in wastewater and for the attenuation of endocrine activity.

Aim 1: Identify androgen-active chemicals from a group of 21 DBPs that are capable of binding to the androgen receptor both individually and in mixtures.

Aim 2: Determine which of 21 DBPs can bind to the estrogen receptor both individually and in mixtures.

Aim 3: Determine the optimal VFSF dose interval for target chemical removal and decreased endocrine activity of seven common anthropogenic contaminants, and explore the value of using endocrine activity assays for measuring changes in water quality resulting from such treatment.

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CHAPTER 2: IDENTIFICATION OF ENDOCRINE ACTIVE DISINFECTION BY-PRODUCTS (DBPS) THAT BIND TO THE ANDROGEN RECEPTOR

2.1. Introduction

Endocrine disrupting compounds (EDCs) are anthropogenic chemicals found in the environment that can interact with homeostatic hormone regulation in humans and animals. These chemicals are able to modulate the hormone systems that are used for basic physiological and developmental control, thus disrupting normal endocrine function (1). Disruption from a diverse set of small molecules can occur through a number of actions. One mechanism is that exogenous EDCs can mimic endogenous hormones to cause alterations in gene transcription by binding to nuclear hormone receptors and initiating signaling pathways. Furthermore, evidence of disruption through androgen signaling has been shown in epidemiological studies which indicate that chemicals including pesticides and other environmental contaminants are responsible for a number of negative health outcomes (2). The androgen system, one of the target endpoints highlighted in the 1996 amendment to the Safe Drinking Water Act, remains largely unstudied in relation to environmental endocrine disruption. Its biological importance, however, includes associated health impacts on male reproductive tract abnormalities, testicular and prostate cancer, and altered male sexual differentiation (3-5).

While it is understood that androgen signaling is part of male sexual differentiation and developmental programing, the effect of environmentally relevant concentrations of EDCs on this pathway is relatively unknown. The androgen hormone pathway plays a pivotal role in male secondary characteristics, including bone mass, musculature, fat distribution, and hair patterning,

among others (6). Androgen hormones primarily modulate the activity of this pathway through interaction with the androgen receptor, a 115 - 120 kD modular protein in the nuclear receptor superfamily, which is expressed in a number of organs including the hypothalamus, pituitary, liver, prostate, and testes (7). Ligand-activated binding occurs with high affinity to two endogenous androgens, testosterone and 5α-dihydrotestosterone (DHT), at the ligand-binding domain (LBD) (8). Exogenous ligand binding is one known route of androgen disruption and the primary target for EDCs, allowing for modulation of the receptor-mediated gene transcription. This binding causes a specific conformational change to the receptor depending on whether the chemical is an agonist or an antagonist, which then allows for recruitment of coactivators or corepressors and thus initiates or suspends gene transcription (9, 10). Understandably, the interaction and binding of environmental contaminants to the androgen receptor has the potential to modulate the androgen signaling pathway and cause disruptions to regular gene transcription levels.

Drinking water disinfection is commonly accomplished through the addition of either chlorine or chloramine. The reaction of these chemicals with natural organic matter already present at very low levels in water results in the formation of DBPs (11, 12). Furthermore, some of these by-products have been associated with bladder cancer and infertility (13-15). While Watson and colleagues found evidence that the androgenic activity of chlorinated wastewaters is partially attributed to DBPs, it has not been fully explained by studies of the currently regulated compounds (16). The specific interaction of unregulated DBPs with the androgen receptor and signaling pathway, however, remains unknown. Moreover, drinking water contains mixtures of DBPs at varying concentrations that should be considered for their potential synergistic, dose additive, or antagonistic activity (17). Previous studies have observed a toxicological response

for a mixture that is different from the predicted effect based on individual tests and in some cases, resultant dramatic biological outcomes are seen, particularly for aquatic species (18, 19). The ability of chemical mixtures to have additive binding and disruption abilities even if the individual chemicals bind with low affinity is, therefore, of interest.

Genotoxic and cytotoxic DBPs, such as those containing chlorine-, bromine-, and/or nitrogen species, are of concern because of their formation from disinfection practices and at concentrations that are dependent on influent water quality. While a few studies have evaluated the effects of low-levels of chemical mixtures on the estrogen system, far less work has investigated the impact of exogenous chemicals on androgen disruption (20, 21). Competitive receptor binding assays, such as the androgen receptor binding assay (ARBA), can establish structure-activity relationships that influence trends in activity of EDCs, for both agonistic and antagonist chemicals. Such chemicals would then be candidates for a number of future health endpoint studies. In the study presented here we set out to identify DBPs that are capable of binding to the androgen receptor, and whether they interact and bind differently in more complex mixtures. Specifically, the receptor affinity and structural trends of DBPs that are able to bind to the androgen receptor individually or in mixtures with the known weakly androgenic chemical, 4-*n*-nonylphenol, are identified. The binding nature, as agonists or antagonists, is then assessed using an *in silico* approach. The model of concentration addition (CA) is used to predict the IC_{50} value of the binary mixture of compounds when added in equal potency and then compared to the experimental value.

2.2. Materials and Methods

2.2.1. Chemicals

Chimp Androgen Receptors (chAR) were supplied by the U.S. Environmental Protection Agency (EPA) in TEDG binding buffer and stored at -80 °C. Chemicals purchased from Sigma Aldrich (St. Louis, MO) included: methyltrienolone (R1881, 98%), chloral hydrate (CH, >98%), DL-dithiothreitol (DTT, >98%), human γ-globulin (>99%), glycerol, dextran-coated charcoal, 2,6-dichloro-1,4-benzoquinone (DCBQ, 98%), dibromoacetic acid (DBAA, 90%), chlorosuccinic acid (CSA, 96%), 2,4,6-trichlorophenol (TCP, 98%), dibromoacetonitrile (DBAN, 97%), mucochloric acid (MCA, 99%), 3,4,5,6-tetrachloro-1,2-benzoquinone (TCBQ, 97%), sodium chloride (NaCl, >99%), 4-*n*-nonylphenol (4-NP, 98.4%), and absolute ethanol. [17 α-methyl-^{3}H]-methyltrienolone ([^{3}H]-R1881, 81.2 Ci/mmol) and Opti-Fluor scintillation cocktail were purchased from Perkin Elmer (Waltham, MA) while 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX, 98%) was supplied by Wako (Richmond, VA) and *N*-nitrosodimethylamine (NDMA, 100%) was from Supelco (Bellefonte, PA). 2-chlorophenol (2-CP, 98%), bromodichloromethane (BDCM, >98%), trichloroacetonitrile (TCAN, 98%), and 3-bromoacetamide (BAM, 98%) were purchased from Acros Organics (New Jersey, USA). Dichloroacetonitrile (DCAN, 99.8%) was obtained from Crescent Chemicals (Islandia, NY), iodoacetic acid (IAA, 99.5%) from Fluka (Buchs, Switzerland), 2-bromopropionitrile (3-BPN, 98%) from Alfa Aesar (Lancs, UK), diiodoacetic acid (DIAA, 90+%) from Cansyn Chemicals (Ontario, Canada), 2,3-dibromopropionitrile (DBPN, 95+%) from Matrix Scientific (Columbia, SC), 2,3-dichloropropionamide (DCP, 97%) from TCI (Tokyo, Japan), and tribromonitromethane (TBNM, 95+%) from Orchid Cellmark (Princeton, NJ). Tris base and hydrochloric acid (ACS grade) were both purchased from Fisher (Fair Lawn, NJ).

2.2.2. Androgen receptor binding assay (ARBA): Single chemical analysis

The androgen receptor binding assay (ARBA) followed published procedures as described by Hartig and colleagues (22). Briefly, initial range finding assays were completed to determine the appropriate receptor concentrations, which would result in 5-15% binding. Saturation binding and Scatchard analyses were completed to determine the amount of receptor that would bind the tritiated methyltrienolone ([^{3}H]-R1881) ligand, showing a dissociation constant (K_d) of 10 nM. A control using unlabeled R1881 ligand was included for each day. The compound 4-*n*-nonylphenol (4-NP), a known endocrine disruptor, was used as a positive control due to its ability to weakly bind to receptors at levels within the range of the test chemical concentrations. TEDG binding buffer (50 mM Tris-HCl, pH 7.4; 2 mM DTT; 10% glycerol; 10 mg/mL human γ-globulin; 0.8 M NaCl) was prepared and used to dilute receptor and chemical stocks. Prior to use, the chARs were diluted 1:100 according to the results of the range finding assay, then kept on ice until addition to the assay wells. Test chemicals were prepared in absolute ethanol well below their individual solubility at concentrations up to 500 mM and stored in amber vials at -20 °C for up to 6 months then vortexed before each use. Unlabeled R1881 was run against [^{3}H]-R1881 in the reference assay and [^{3}H]-R1881 was tested for competitive binding against varying concentrations of test chemicals in the binding assays. Each assay concentration was run in triplicate on the day of the experiment and repeated on at least two separate days.

A 0.1 μM stock of the [^{3}H]-R1881 and a 10 μM stock of the unlabeled R1881 were prepared from the neat standards in absolute ethanol, and stored at -20 °C in amber vials. Aliquots of each were dried down in borosilicate glass tubes on a SpeedVac (Thermo Scientific Savant Model SPD1010; Waltham, MA) then reconstituted in TEDG buffer and 1 μL absolute

ethanol to 400 nM R1881 and 8 nM [^{3}H]-R1881. For the assay, 50 µL of working test chemical dilutions in TEDG buffer were added to each of three wells in a 96-well plate (Fisher; Fair Lawn, NJ) resulting in final concentrations from 2 mM to 0.1 nM following the addition of 25 µL of 8 nM [^{3}H]-R1881, at a final concentration of 2 nM in the plate. Plates were allowed to gently shake (Thermo Scientific Barnstaed Lab Line Rotator; Waltham, MA) for 5 min at 4 °C before 25 µL of 10,000 cell equivalents chAR was added to all wells. One set of three wells for non-specific binding was prepared by adding 50 µL of 100-fold excess R1881 over [^{3}H]-R1881 instead of test chemical; another set of three wells was used to measure total binding by adding 50 µL of TEDG buffer instead of test chemical. Separately, a 5% dextran-coated charcoal slurry was prepared in TEDG buffer. The sealed prepared plates and slurry were placed on a shaker and incubated overnight at 4 °C.

Following incubation, 50 µL of the charcoal slurry was added to each well and the plates were sealed and shaken an additional 20 min then centrifuged (International Equipment Company; Needham Heights, MA) at 1000 relative centrifugal force (rcf) at 4 °C for 5 min. 50 µL of the supernatant was removed from each well and placed into a scintillation vial (Fisher; Fair Lawn, NJ) containing 4.5 mL Opti-Fluor scintillation cocktail then read on a Packard 1900 TR scintillation (Palo Alto, CA).

2.2.3. In silico binding prediction

The DBPs were screened for their binding affinity to the receptor using the Endocrine Disruptome docking program http://endocrinedisruptome.ki.si/ as previously described (23). The software was used to predict the likelihood of whether the compounds would bind in the agonist or antagonist confirmation and their free binding energy, shown in kcal/mol.

2.2.4. ARBA binary assay

Binary mixtures were prepared by combining equipotent amounts of test chemical and 4-NP into one stock solution in absolute ethanol. This means that compounds of unequal potency would be adjusted in concentration so that their impact on the mixture binding affinity, as measured by a sigmoidal dose response curve, could be predicted using the model of concentration addition (CA) (24). Working solutions were then prepared and handled as described in the previous section. For comparison, the non-binding chemical, mutagen X (MX), was added to 4-NP at equal concentration to determine whether a non-binding chemical would influence its binding ability.

2.2.5. Data Analysis

The data obtained from the scintillation counter (in units of dpm) were analyzed using GraphPad Prism 4 software (Version 4.03, La Jolla, CA). Specific binding was calculated for each chemical concentration as shown in Equation 2.1 and then plotted against concentration to produce dose response curves:

$$\left(\frac{Measured\ Binding - Non\text{-}specific\ Binding}{Total\ Binding}\right) * 100 = Specific\ Binding \quad (2.1)$$

Linear regression analysis was used for the dose response curves to examine whether each test chemical induced a response that deviated from a mean slope of zero with a p-value confidence level of 0.05. Chemicals that showed significant deviation were then fit with a log[inhibitor] vs. response (variable slope) curve to determine the chemical's binding affinity, as described by its log(IC_{50}) value, with the bottom constrained to zero. The relative binding affinity (RBA) to R1881 was calculated by dividing the IC_{50} of R1881 by the IC_{50} of the test chemical, and then multiplied by 100 to be expressed as a percent. For R1881, the RBA was set to 100%. The IC_{50} values represent the mean values for a set of test chemical results. Nonbinders

(NBs) were determined as those chemicals that did not result in a $p<0.05$ for the linear regression analysis. Slight binders (SBs) were considered to be chemicals whose dose response curves did not reach 50% binding at the maximum concentration tested (25).

Since all of the chemicals tested are assumed to bind at the same active site, the CA model as shown in Equation 2.2 was used to determine whether the combination of the chemicals enhanced or suppressed the predicted response as measured by IC_{50} values for the binary mixtures: [24]

$$\frac{[A]}{[A_E]} + \frac{[B]}{[B_E]} = X \qquad (2.2)$$

$[A]$ and $[B]$ refer to the concentrations of the test chemical and 4-NP present, respectively, in the binary mixture at the designated effect (IC_{50}), $[A_E]$ and $[B_E]$ refer to the concentrations of the individual test chemicals that are required to cause the same 50% binding, and X refers to the resulting effect. According to Loewe synergism, additive mixtures have a combined effect, X, of 1, synergistic mixtures will have X less than 1, and in antagonistic mixtures X will be greater than 1 (26). Effectively, this means that lower concentrations of those chemicals in a mixture are required to induce the same effect for synergistic mixtures and higher concentrations are required to result in the same effect for antagonistic mixtures (27).

2.3. Results

2.3.1. Individual chemical binding

Individual chemical assays were completed for the R1881 standard, the weak androgen (4-NP) as the positive control, and the 21 test compounds shown in Figure 2.1, with results shown in Table 2.1.

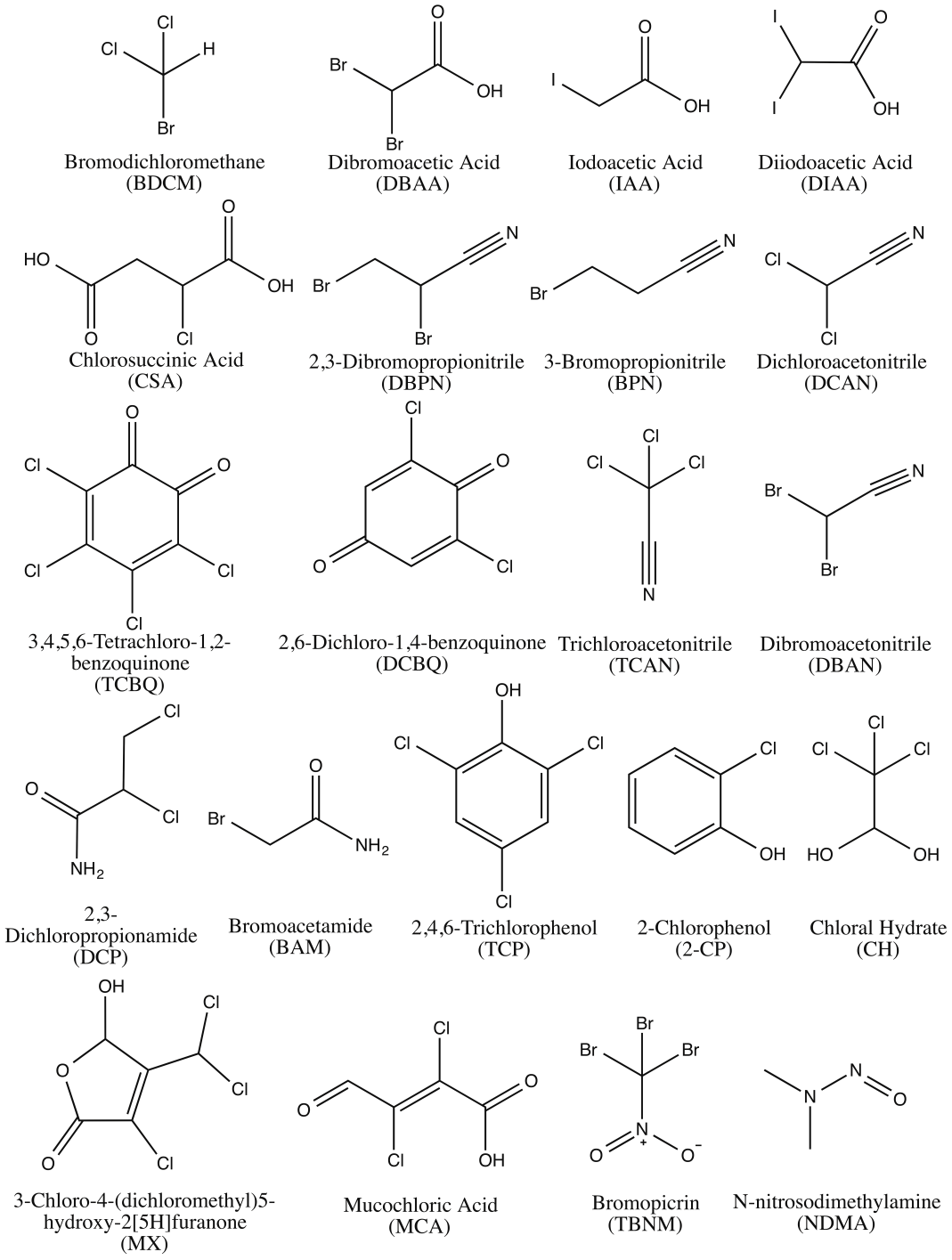

Figure. 2.1. Chemical structures of the twenty-one DBPs tested with the androgen receptor binding assay (ARBA).

The log(IC_{50}) for R1881, -8.46 ± 0.22 M, was in close agreement with a previously reported value of -9.02 M by Hartig and colleagues (22). The test chemicals that bound to the receptors showed dose response curves generally spanning a drop in specific binding from 90% to 10% over two orders of magnitude in concentration which, along with the measured R1881 IC_{50} value, supports the validity of these findings for a single receptor site competition (28). Of the compounds tested, 14 bound to the androgen receptor with one compound, TCBQ, binding more strongly than the positive control 4-NP, with log(IC_{50}) values of -4.87 ± 0.293 M vs -4.50 ± 0.032 M, respectively. DBPN showed a similar binding affinity as 4-NP. An example of a binding and non-binding chemical are shown in Figure 2.2.

Table 2.1. Androgen receptor-binding assay results for 21 potential DBPs, the positive control 4-*n*-nonylphenol (4-NP), and the androgen standard, R1881, n=3.

Chemical[a]	Molecular Weight (g/mol)	Log[IC_{50}] ± log[SD][b] (M)	IC_{50} (mg/L)	RBA[c] (Percent Inhibition)
R1881	284.39	-8.46 ± 0.219	9.86E-04	100
TCBQ	245.88	-4.87 ± 0.293	3.31	0.026
4-NP	220.35	-4.50 ± 0.032	7.01	0.011
DBPN	212.87	-4.40 ± 0.075	8.59	0.009
MCA	168.96	-4.38 ± 0.154	7.01	0.008
DCBQ	176.98	-4.24 ± 0.029	10.2	0.006
DBAN	198.84	-3.80 ± 0.085	31.4	0.002
IAA	185.95	-3.81 ± 0.104	28.9	0.002
TBNM	297.73	-3.71 ± 0.007	58.7	0.002
DCAN	109.94	-3.63 ± 0.397	25.9	0.001
BAM	137.96	-3.58 ± 0.042	36.4	0.001
TCP	197.45	-3.36 ± 0.022	87.2	0.001
DCP	141.98	-2.73 ± 0.047	2.65E02	0.0002
DBAA	217.84	-2.41 ± 0.168	8.50E02	SB[d], 0.0001 (29.1)
CSA	152.53	-2.69 ± 0.444	3.09E02	SB[d], 0.0002 (29.1)
DIAA	311.85	-1.91 ± 0.750	3.84E03	SB[d], 0.00003 (24.4)
MX	217.43	NB[d]	NB[d]	NB[d]
CH	165.4	NB[d]	NB[d]	NB[d]
BDCM	163.8	NB[d]	NB[d]	NB[d]
2-CP	128.56	NB[d]	NB[d]	NB[d]
NDMA	74.08	NB[d]	NB[d]	NB[d]
TCAN	144.39	NB[d]	NB[d]	NB[d]
3-BPN	133.97	NB[d]	NB[d]	NB[d]

a. See Figure 2.1 for identity of chemical acronym.
b. SD = standard deviation.
c. RBA = relative binding affinity.
d. NB = nonbinder, SB = slight binder.

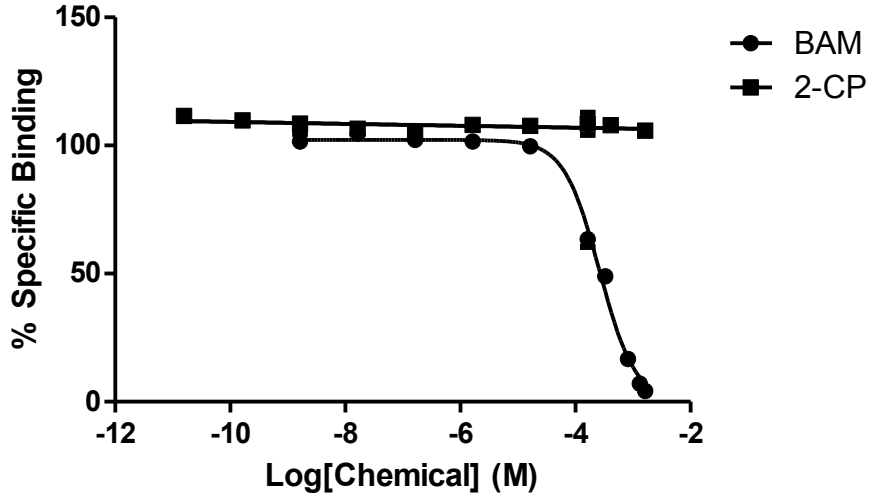

Figure 2.2. Example binding curve of binding compound (BAM) and non-binding compound (2-CP) from individual chemical binding assays plotted as mean values with standard deviations.

The only tested chemicals that did not show a significant deviation from linearity and were, therefore, not able to bind to the androgen receptor at the concentrations tested were MX, CH, BDCM, 3-BPN, 2-CP, NDMA, and TCAN. Binding affinities for the other compounds tested ranged in log(IC_{50}) concentrations from -4.38 ± 0.154 M for MCA to -1.91 ± 0.750 M for DIAA. The IC_{50} values for the compounds tested did not follow a pattern based on molecular weight, halogen type or abundance, or relative size. It is, however, expected that these compounds bind to the threonine (Thr877), asparagine (Asn 705), and arginine (Arg752) residues in the ligand-binding domain as shown in Figure 2.3, competitively against [^{3}H]-R1881, through hydrogen bonding or hydrophobic interactions.

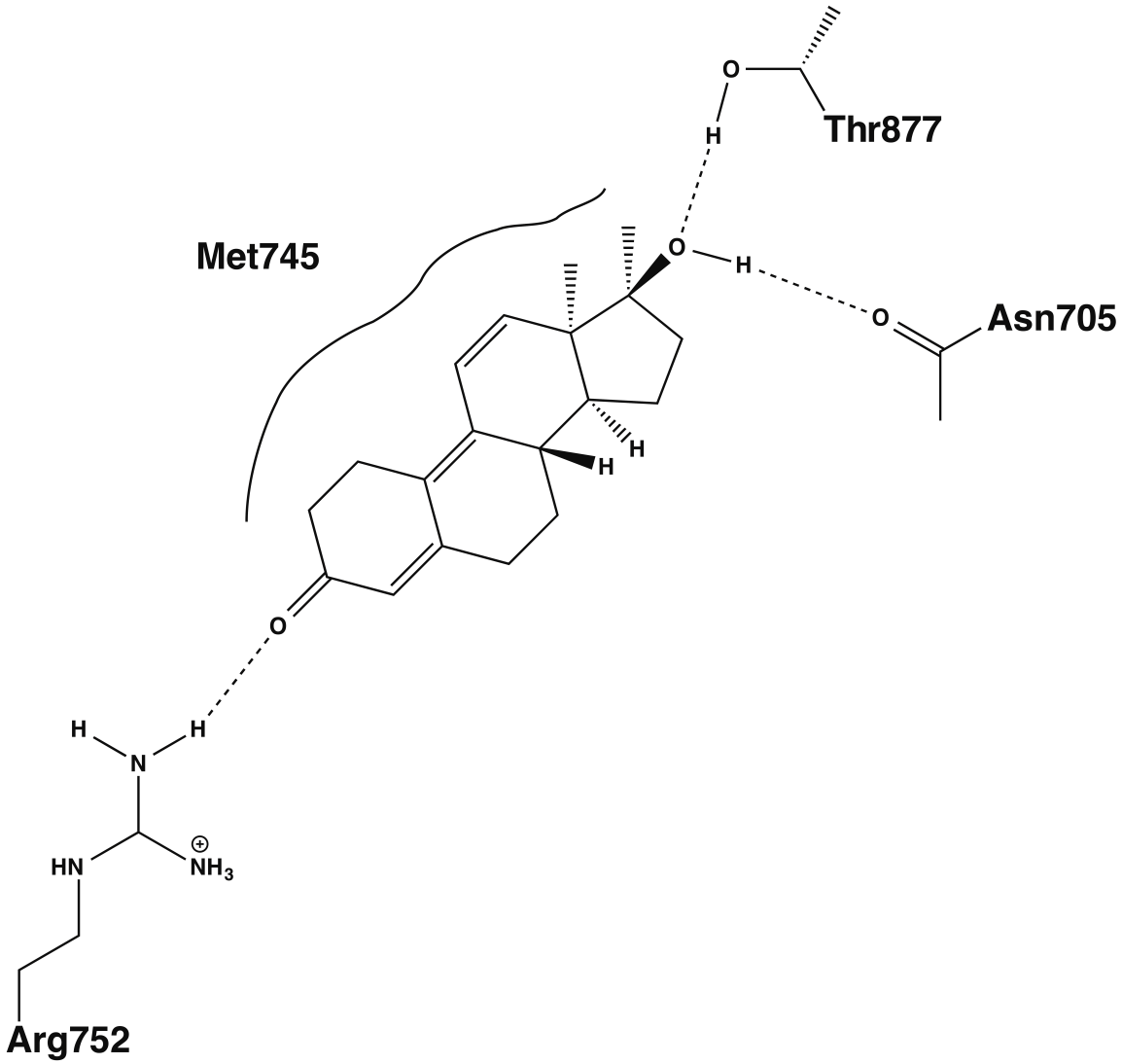

Figure 2.3. The AR ligand binding domain with the hydrogen bonding network shown for methyltrienolone with threonine (Thr), asparagine (Asn), and arginine (Arg). The crystal structure for the AR binding domain is from Protein Data Bank entry 1E3G.

Using the groupings originally described by Fang et al., chemicals that bound were compared to R1881 based on their RBA values and considered as either a strong binder (RBA > 1), a moderate binder (1 > RBA > 0.01), or a weak binder (0.01 > RBA > 0.0001) (25). Of the 21 chemicals tested, there were no strong binders but there was one moderate binder, TCBQ (RBA = 0.026%), in addition to the positive control, 4-NP (RBA = 0.011%). Ten of the chemicals were considered weak binders, namely DBPN (RBA = 0.009%), DCBQ (RBA = 0.006%), MCA (RBA = 0.008%), DBAN, IAA, and TBNM (RBA 0.002% each), DCAN, BAM and TCP (RBA

= 0.001% each), and DCP (RBA = 0.0002%). While DBAA and CSA had weak RBA values (0.0001% and 0.0002%, respectively), they did not reach 50% inhibition over the concentrations tested (29.1% binding response induced for each), and were, therefore, categorized as slight binders. DIAA showed some displacement of R1881 (24.4%) but had an RBA of 0.00003%.

2.3.2. In silico binding predictions

The results from the Endocrine Disruptome molecular docking predictions are shown in Table 2.2. The predictions indicate favorable antagonistic binding for all of the chemicals that bound, denoted by an asterisk, to the receptor except IAA, but also suggested potential antagonistic binding for the chemicals that did not bind across the tested concentration range. None of the compounds were predicted to bind in the agonist conformation.

Table 2.2. Binding predictions for the 21 DBPs as antagonists or agonists to the Androgen Receptor using Endocrine Disruptome. (*) indicates favorable binding.

DBP	AR Agonism Free Binding Energy (kcal/mol)	AR Antagonism Free Binding Energy (kcal/mol)
TCBQ	-6.1	-5.8*
DBPN	-3.7	-3.7*
MCA	-4.9	-4.4*
DCBQ	-5.5	-5.6*
DBAN	-3.3	-3.2*
IAA	-3.4	-3.1
TBNM	-4.4	-4.3*
DCAN	-3.3	-3.3*
BAM	-3.5	-3.5*
TCP	-5.4	-5.7*
DCP	-4.3	-4.3*
DBAA	-3.8	-3.7*
CSA	-4.7	-4.5*
DIAA	-3.8	-3.6*
MX	-6.2	-5.9*
CH	-4.6	-4.7*
BDCM	-3.2	-3.1
2-CP	-5.3	-5.7*
TCAN	-3.9	-3.8*
NDMA	-3.5	-2.8
3-BPN	-3.3	-3.1

2.3.3. Binding as mixtures

The androgenic activity of eight of the chemicals tested from the individual assays, seven of the strongest binding affinities in the individual assays and one non-binder, were evaluated in binary mixtures with 4-NP using the CA model. This chemical was chosen as the positive control because it is known to be a weakly androgenic chemical and a source of environmental endocrine disruption. When placed in a binary mixture with one of the test chemicals that are equipotent or weaker binders for the androgen receptor, it was expected that changes in the binding affinity for the 4-NP would be due to the presence of the new chemical. Using

equipotent mixtures meant that compounds were added at concentrations that should produce the same effect as each other, as measured by their IC_{50} values from the individual chemical assays. When compounds act independently but with similar modes of action, such as binding to the same active site of a receptor, the results should be additive because one compound could essentially replace the other in the mixture, thus behaving as if it were the same compound. Each experiment was run as a mixture of one test compound with 4-NP across the same concentration range as the individual assays and the resulting data are presented in Table 2.3. The IC_{50} values were all in the micromolar range, from approximately 31.5 μM for TCBQ with 4-NP to 178 μM for BAM with 4-NP. To determine the effect of one chemical on the binding affinity of the other in the mixture, (X in equation 2), the CA model was used on the mean (n=3) IC_{50} concentrations (27, 29). While the model from the mean values shows enhanced and suppressed binding affinity of these compounds in mixtures, when the standard deviation of the individual and binary assay results are considered, none of the mixture results for CA model are statistically significant for Loewe synergism. The trend in the mean values, however, indicates that this warrants further investigation.

Table 2.3. Equipotent binding assays presented as mean values of log(IC_{50}) and standard deviation (SD), individual concentrations at mixture IC_{50}, potency ratio, and result of the concentration addition test, n =3.

DBP + 4-NP	Log[IC_{50}] ± Log[SD] (M)	DBP[b] [IC_{50}] (mg/L)	4-NP[b] [IC_{50}] (mg/L)	Potency Ratio In Mixture	CA Effect[c]
MCA	-4.42 ± 0.147	3.38	3.93	1.12:1	1.12
IAA	-4.11 ± 0.168	12.2	2.62	5.5:1	0.79
DBAN	-3.98 ± 0.272	17.3	3.99	4.8:1	1.12
DBPN	-4.29 ± 0.405	6.50	4.48	1.1:1	1.40
TCBQ	-4.50 ± 0.573	2.69	4.54	0.53:1	1.46
BAM	-3.75 ± 0.038	21.9	4.27	8.2:1	1.21
TBNM	-4.10 ± 0.291	20.4	2.56	5.9:1	0.71
MX[a]	-4.56 ± 0.018	3.02	3.06	1:1[a]	0.44

a. Chemical mixture tested as negative control by using 1:1 concentration ratio.
b. Represents the concentration of the contributing chemical in that mixture at the IC_{50}.
c. Value represents 'X' in Equation 2.2.

2.4. Discussion

Of the over 800 DBPs that have been identified in various types of drinking water, the vast majority have unknown biological activities, which has spurred the need to understand how exposure to these chemicals may impact human health (30). Endocrine disruption due to chemicals in the environment highlights the ability of low-level and chronic exposures to influence gene activity through chemical binding to receptors in a myriad of systems, sometimes with non-monotonic responses, and induce a variety of diseases (31). The results of this study showed that 14 of the 21 tested DBPs could bind to the androgen receptor, with some trends within structural classes but not between them, and that they had comparable binding affinities to the observed results for other active chemicals and known endpoints (32). Chemicals that were found to bind individually were then tested in binary mixtures, since environmental exposures

are not to single compounds but complex mixtures such as through consumption of drinking water. The additive effect observed for the tested binary mixtures is consistent with other published reports, and supports the impetus for evaluating chemical mixtures in water for their influence on endocrine activity, beyond the currently regulated 11 chemicals (33). In particular, further exploration into the structure-activity relationships with the androgen receptor and DBPs should be evaluated.

For the tested chemicals, ring structures showed favorable binding with interactions that are generally through hydrophobic residues. Possible limitations to binding could be based on steric hindrance, as in the case of DCAN binding ($\log(IC_{50})$ of -3.63 M) compared to TCAN which did not show any binding affinity. Similarly, IAA was a moderate binder ($\log(IC_{50})$ of -3.81 M), while DIAA was only a slight binder ($\log(IC_{50})$ of -1.91 M). For planar molecules the trend appears to reverse, as the more halogenated structures showed stronger binding affinities, which is similar to the toxicity trend that has been previously observed for quinones (34). For example, TCP ($\log(IC_{50})$ of -3.36 M) bound more strongly than 2-CP, a non-binder, and TCBQ ($\log(IC_{50})$ of -4.87 M) bound more strongly than DCBQ ($\log(IC_{50})$ of -4.24 M). These results are likely because of the large size of the binding pocket, which favors phenolic structures but accommodates a variety of small molecules through its hydrogen bonding network (9). For the androgen receptor, the binding site of the ligand binding domain (LBD) is comprised of a similar structural motif to other nuclear receptors, including the estrogen receptor. The LBD consists of the helices 3, 5, 7 and 10, and β1, which provide hydrogen bonding networks and hydrophobic interactions for androgens (35). Once the ligand binds, a conformational change is induced that forms a hydrophobic cleft from the movement of helix 12 (36). It is expected that the chemicals that bound were able to interact through this hydrogen bonding network when present

individually. The predicted docking position for each chemical in Endocrine Disruptome indicates that these chemicals will bind in the antagonist conformation, and thus likely correspond to decreased gene transcription, as was shown for some flame retardants (37). None of the compounds tested were predicted to bind as agonists.

Two of the three chemicals capable of binding to the androgen receptor with the strongest affinity in the individual assays, TCBQ and DCBQ, belong to the emerging class of DBPs known as haloquinones (HQs), which occur in treated waters at nanogram per liter levels and have been implicated as potential bladder carcinogens (38). In one study, DCBQ was present in all of the aqueous samples tested (4.5 to 274.5 ng/L), with the highest median concentrations of the tested HQs at 23.0 ng/L, but over 100 ng/L in 5 of the 16 tested samples (39). Using a quantitative structure-toxicity relationships analysis as well as toxicological and occurrence data, HQs were predicted to be important potential carcinogens with toxicity levels as high as 1000 times those of the currently regulated DBPs (40). Furthermore, the use of chloramines for disinfection favors the formation of haloquinones, such as TCBQ, as a result of its reaction with naturally occurring organic compounds including lignins, tannins, or catechol (12). The relatively strong affinity of TCBQ and DCBQ for the androgen receptor, and their known influence in other disease states, indicates that these compounds should be included in more widespread occurrence surveys. For example, halobenzoquinones have shown cytotoxicity to T24 bladder cancer cells in the micromolar range (41). Also of concern, dibromoacetic acid (DBAA) has been shown to have liver and testes toxicity, bromodichloromethane (BDCM) is listed as a probable human carcinogen and shown to produce liver and kidney tumors in rats as well as alter sperm motility (42-44). Dibromoacetonitrile (DBAN) has shown a number of

negative outcomes including DNA damage in mammalian cell assays and altered hepatic activity (45).

In occurrence studies, the tested DBPs have generally been found in the μg/L to ng/L range in drinking water. Tribromonitromethane (bromopicrin) was found at the highest levels within the class of nine different halonitromethanes, which were detected at 0.1 – 5 μg/L in the U.S. Nationwide DBP Occurrence Study (17, 46). Iodoacetic acid, identified in drinking water from high bromide/iodide source water that was disinfected with chloramines and found at low parts per billion (ppb) levels in some cities, is another example of an individual DBP that could contribute to the overall endocrine activity of a finished water (47). Haloamides, including bromoacetamide, were detected in distribution system samples at up to 7.6 μg/L. Similarly to iodo-acids, there is some evidence that indicates haloamides form more preferentially with the use of chloramine disinfectant. Haloacetonitriles (HANs), including DBAN, DCAN and TCAN, as well as others, have been detected in U.S. and Canadian drinking waters at up to 41.0 μg/L (48, 49). These occurrence levels in conjunction with the measured androgen binding affinities from this study indicate that it is important to consider the impact of more complex mixtures of compounds. The observed binding across structural classes further indicates the importance of assessing how a mixture in different waters could influence androgen disruption.

Although the compounds were tested mostly at concentrations above environmentally relevant levels, their ability to bind to the receptors at all lends credence to their potential as endocrine disruptors, particularly when occurring in more complex mixtures. The persistence of the compounds in disinfected drinking water is also of note, as these exposures occur over the long-term at low-levels instead of at acute high-levels. In drinking or recreational waters there is a much more complex mixture of a large number of compounds, which means that compounds

that are able to bind but are present at what would be weak acute individual effect levels (below their IC_{50} level) could combine to induce an endocrine response (50). Furthermore, drinking water regulations for DBPs currently set a maximum contaminant level (MCL) for only a small subset of all DBPs, including the sum of four trihalomethanes (including the listed BDCM tested in this study), the sum of five haloacetic acids, and bromate, and all at sub-micromolar levels.

2.5. Conclusions

As this study has shown, a number of known and suspected DBPs that are not currently regulated are capable of binding to the androgen receptor including haloquinones, haloacetonitriles, haloacids, and halofuranones. The structural diversity of these chemicals together with their demonstrated CA binding indicates that chemicals present beyond those currently regulated need to be considered when assessing potential health effects, particularly endocrine disruption. While the MCL concentrations for regulated DBPs are well below the reported IC_{50} for androgen receptor activity, the ability of these compounds to bind at all is still of concern. For comparison, flame-retardants well below no observed adverse effect levels from toxicological studies result in adverse health effects when low-level chronic exposures occur (51). Further experiments are required to not only understand the behavior of mixtures with biological systems, but also what altered physiological states result from low-level binding to the androgen receptor and whether the active DBPs detected here are cause for greater concern. These results indicate that a wide variety of DBPs are capable of binding to the androgen system preferentially as antagonists and that this pathway should be investigated more as cause of the negative health impacts identified from DBP exposure.

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CHAPTER 3: DISINFECTION BY-PRODUCTS (DBPS) AS ESTROGEN ACTIVE SUBSTANCES

3.1. Introduction

Most conventional water treatment in the U.S. is accomplished through the use of disinfectants including chlorine and chloramine that inactivate bacteria and pathogens, but also react with low-levels of natural organic matter and form disinfection by-products (DBPs) (1). While this practice is used for both drinking and wastewater, laboratory-based and epidemiological studies have shown that there is an established link between exposure to disinfected water and a number of toxicological outcomes, including bladder cancer and impaired reproductive health (2). Endocrine disruption, a primary toxicological endpoint of concern, is the result of non-hormonal compounds interfering with normal hormonal signaling processes through receptor binding and other mechanisms (3, 4). Exogenous compounds, that are able to bind to these receptors or otherwise interfere with normal signaling levels, are known as endocrine disrupting compounds (EDCs) (5). The environmental impact of EDCs as estrogen active compounds is particularly important for public health as improper estrogen signaling is linked to a number of negative public health outcomes including infertility, breast cancer, and obesity (6, 7). This is due to the fact that estrogens cause cellular changes that influence reproduction, bone development, impaired cognition, stress responses and cardiovascular health and are, therefore, prone to cause a number of diseases when disrupted in either males or females (8). Due to the importance of the estrogen signaling pathway in the development and control of

such diverse outcomes, the ability of both regulated and unregulated DBPs to act as EDCs is an important and as yet unresolved question.

Estrogenic disruption in humans occurs primarily through nuclear hormone receptors (5). Two major estrogen receptor subtypes, ERα and ERβ, exist in humans as coded by the ESR1 and ESR2 genes, respectively, and act in tissue-dependent roles (8). These variances mean that EDCs which interact with the estrogen system may not show the same affinity for each receptor subtype and, as a result, the binding of EDCs with one subtype over another could be more indicative of a specific disease response (8-10). For example, ERα is found in the mammary glands, kidney, lung bronchi, and gonads and promotes cell proliferation, whereas ERβ has an anti-proliferative effect in mammary tissues and is found primarily in bone, lung alveoli and prostate tissues (4, 8, 9). Furthermore, within the family of nuclear receptors, ERs are known to have highly flexible and large binding pockets that lack specificity and are thus able to bind ligands in multiple orientations, which then act as agonists, partial-agonists, or antagonists (11). These variances are due to the structural changes that result at the ligand binding domain, which change the conformation of the receptor and its recruitment for co-regulator proteins (3, 12). It should be mentioned that these structural changes are tissue-specific, which help to determine the role of the estrogen receptor in subsequent pathways and its involvement in different diseases (13). These receptors are found in the nucleus of the cell and once bound to a hormone or EDC will bind to the estrogen response element and begin gene transcription, thus imparting physiological changes.

As a result of the promiscuous nature of estrogen receptors, the number and variety of anthropogenic compounds that can bind to them and cause disruption is extensive and include phthalates, phytoestrogens, pesticides, polycyclic aromatic hydrocarbons, and plasticizers (7).

Molecular docking studies have shown that even other steroids are able to bind at the ligand binding domain of ERs in multiple orientations, in part because of their symmetry, which makes it more difficult to predict whether specific chemicals will be estrogen binders (10).

One area of human exposure to EDCs that has garnered little attention is through consumption of drinking water much of which is disinfected with chemicals that react with the background natural organic matter to produce DBPs. There is increasing evidence that some of the over 800 DBPs identified to date are EDCs, and comprise a group of structurally diverse compounds that often contain halogens and aromatic ring structures (14). Currently, only 11 of these DBPs are regulated in the United States while the European Union and World Health Organization have expanded the list to include an additional 7 compounds (15). Previous research has exposed a gap between the observed toxicity of the regulated DBPs and the overall activity of drinking water, which suggests that unregulated compounds are involved but their identity remains largely unknown. For example, studies of some of the unregulated DBPs have shown higher genotoxicity than their regulated counterparts (16). Furthermore, the potential ability of DBPs to bind to the estrogen, androgen, or thyroid nuclear hormone receptors (ER, AR, TR, respectively) and initiate transcription of downstream gene targets was raised in the 1996 amendment to the Safe Drinking Water Act (17). As a result of the already observed estrogenic activity of disinfected wastewaters and finished drinking water (18) and the known promiscuity of ERs, it is important to determine which DBPs are contributing to this activity and whether a particular type of disinfection might need to be reconsidered. Due to the differences in the formation of DBP classes and their related genotoxicities as a result of the disinfectant type, the influence that treatment type could have on estrogenic activity should also be evaluated (19).

In this study, a group of 21 structurally diverse DBPs, shown in Figure 3.1, were tested for their ability to bind to the human estrogen receptor (hER) as measured by their half inhibitory concentration (IC_{50}) in a competitive binding assay. These chemicals were also tested using an *in silico* binding approach to determine whether their binding activity or conformation could be predicted. A subset of 7 of the active compounds was then tested in binary mixtures containing the known water contaminant and weak endocrine disruptor, 4-*n*-nonylphenol, to determine whether their behavior is dose-additive. The behavior of these compounds in mixtures is of particular importance because environmental exposures occur to mixtures of EDCs, such as those found in drinking water, and understanding whether their toxicity as a mixture can be predicted is important for understanding the overall risk from exposure to the estrogen signaling pathway.

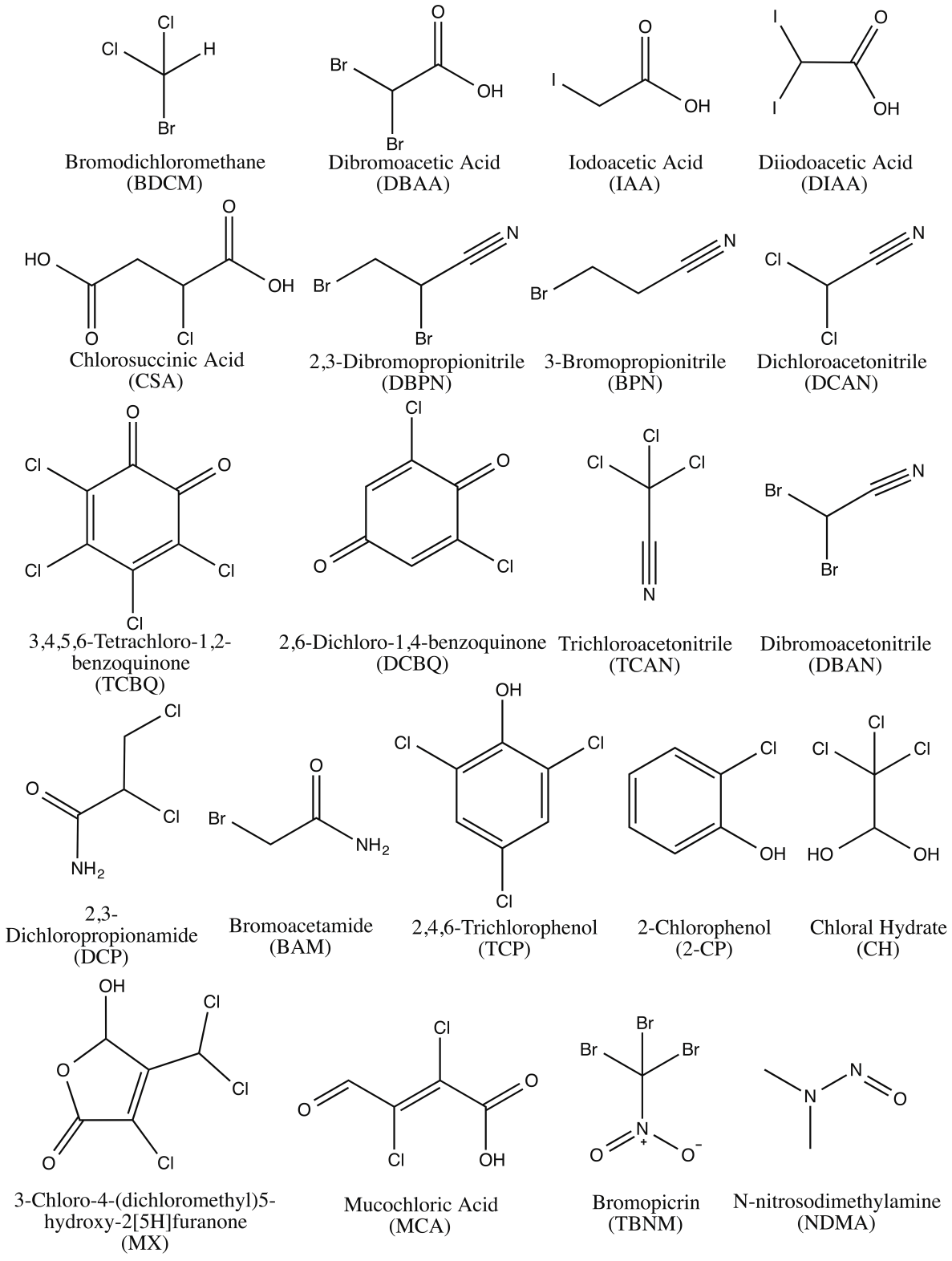

Figure 3.1. Chemical structures of the twenty-one chemicals tested with the estrogen receptor binding assay (ERBA).

3.2. Materials and Methods

3.2.1. Chemicals

Recombinant human Estrogen Receptors-α (hER-72) were supplied by the U.S. Environmental Protection Agency (EPA) in TEDG binding buffer and stored at -80 °C. Chemicals purchased from Sigma Aldrich (St. Louis, MO) were: bovine serum albumin (BSA), phenylmethyanesulfonyl fluoride (PMSF, 98%), sodium molybdate dehydrate (>95%), 17β-estradiol (E2, >98%), chloral hydrate (CH, >98%), DL-dithiothreitol (DTT, >98%), ethylenediaminetetraacetic acid (EDTA), glycerol, dextran-coated charcoal, 2,6-dichloro-1,4-benzoquinone (DCBQ, 98%), dibromoacetic acid (DBAA, 90%), chlorosuccinic acid (CSA, 96%), 2,4,6-trichlorophenol (TCP, 98%), dibromoacetonitrile (DBAN, 97%), mucochloric acid (MCA, 99%), 3,4,5,6-tetrachloro-1,2-benzoquinone (TCBQ, 97%), sodium chloride (NaCl, >99%), 4-*n*-nonylphenol (4-NP, 98.4%) and absolute ethanol. [^{3}H]-17β-estradiol ([^{3}H]- E2, 140 Ci/mmol) and Opti-Fluor scintillation cocktail were purchased from Perkin Elmer (Waltham, MA) while 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX, 98%) was supplied by Wako (Richmond, VA) and *N*-nitrosodimethylamine (NDMA, 100%) was from Supelco (Bellefonte, PA). From Acros Organics (New Jersey, USA) the chemicals 2-chlorophenol (2-CP, 98%), bromodichloromethane (BDCM, >98%), trichloroacetonitrile (TCAN, 98%), and 2-bromoacetamide (BAM, 98%) were purchased. Dichloroacetonitrile (DCAN, 99.8%) was obtained from Crescent Chemicals (Islandia, NY), iodoacetic acid (IAA, 99.5%) from Fluka (Buchs, Switzerland), 2-bromopropionitrile (3-BPN, 98%) from Alfa Aesar (Lancs, UK), diiodoacetic acid (DIAA, 90+%) from Cansyn Chemicals (Ontario, Canada), 2,3-dibromopropionitrile (DBPN, 95+%) from Matrix Scientific (Columbia, SC), 2,3-dichloropropionamide (DCP, 97%) from TCI (Tokyo, Japan), and tribromonitromethane

(TBNM, 95+%) from Orchid Cellmark (Princeton, NJ). Potassium chloride (99%), Tris base and hydrochloric acid (ACS grade) were purchased from Fisher (Fair Lawn, NJ).

3.2.2. Estrogen receptor binding assay (ERBA): Single chemical analysis

The estrogen receptor binding assay (ERBA) followed published procedures as previously described for the androgen receptor, with some modifications (20). Briefly, initial range finding assays were completed to determine the appropriate receptor concentrations, which would result in 5-15% binding. Saturation binding and Scatchard analyses determined the dissociation constant (K_d) of the receptor to the potent tritiated 17β-estradiol ([^{3}H]-E2) ligand, resulting in a value of 1.0 nM, which corresponds well to the literature value (21). For each set of competitive binding assays, an unlabeled E2 control ligand was included along with tests for non-specific and total binding of the [^{3}H] ligand to the receptor. The known endocrine disruptor and weak binder, 4-NP, was used as a positive control for test chemicals to show a measurable affinity at the test concentrations. TEDG binding buffer (10 mM Tris-HCl, pH 7.4; 1 mM DTT; 10% glycerol; 10 mg/mL bovine serum albumin; 400 mM KCl; 1 mM PMSF; 1mM sodium molybdate; 1.5 mM EDTA) was prepared and used to dilute receptor and working chemical stocks. Prior to use, the hERs were gently mixed by pipette and diluted to 625 cell eq/ml according to the range finding assay, then kept on ice until addition to the assay wells. All assays were kept on ice during use and sealed with plate-sealing film during overnight shaking and mixing. Test chemicals were prepared in absolute ethanol well below their solubility concentrations and stored in amber vials at -20°C for up to 6 months. These were vortexed briefly before use in the assay. Unlabeled E2 was run against [^{3}H]-E2 in the reference assay and [^{3}H]-E2 was tested for competitive binding against varying concentrations of test chemicals in

the binding assays. Each assay concentration was run in triplicate on the day of the experiment and repeated on at least two separate days.

Stocks of 0.1 μM [^{3}H]-E2 and 10 μM unlabeled E2 were prepared from the neat standards and stored at -20 °C in amber vials. Aliquots of unlabeled 10 μM E2 and 0.1 μM [^{3}H]-E2 were dried down in borosilicate glass tubes on a SpeedVac (Thermo Scientific Savant Model SPD1010; Waltham, MA) then reconstituted in TEDG buffer and 1 μL absolute ethanol prior to addition to the plates. For the assay, 50 μL of freshly prepared working test chemical were added to each of three wells in a 96-well plate (Fisher; Fair Lawn, NJ) resulting in final concentrations from 2 mM to 0.1 nM following the addition of 25 μL of 8 nM [^{3}H]-E2, at a final concentration of 2 nM in the plate. Prepared plates were allowed to gently shake (Thermo Scientific Barnstaed Lab Line Rotator; Waltham, MA) for 5 min at 4 °C before 25 μL of the prepared hER was added to all wells. One set of three wells for non-specific binding was prepared by adding 50 μL of the 400 nM unlabeled E2 which is equivalent to a 100-fold excess E2 compared to [^{3}H]-E2 instead of test chemical; another set of three wells was used to measure total binding by adding 50 μL of TEDG buffer instead of test chemical. Separately, a 5% dextran coated charcoal slurry was prepared in TEDG buffer. The sealed prepared plates and slurry were placed on a shaker and incubated overnight at 4 °C.

Following incubation, 50 μL of the charcoal slurry was added to each well and the plates were sealed and shaken an additional 20 min. They were then centrifuged (International Equipment Company; Needham Heights, MA) at 1000 relative centrifugal force (rcf) at 4 °C for 5 min, placed on ice, and then 50 μL of the supernatant was removed from each well and placed into a 20 mL scintillation vial (Fisher; Fair Lawn, NJ) containing 4.5 mL Opti-Fluor scintillation

cocktail. The vial was then briefly shaken and read on a Packard (Palo Alto, CA) 1900 TR scintillation counter.

3.2.3. In silico binding predictions

The quick and efficient docking program, Endocrine Disruptome (http://endocrinedisruptome.ki.si/), was used as previously described to determine the likelihood of DBP binding to the estrogen receptor subtypes and their other nuclear receptor counterparts (22). The program utilizes previously published crystal structures of the nuclear receptors and AutoDock Vina molecular docking software to determine the potential for the ligands to bind as agonists and antagonists. The likelihood of binding is indicated by an asterisk (*) for favorable binding, and then their free binding energy (kcal/mol) is calculated (23).

3.2.4. ERBA binary assay

For the binary binding assays, mixtures were prepared by combining equipotent amounts of test chemical and 4-NP into one stock solution in absolute ethanol. This means that compounds of unequal potency would be adjusted in concentration so that their impact on the mixture binding affinity, as measured by a sigmoidal dose response curve, could be predicted using the model of concentration addition (CA) (24). Working solutions were then prepared and handled as described in 3.2.2.

3.2.5. Data Analysis

Data obtained from the scintillation counter (in units of dpm) were analyzed by first calculating the specific binding and comparing that to the test concentration. Specific binding was calculated for each chemical concentration as shown in Equation 3.1 and then plotted against concentration using GraphPad Prism 4 software (Version 4.03, La Jolla, CA):

$$\left(\frac{Measured\ Binding - Non\text{-}specific\ Binding}{Total\ Binding}\right) * 100 = Specific\ Binding \quad (3.1)$$

Using a p-value confidence level of 0.05 to determine whether each DBP was able to displace [^{3}H]-E2, a linear regression analysis was fit to each response curve to examine whether the slope deviated from a mean of zero. Chemicals without a significant p-value (> 0.05) were considered to be non-binding (NB), while a slight binder (SB) refers to a chemical that did not reach 50% binding over the tested concentrations (25). Chemicals that showed significant deviation were then fit with a log[inhibitor] vs. response (variable slope) curve to determine the chemical's binding affinity, as described by its log[IC_{50}] value, with the bottom constrained to zero. The relative binding affinity (RBA) to E2 was calculated by dividing the IC_{50} of E2 by the IC_{50} of the test chemical, and then multiplied by 100 to be expressed as a percent. For E2, the RBA was set to 100%. IC_{50} values that are smaller indicate more potent binding of the chemical to the receptor and, therefore, a larger RBA to E2.

The CA model as shown in Equation 3.2 was used to determine whether the combination of the two chemicals was synergistic, antagonistic, or dose-additive as compared to the IC_{50} for the binary mixtures:

$$\frac{[A]}{[A_E]} + \frac{[B]}{[B_E]} = X \qquad (3.2)$$

In this equation, [A] and [B] refer to the concentrations at the IC_{50} of the test chemical and 4-NP present, respectively, in the binary mixture, [A_E] and [B_E] refer to the concentrations of the individual test chemicals that are required to cause the same 50% binding, and X refers to the resulting effect. According to Loewe synergism, if the chemical mixture is additive then the combined effect, X, will be 1, if it is synergistic X will be less than 1, and if it is antagonistic then X will be greater than 1 (24, 26). Effectively, this means that lower concentrations of those chemicals in a mixture are required to induce the same effect for synergistic mixtures and higher concentrations are required to result in the same effect for antagonistic mixtures (27).

3.3. Results

3.3.1. Individual chemical binding

Competitive binding curves were completed for 21 structurally diverse DBPs with ERα, shown in Figure 3.1, to concentrations up to but below their solubility limit. The measured IC_{50} values for the individual ERBA experiments showed that 9 of the tested DBPs were able to weakly bind to the receptor. The results from these assays along with the affinities measured for the weak estrogen (4-NP) and natural ligand (E2) are reported in Table 3.1. These binding chemicals were TBNM, BDCM, DBPN, TCBQ, DBAN, CSA, TCP, BAM, and 2-CP, with log[IC_{50}] values ranging from -3.83 ± 0.064 M for TBNM to -2.19 ± 0.216 M for 2-CP, compared to the weak positive control 4-NP that had a log[IC_{50}] of -5.67 ± 0.346 M. The measured log[IC_{50}] of E2 was -9.13 M, or an IC_{50} value of 7.46 E-10 ± 0.636 E-10 M, was in close agreement to the published value of 8.77 E-10 (28). Chemicals that bound showed a drop from 90% to 10% inhibition over two orders of magnitude, which confirms the expectation that the chemicals interacted with a single binding pocket (20).

Table 3.1. Estrogen receptor-binding assay results for 21 test compounds, the positive control 4-*n*-nonylphenol (4-NP), and the estrogen standard, E2. NB = nonbinder, SB = slight binder.

Chemical from Figure 3.1[1]	Molecular Weight (g/mol)	Log[IC_{50}] ± log[SD] (M)	Mean IC_{50} (mg/L)	RBA (Percent Inhibition)
E2	284.39	-9.13 ± 0.197	2.12E-04	100
4-NP	220.35	-5.67 ± 0.346	0.547	0.0331
TBNM	297.73	-3.83 ± 0.064	44.1	0.0006
BDCM	163.8	-3.26 ± 0.053	89.5	0.0002
DBPN	212.87	-3.23 ± 0.075	126	0.0001
TCBQ	245.88	-2.89 ± 0.023	399	0.0001
DBAN	198.84	-2.84 ± 0.328	351	0.0001
CSA	152.53	-2.83 ± 0.092	228	SB, 0.0001
TCP	197.45	-2.73 ± 0.021	371	SB, 0.00004
BAM	137.96	-2.41 ± 0.107	540	SB, 0.00002
2-CP	128.56	-2.19 ± 0.216	886	SB, 0.00001
IAA	185.95	NB	NB	NB
MCA	168.96	NB	NB	NB
NDMA	74.08	NB	NB	NB
DCAN	109.94	NB	NB	NB
TCAN	144.39	NB	NB	NB
MX	217.43	NB	NB	NB
CH	165.4	NB	NB	NB
DCP	141.98	NB	NB	NB
DIAA	311.85	NB	NB	NB
DBAA	217.84	NB	NB	NB
DCBQ	176.98	NB	NB	NB
3-BPN	133.97	NB	NB	NB

The DBPs CSA, TCP, BAM, and 2-CP were classified as slight binding chemicals because they did not reach at least 50% inhibition over the tested assay concentration ranges, with relative binding affinities of 0.0001%, 0.00004%, 0.00002% and 0.00001%, respectively. TBNM had a RBA of 0.0006%, BDCM had an RBA of 0.0002%, and DBPN, TCBQ, DBAN, and CSA had RBA values of 0.0001%. Groupings of RBA values defined by Fang and colleagues, classify these DBPs as weak binders because they showed relative binding affinities (RBA) of less than 0.001% when compared to the IC_{50} of E2 (25). Of the chemicals tested, 12

were not able to bind to the estrogen receptor as determined by the fact that their dose addition curves did not depart from linearity with a $p \leq 0.05$ over the concentration range tested which was depending on compound solubility. The non-binding chemicals included IAA, DIAA, MCA, NDMA, DCAN, TCAN, MX, CH, DCP, DBAA, DCBQ, and 3-BPN. An example of a binding and non-binding curve are shown in Figure 3.2.

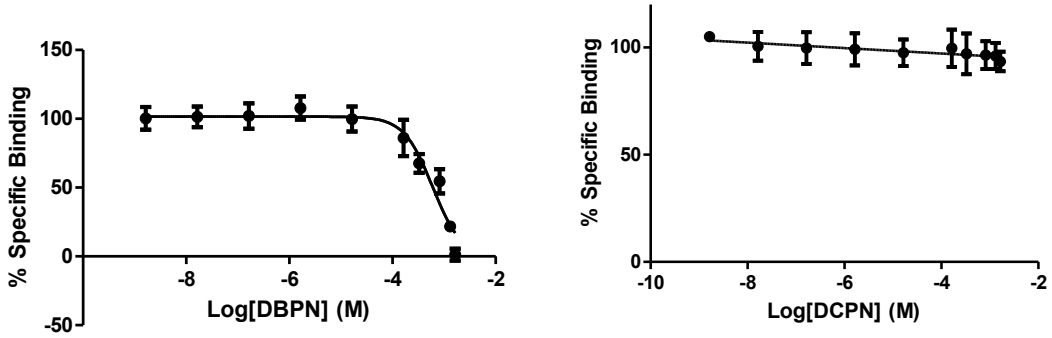

Figure 3.2. Examples of binding (DBPN) and non-binding (DCPN) dose response curves.

3.3.2. In silico binding predictions

The results from the molecular docking predictions to ERα and ERβ as agonists and antagonists are shown in Table 3.2 indicating likely binding probability, denoted by an asterisk, and the free binding energy presented in kcal/mol. The *in silico* molecular docking predictions for these compounds suggest a low probability of binding for all 21 tested DBPs to the receptors, including the known weak estrogen 4-NP, which is why none of the results have been marked with an asterisk. The software used for these predictions allows for ligand flexibility but limited receptor flexibility, which is primarily restricted to receptor side chains, and could account for difference in the observed binding for the individual assays but not in the molecular docking predictions. This is attributed to the well-established binding pocket flexibility of ERs, which is especially influential for estrogen receptor binding as the pocket is known to be highly promiscuous.

Table 3.2. The results of the molecular docking predictions for the 21 DBPs and weak positive control with the ERα and ERβ as agonists and antagonists, with all indicating low probability and the free binding energy indicated in kcal/mol.

	Free Binding Energy (kcal/mol)			
DBP	ERα agonist	ERα antagonist	ERβ agonist	ERβ antagonist
4-NP	-6.8	-7.0	-7.1	-7.0
TBNM	-3.5	-3.6	-4.0	-3.6
BDCM	-2.9	-2.9	-3.0	-3.0
DBPN	-3.1	-3.1	-3.2	-3.2
TCBQ	-5.8	-5.4	-5.8	-5.9
DBAN	-3.0	-2.9	-3.1	-2.9
CSA	-3.8	-4.1	-4.4	-4.1
TCP	-5.2	-5.4	-5.3	-5.4
BAM	-3.4	-3.4	-3.4	-3.3
2-CP	-5.4	-5.1	-5.1	-5.1
IAA	-3.0	-2.8	-3.3	-2.9
MCA	-4.4	-4.2	-4.5	-4.1
NDMA	-2.8	-2.5	-3.1	-2.5
DCAN	-3.1	-2.8	-2.9	-3.1
TCAN	-3.1	-3.1	-3.3	-3.2
MX	-5.7	-5.2	-5.5	-5.3
CH	-3.9	-3.9	-4.0	-3.9
DCP	-4.2	-4.1	-4.3	-4.0
DIAA	-3.3	-3.5	-3.5	-3.4
DBAA	-3.5	-3.3	-3.8	-3.3
DCBQ	-5.2	-5.1	-5.4	-5.3
3-BPN	-3.0	-2.9	-3.0	-3.0

3.3.3. Binding as binary mixtures

Of the 21 initial DBPs tested, 6 chemicals that were able to bind in the individual competitive binding assays and one nonbinding chemical were then examined in binary mixtures with 4-NP. Equipotent mixtures were used to determine whether, at concentrations equal to their relative activity, the chemicals would be additive, synergistic or antagonist to binding. These chemicals included BAM, TCP, 2-CP, TCBQ, DBPN, TBNM, and the non-binding chemical DIAA, and their results are presented in Table 3.3. The exception was for DIAA, which was a non-binding chemical and was added at equal concentrations to the 4-NP. The RBA values showed that all of the chemicals could be classified as weak binders, as expected based on the

individual assay results. The concentration addition values were additive according to the CA model when the standard deviation of the individual and binary assay concentrations were taken into account (27).

Table 3.3. Equipotent binding assays presented as log(IC_{50}), IC_{50}, RBA, potency ratio, and result of the concentration addition test.

Chemicals + 4-NP	Log([IC_{50}] ± SD) (μM)	Mixture IC_{50} (μM)	RBA (%)	Potency Ratio In Mixture	Concentration Addition
DIAA	-5.43 ± 0.115	3.70	2.22E-02	1:1[a]	0.75
TCP	-4.91 ± 1.092	12.4	6.06E-03	758:1	0.01
2-CP	-3.72 ± 1.027	188	4.36E-04	2778:1	0.05
BAM	-3.56 ± 0.244	277	2.97E-04	1578:1	0.14
DBPN	-3.37 ± 0.023	426	1.92E-04	239:1	1.43
TCBQ	-2.82 ± 1.394	1530	5.37E-05	655:1	1.88
TBNM	-2.73 ± 0.787	316	4.37E-05	60:1	24.89

[a] Equal concentration mixture of a non-binder (DIAA) with 4-NP.

3.4. Discussion

DBPs are structurally diverse, often halogenated, structures that form in a highly dependent matter based on source water quality and disinfectant type during drinking water treatment (15). In order to determine which structural classes are the most potent potential estrogens, a subset of 21 DBPs was evaluated that included regulated DBPs, haloacids, haloaldehydes, halofuranones, cyanogenic compounds, and by-products that are predicted based on the structure of natural organic matter in water. The high number of DBPs identified thus far, coupled with the observed estrogenic activity and endocrine disrupting potential of conventionally disinfected waters, suggest a need to explore the identity or type of DBP responsible for this activity so that they can be mitigated (14, 18, 29). In this work, nine DBPs

formerly uncharacterized for endocrine activity, were shown to weakly bind to the estrogen receptor and it was demonstrated that they behave in an additive manner when in mixtures with 4-NP, a known weak endocrine disruptor that is found as a surfactant degradation product in some surface waters. This supports the notion that estrogenic activity in waters disinfected with chlorine or chloramine could, in part, be attributed to DBPs. For example, BAM and DBAN both bound to the receptor and have been found to form in some drinking waters containing bromide with chloramination. TBNM, another weak binder, can form under multiple disinfection scenarios using chlorine, chloramine, ozone-chlorine, or ozone-chloramine (15). While all of these DBPs were only able to bind to the receptor weakly when compared to 17β-estradiol, their ability to bind at all indicates their potential to disrupt the long-term balance of the endocrine system. A number of diseases have already been associated with chronic exposure to low-level contaminants in the environment and as these chemicals have been shown to influence gene expression, at times with non-monotonic responses (30). The additive effect of these DBPs further supports the need to identify which emerging contaminants that can bind to the estrogen receptor and, therefore, interfere with the homeostatic balance of the estrogen pathway and those pathways in which it is involved (6, 31). Due to the extensive number of DBPs formed during disinfection, the ability of these DBPs to bind at all despite being at such low-levels is an important finding for understanding the estrogenic nature of this class of compounds.

The 9 DBPs that were able to bind to the estrogen receptor in this study ranged in IC_{50} concentrations from 44.1 mg/L for TBNM to 886 mg/L for 2-CP. While these concentrations are clearly above the sub- to low-μg/L levels occurrence levels detected in drinking waters (15, 32) they still indicate the ability of the chemicals to bind to the estrogen receptor and the implications of chronic exposures are unknown. Five of the nine binding compounds were

bromine-containing species, which is consistent with previously published work that suggests higher toxicity for brominated DBPs. Neither of the iodinated compounds were able to bind despite the observation that they are the most genotoxic of the halogenated DBPs (33). Bromide and iodide concentrations in influent water enhance the formation of bromine- or iodine-containing DBPs relative to those containing just chlorine as the halogen (15). When considering toxicity, the predominant estrogen affinity observed for bromine-containing DBPs supports the claim that higher levels of these compounds could lead to greater toxicity (34). The most potent DBP in this study, TBNM, was found at levels from 0.1 to 5 μg/L in the U.S. Nationwide DBP Occurrence Study, which were the highest levels within the class of 9 different halonitromethanes (32, 35). BAM, a chemical in the class of haloamides and another emerging class of DBPs, was detected in distribution system samples at concentrations up to 7.6 μg/L (35). TCBQ, which was able to bind despite DCBQ being unable to, has also been detected in drinking water samples at ng/L levels and is part of the class of halobenzoquinones which are suspected bladder carcinogens (36). Effectively, the generally low-levels of individually detected DBPs can logically be considered to contribute to a higher concentration of the total DBP presence in disinfected waters.

Limited structural trends were observed between the DBPs tested, with no trends appearing based on molecular weight or size. This is most likely due to the large and accommodating binding pocket, which is able to accept ligands in multiple binding modes (4). NDMA, a commonly studied emerging contaminant and a chloramine byproduct, also showed no binding affinity. Aromatic ring structures, with the exception of DCBQ, were all able to bind which is a common outcome with these chemicals because they are more structurally similar to the aromatic rings of the natural estrogens, such as E2 and estrone.

The group of chemicals selected for this study was based on their structural diversity as well as suspected reproductive and other toxicity endpoints. For example, BDCM, a regulated DBP, was shown to induce a toxicogenomic response in embryo development specifically in the estradiol pathway, at ppb (μg/L) levels when the exposure occurred during embryo development. TCP has been shown in previous studies to cause disruption through the retinoid X receptor, which is another member of the same nuclear receptor superfamily as the estrogen receptor (37). 2-CP, which was the weakest binder of the estrogen active compounds in this study, has been shown to have dose-dependent toxic and genotoxic effects in bacteria, fish and human cell lines previously (38). Another chemical with established toxicity is DBAN, shown in this study to have a log[IC_{50}] value of -2.84 ± 0.328 M and has been identified as capable of inducing DNA damage and altering tumor-initiating genes causing hepatotoxicity (39). In all of these instances, the measured estrogenic activity of the individual compounds adds to the body of knowledge of the overall risk to these specific DBPs, as these chemicals are shown to induce multiple toxicity pathways.

As binary mixtures with 4-NP these chemicals showed binding affinities in the range of 1880 μM for TBNM to 3.7 μM for DIAA. The concentration addition values, which crossed from below 1 (synergistic) to above 1 (antagonistic) when standard deviations of the individual and binary IC_{50} values were taken into account, support the assumption of additive activity with these compounds to the receptor. The mixtures here are particularly important since 4-NP has been detected in waterways as a byproduct of surfactant degradation and largely recalcitrant in treatment processes (18, 29). Additionally, drinking water regulations are set as a maximum contaminant level for groups of a subset of the known DBPs, such as sum of the concentration of the four trihalomethanes (including the listed BDCM), the sum concentrations of 5 haloacetic

acids, and bromate, and all at sub-micromolar levels. The ability of 7 of the tested, but unregulated, DBPs to bind to the estrogen receptor in an additive manner suggests that unregulated compounds even well below their IC_{50} concentrations in water, could still account for some of the observed estrogenic activity.

This study identified new DBPs capable of targeting the estrogen receptor both individually and in mixtures. These compounds have previously been shown to be toxic for other endpoints and, therefore, their ability to also impact the estrogen pathway further emphasizes the risk from exposure. Additional work needs to be completed, however, to show how the binding of DBPs to the estrogen receptor will influence gene transcription in a tissue-specific manner, as the binding nature with the estrogen receptor has been established as variable and dynamic (4). Specifically, chronic low-level environmental exposure to these compounds is also of concern, and their implications on long-term gene transcription and disease progression should be investigated. Other chemicals, such as flame-retardants, have been shown to have similarly safe levels in toxicological studies but result in adverse health effects when long-term exposures occur (40). Future research should include an expansion of the structural groups in this study to include other compounds from the most potent groups identified, namely halobenzoquinones and nitromethanes.

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CHAPTER 4: OPTIMIZATION STUDIES FOR ENDOCRINE ACTIVITY REMOVAL IN A VERTICAL FLOW STALITE FILTER

4.1. Introduction

Wastewater treatment is designed to remove the biological activity and chemical contaminants inherently present. The treatment steps vary depending on the nature of the influent and the capabilities of the system, with the goal of producing effluent water that can be released back into the environment without causing harm. As water scarcity increases, however, efforts to expand the reuse of this treated wastewater are being developed (1). In designs for decentralized domestic wastewater treatment systems, the removal of chemical and microbial contaminants would have to be considered if the water is to be reused for agricultural or other purposes (2). Vertical sand filters and constructed wetlands are two types of treatment that can be applied in rural settings and evaluated for potential reuse (3-6).

Common contaminants found in wastewater treatment plant and septic tank effluents, receiving streams, and stormwater runoff include personal care products, pesticides, and hormones (7). Many of these compounds are recalcitrant and can, therefore, persist in the environment into which they are disposed, potentially causing harm to aquatic life or entering drinking water treatment plants downstream (8). Although at low micro- or nanomolar levels in wastewater (in the microgram to nanogram per liter range) these compounds are environmental contaminants of concern because of their chronic exposure to aquatic organisms or humans (9-12). In particular, endocrine disruption is a known area of concern for both raw and treated wastewater (11, 13, 14). Endocrine disruptors, which include hormones, plasticizers, and

pesticides, are able to mimic natural hormones and influence signaling and gene transcription, resulting in diseases such as breast and prostate cancer as well as infertility (15-17). The risk of exposure to these compounds also exists for aquatic life where intersex, fecundity, and birth defects are of concern (15, 18).

Besides the sand filter design mentioned in Chapter 1, section 1.2, other onsite constructed wetland wastewater treatment options include subsurface flow (SFCWs), free water surface (FWSCWs), and vertical flow constructed wetlands (VFCWs). SFCWs and FWSCWs have a tendency towards anaerobic conditions while VFCWs have a larger aerobic zone (19). These Hybrid Constructed Wetland systems utilize multiple treatment environments to enhance wastewater treatment, with various goals and related performances. Generally, constructed wetlands contain shallow ponds or beds in which emergent wetland vegetation grows, and a constant water level can be maintained. Similar to vegetative sand filters, the microbes present will be attached to the root structure of vegetation or soil and help to increase the aerobic area of treatment depending on the depth, saturation, and other factors, while the plants can also uptake contaminants. Loading rates and hydraulic retention times can be tailored depending on the types and concentrations of contaminants. FWSCWs have inflow into a basin, generally 2-3 ft or up to 4 ft deep, which is planted with dense vegetation from which the treated water is released after about 3 days. The major difference between vegetative sand filters and CWs is the water level, which changes how the aerobic portion is maintained and the biological oxygen demand (BOD), and subsequently how effectively the carbon and nitrogen are treated. To maintain some level of aeration, SFCWs and FWSCWs require large amounts of land to be in contact with air, which is a limitation of their use when compared to vegetative vertical flow sand filters. While these systems receive spaced doses of water and rely on that spacing to keep some dissolved oxygen

from the air in the upper levels of the system, CWs use plant systems and dissolution into the water layer to maintain an aerobic portion, which is where aerobic microbes exist and are needed for nitrification. Anaerobic portions of both CWs and vegetative sand filters are where denitrification occurs by heterotrophic bacteria, but this is most effective when there is enough organic content present. In both types of systems, recirculation of effluent allows for denitrification followed by nitrification (breaking down organic carbon and ammonia), then denitrification again to remove the remaining N by releasing N_2 (19). Alternating between aerobic and anaerobic stages, which is increasingly common in hybrid systems, significantly aids in nitrogen removal and, therefore, is becoming a more widely used process (19, 20).

VFCWs are also highly efficient at removal of emerging contaminants because they operate under aerobic conditions and require less space as a result (21). Because VFCWs operate with a similar dosing scheme to vegetative sand filters, the hydraulic retention time is only a few hours compared to a few days for horizontal flow constructed wetlands (HFCWs), like those discussed above. In a study by Matamoros and colleagues, VFCWs were compared to unplanted sand filters (SFs) and flooded systems, such as HFCWs, for their ability to remove pharmaceuticals (22). HFCWs, including SFCWs and FWSCWs, have lower levels of oxygen than VFCWs or vegetative sand filters and, therefore, require a longer contact time for pollutants to be degraded by microbial communities since a smaller population of aerobic microbes and smaller concentration of O_2 for consumption will exist (22). The researchers found that the VFCW was more robust over time, with less impact caused by overloading conditions, and that the presence of vegetation and a specific hydraulic loading rate were crucial for the removal of pharmaceuticals and personal care products, BOD_5, and ammonia from the wastewater (22). Overall, VFCWs have also been shown to be more effective for wastewater treatment than other

constructed wetland configurations, including SFCWs and unplanted sand filters. Ultimately, introduction of aerobic periods by maintaining a specified hydraulic loading rate and retention time that alternates with anaerobic periods is understood to be important for the treatment of chemicals of emerging concern. These periods allow for changes in the microbial communities, dissolved oxygen (DO) concentration, and the time that compounds are in contact with the microbial communities themselves, which make VFCWs and vegetative sand filters possibly more efficient for emerging contaminant treatment than traditional HFCWs or FWSCWs.

The Jordan Lakes Business Center (JLBC) is a small business park that includes a school and totals around 60 people in Apex, North Carolina, in which a vegetated sand filter connected to a greenhouse is used to treat its domestic wastewater. This site is designed to include a sedimentation tank, from which the wastewater flows into the anaerobic zone of a vegetated sand filter and is allowed to move down through the soil before being collected and reapplied to the aerobic zone (23). The water then passes through the anaerobic zone again before being pumped to the greenhouse, which grows tropical plants, before disinfection and reuse for toilet flushing and landscaping. This system, which includes combinations of vegetated sand filters and subsurface flow constructed wetlands, is the reference site for this study and includes a combination of treatment types that provide a range of physical, chemical, and biological processes. While primarily aerobic vegetated sand filters are effective for nitrification, phosphorus storage, and the oxidation of organic compounds, constructed wetlands can be hypoxic or anaerobic and are instead useful for denitrification, physical removal, and the anaerobic metabolism of organic compounds (24, 25). The domestic wastewater at this hypbrid wetlands site was used for the duration of this work, after it had passed through the anaerobic-aerobic-anaerobic stages of circulation of the vegetated sand filter but before it has been

disinfected or reached the greenhouse. The addition of wastewater to this system occurs in pulses, which aids in the dispersion of oxygen in the aerobic layer and helps to prevent clogging from excessive exopolymeric substances, which are produced by the biofilm present on the packing material (26).

The use of vertical flow filters for wastewater treatment has been well studied, as discussed in Chapter 1, section 1.2, but the optimization of these systems for the removal of a range of persistent pollutants has not yet been accomplished. At the JLBC, the system uses Stalite as the packing medium. Stalite is a lightweight, expanded-slate aggregate that has a high porosity allowing for biofilm formation with the potential to act as a suitable packing material in a vertical flow filter, as an alternative to commonly used substrates such as gravel and soil. It is an example of a locally sourced, cheap material that can serve as a guide for expanding on the materials used in constructed wetlands for wastewater reuse. In this study, varying sizes of this material were packed into three sequential columns for the treatment of a domestic wastewater collected from the JLBC. The removals of seven persistent wastewater pollutants that are also commonly found in surface waters were measured using liquid chromatography- electrospray ionization-mass spectrometry (LC-ESI-MS), along with the effects on endocrine activity. The concentrations of the target chemicals were elevated through the addition of standards at the start of experiments in order to allow for their detection after treatment. The target chemicals in this study were selected to represent a wide range of wastewater pollutants with varied degradation pathways. Included were the herbicide atrazine, the stimulant caffeine, the anti-epileptic carbamazepine, the insect repellent DEET, the hormone 17β-estradiol, the surfactant degradation product nonylphenol, and the antimicrobial agent triclosan. These compounds are found in microgram to nanogram per liter ranges in wastewater effluent, have varied known degradation

pathways, and are considered emerging contaminants of concern. Some of these chemicals are also known to cause endocrine disruption, with 17β-estradiol acting as a particularly potent estrogen.

To determine the most effective treatment for this column system, wastewater was dosed with the target chemicals and added at unsaturated dose intervals of 8, 12, 24, 36, 48, and one saturated 8h interval. These experiments varied the amount of water added to each column based on that time period, and were tested using the three sequential columns acclimatized for more aerobic and more hypoxic levels of oxygen, then run in the order of hypoxic-aerobic-hypoxic and aerobic-hypoxic-aerobic. The optimal dose interval of a Stalite-packed vertical flow filter was determined based on the overall removal of the target chemicals and the measured reduction in estrogenic activity.

4.2. Materials and Methods

4.2.1. Chemicals

Atrazine (99.1%), DEET (98.1%), anhydrous caffeine, and D(+) glucose were purchased from Fluka (St. Louis, MO). From Sigma Aldrich (St. Louis, MO), citric acid monohydrate, ACS grade methyl-tert-butyl ether (≥99%), chlorophenol red-β-Dgalactopyranoside, cupric sulfate pentahydrate, thiamine, erythromycin, pyridoxine, D-pantothenic acid hemicalcium salt, potassium hydrogen phthalate (99.95%), 4-nonylphenol (technical grade), 17β-estradiol (98.1%), and triclosan were acquired. Potassium dihydrogen phosphate, potassium hydroxide pellets, liquid ammonium hydroxide (ACS grade), casamino acids, agarose, anhydrous dextrose, Luria-bertani (LB) powder, and ethanol (absolute) were purchased from Fisher Scientific (Fair Lawn, NJ). From MP Biomedicals (Santa Ana, CA), atenolol, iron(III) sulfate, L-aspartic acid, and the FastDNA Spin Kit for Soil were purchased. L-threonine, magnesium sulfate septahydrate,

inositol, carbamazepine (99%), and adenine sulfate were from Acros Organics (New Jersey, NJ), and biotin was from Kodak (Rochester, NY). Ammonium sulfate was from Mallinkrodt (Paris, KY), adenine from Alfa Aesar (Ward Hill, MA), and bacto agar and Difco yeast nitrogen base from BD (Sparks, MD). ACS grade methanol was from Honeywell Burdick & Jackson (Muskegon, MI), potassium nitrate was from EM (Gibbstown, NJ), and laboratory grade water (LGW) (> 17 MΩ) prepared using a Dracor (Durham, NC) system that passes chlorinated tap water through ion exchange resins and activated carbon before 0.2 μm filtration. HPLC grade methanol was purchased from VWR (Radnor, PA). SYBR SAFE DNA Gel Stain (Invitrogen, Carlsbad, CA), agarose (Fisher, St. Louis, MO), and some tests for the MP Biomedicals FastDNA Spin Kit for Soil (Santa Ana, CA) were generously provided by Dr. David Singleton.

4.2.2. Solid Phase Extraction (SPE)

SPE was used to concentrate the analytes in effluent samples for chemical and endocrine activity. This was completed for all column 3 and the final column 1 and 2 time points for each study, as previously described (27). Briefly, to concentrate the target analytes, 200 mg/6 mL Strata-X, 500 mg/3 mL Strata-SAX, and 200 mg/3 mL Si-1 cartridges (Phenomenex, Torrence, CA) were used. The Strata-X cartridges were conditioned with 3 mL methyl tert-butyl ether, 3 mL methanol, and 6 mL LGW, while the Strata-SAX cartridges had an additional wash of 3 mL of 0.2 M citric acid and were connected to the top of the Strata-X cartridges. Each new JLBC unspiked wastewater was measured into 250 mL volumes and spiked with increasing concentrations (5 – 150 μg/L) of each of the target analytes using the standards from the column study to obtain a standard addition calibration curve. 250 mL, in duplicate for each sample, of the filtered wastewater samples were spiked with either 50 or 100 μg/L atenolol internal standard (consistently between dose interval extraction) and then passed over the conditioned cartridges

on a vacuum manifold at a flow of less than 8 mL/min. The SAX cartridges were then discarded and the Strata-X cartridges were washed with 3 mL of 40% HPLC grade methanol in LGW, 3 mL of LGW, and 3 mL of 10% HPLC grade methanol and 2% ammonium hydroxide in LGW, then dried under vacuum for 1 h. Additionally, 200 mg/3 mL Si-1 cartridges were conditioned with 2 mL of ACS grade methyl tert-butyl ether: HPLC grade methanol (9:1) and then the Strata-X cartridges were eluted slowly with 5 mL of the same mixture directly onto the Si-1 cartridges and collected directly into conical vials. These vials were subsequently placed on a heating block at 40 °C and the extracts blown down to dryness under a gentle flow of high purity N_2 (Airgas, Radnor, PA) using a Pierce (Rockford, IL, USA) Reacti-Vap Model 18780. Samples were reconstituted into 2 mL HPLC grade methanol for chemical analyses. Samples that were to be used for both chemical and estrogenic activity analyses were split equally following elution and then blown down separately in order to reconstitute in 1 mL of methanol for chemical analysis and 1 mL of ethanol for estrogenic activity analysis, and stored at -20°C in 2 mL amber vials (Fisher, St. Louis, MO).

4.2.3. Chemical Analysis

Reconstituted SPE extracts were diluted by pipetting 20 µL into 1 mL HPLC grade methanol containing 100 µg/L of erythromycin and analyzed using reverse phase liquid chromatography with a 3.0mm x 50 mm x 1.7µm ACQUITY UPLC CSH (Charged Surface Hybrid Particle) column (Waters, Milford, MA) connected to a Thermo Scientific (Waltham, MA) TSQ Quantum Ultra triple-quadrupole mass spectrometer (LC/MS). Compound detection was achieved via electrospray ionization for ion transitions in positive mode for atrazine (m/z: 216 ➔ 146), caffeine (m/z: 195 ➔ 138, 195 ➔ 110), carbamazepine (m/z: 237 ➔ 194), DEET (m/z: 192 ➔ 119), and transitions in negative mode for 17β-estradiol (m/z: 271 ➔ 145),

nonylphenol (m/z: 219 ➔ 133), and triclosan (m/z: 287 ➔ 142). The column temperature was maintained at 30 °C and the flow rate was kept at a constant 0.20 mL/min. A gradient elution was employed with the mobile phase initially consisting of 99.9% of a 0.05% ammonium hydroxide solution in LGW water and 0.1% HPLC grade methanol, the latter then increased to 5% by 0.3 minutes and held for 1.4 minutes, then increased to 50% over 1 minute and held for 1.5 minutes. The methanol concentration was then increased to 80% over 1.7 minutes and held for 1.6 minutes, increased again to 99.9% over 1.4 minutes and held for 5 minutes. At this point, the methanol was decreased to 0.1% over 0.7 minutes and then held, amounting to a total method run time of 21 minutes.

4.2.4. Yeast Estrogen Screen

Estrogenic activity of the ethanol-reconstituted SPE extracts was measured using the yeast estrogen screen (YES) assay. These were brought to room temperature and diluted into 10% ethanol in LGW and run on the assay as previously described (28) and given in Appendix E, with the following changes. LGW was filtered sterile prior to its use for preparing chlorophenol red-β-D-galactopyranoside (CPRG), the 10% ethanol solution, and chemical stocks because of prior issues with microbial contamination. Yeast colonies were grown on solid Ura-Trp agar plates to prevent contamination of this yeast strain because it is differential and selective and will not allow for the growth of non-targeted strains. The column-treated wastewater samples that were tested were the source (unspiked), spiked, early, mid, and final time points. The early, mid and final time points are relative for each study due to the difference in the total number of collected samples; for example, the 36h study has fewer samples than the 8h study due to the longer dose interval.

The cytotoxicity absorbance (620 nm), which is a measure of the turbidity of the suspension, from the negative control (10% ethanol), and the cytotoxicity absorbance (620 nm) measured at each concentration in the assay were subtracted from the absorbance reading (540 nm) that resulted from estrogenic chemicals. The estrogenic samples bound to the estrogen receptor in the nucleus of the yeast, which then bound to the estrogen response element and allowed for transcription of *lac*-Z, the reporter gene. *Lac*-Z causes the expression of β-galactosidase, which hydrolyses the galactosidic bond in CPRG, causing a color change from yellow to red, which can be measured by absorbance at 540 nm. Duplicate absorbance measurements of each well from the samples and the positive control (E2) standard plates were graphed using GraphPad Prism 4.3 (GraphPad Software, La Jolla, CA). The corrected absorbance values were plotted against the volume of extract added to each well and fitted with a sigmoidal dose curve (variable hillslope), which gives an EV_{50} (effect concentration at 50% activation) in terms of liters of sample added per well. The E2 standard was prepared in 10 % ethanol and diluted across 11 wells which, after processing, generate absorbance values which are plotted against concentration in terms of ng/well, producing an EC_{50} value (ng/well). Results of analyses are presented as an estradiol equivalent factor (EEF), shown in Equation 4.1 (29). Samples were run in triplicate and results are presented as the mean ± standard deviation.

$$EEF\ \left(\frac{ng}{L}\right) = \frac{EC_{50}\ (\frac{ng}{well})}{EV_{50}(\frac{L}{well})} \qquad (4.1)$$

The results of the YES assays, presented as estradiol equivalents (EEQs) (ng/L), were then compared to results of predicted EEQs, which are the result of the potency of each individual compound (measured by EEF) multiplied by the concentration of that compound obtained from the chemical analysis in that sample (see Equation 4.2). These comparisons are

used to indicate the difference between the measured estrogenic compounds and the observed estrogenic activity of the wastewater.

$$EEQ = \sum_{i=1}^{n} EEF_n \cdot [Concentration]_n \quad (4.2)$$

4.2.5. Other Analyses

Ammonia, nitrate, and nitrite measurements used Hach® kit methods numbers 64, 60, and 54, respectively (Hach; Loveland, CO). Samples were first filtered using a Whatman GF/D (Little Chalfont, UK) (2.7 μm, 4.7 cm) glass fiber filter stacked on top of a Whatman GF/D (Little Chalfont, UK) 934-AH (1.5 μm, 5.5 cm) glass fiber filter in a vacuum filtration apparatus, then the filtrate was filtered again using a 0.45 μm, 47 mm nylon filter (Fisher, St. Louis, MO). The final filtrate was then diluted by pipette into LGW to fall within the kit concentration range and protocols for the kits were followed. For TOC, TN, DOC, and DN measurements, samples were diluted by pipette with LGW into an estimated range of 0.5 – 10 mg/L as C or N as appropriate based on a reading of the source wastewater. Standards of 0.5, 5, and 10 mg/L as carbon and nitrogen were prepared from 1,000 mg/L stock solutions of potassium hydrogen phthalate as C, and potassium nitrate as N. These stocks were stored for up to 2 months at 5 °C. The samples and standards were acidified to pH 2-2.5 using 2N HCl and measured using a TOC-V organic carbon analyzer and TNM-1 total nitrogen measuring unit (Shimadzu; Atlanta, GA).

Total coliform and *Escherichia coli* were measured as colony forming units (CFU) for the source wastewater and final effluent from each study using IDEXX Colilert Quanti-Tray kits (Westbrook, ME). Unfiltered samples were diluted 1:10 into LGW, combined with the Colilert chemical indicator, and poured into 96 well trays which were then incubated (Thermo Electron Corporation, Marietta, Ohio) for 24 h at 37 °C. Wells that turned yellow were considered positive for total coliform and those that were yellow and then fluoresced blue under UV light

were considered positive for *Escherichia coli* by the IDEXX protocol and converted into CFU using the IDEXX most probable number (MPN) table.

4.2.6. Column Study

4.2.6.1. Column Setup

The columns used in this study were filled with wastewater collected from the JLBC, which is a small (~60 person) site that contains a school and small businesses with onsite wastewater treatment through a constructed wetland. A schematic of the JLBC treatment setup is shown in Figure 4.1, which includes treatment with stacked vegetated filters and anaerobic wetlands. The wastewater for use in this study was collected in batches of 20 - 50 L from after the VSFs and anaerobic wetlands after partial treatment, which included passage through a settlement tank and vegetated constructed wetland, then was stored at 5 °C for use in the column study. It still had biological activity, organic matter and nitrogen content, but because of low or non-detectable levels of our target compounds the water was spiked with a mixture of these chemicals before use. It was expected that the small size of this site would allow for fairly consistent wastewater quality throughout the course of the study.

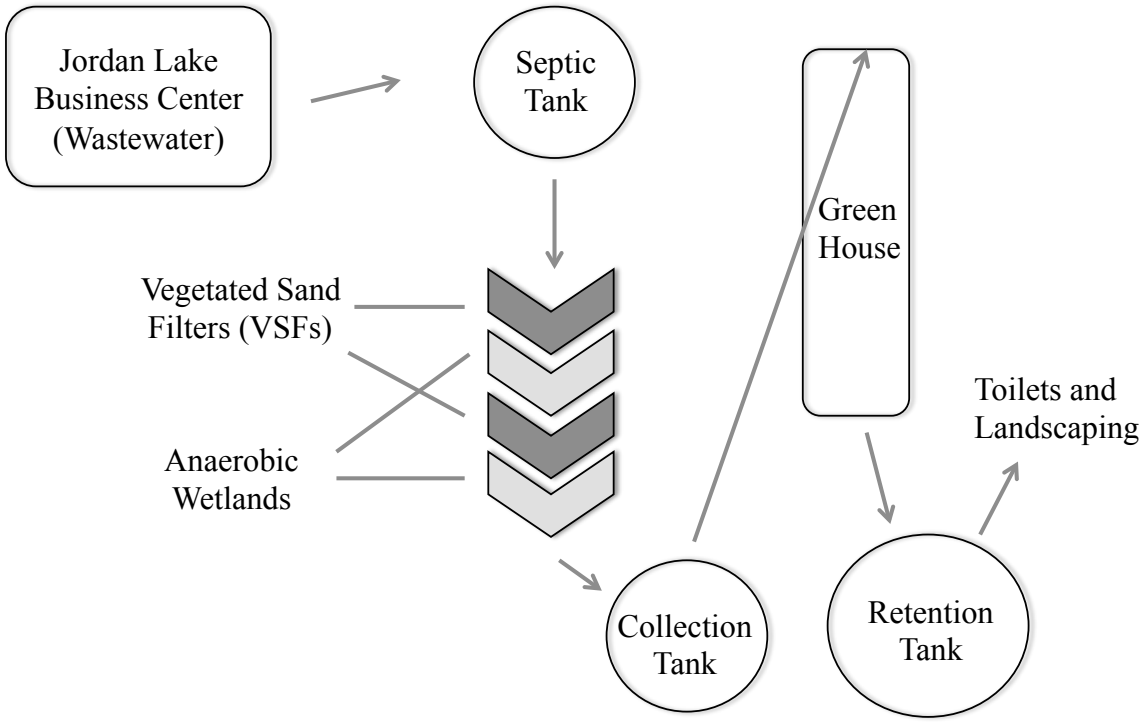


Figure 4.1. A schematic of the Jordan Lake Business Center on-site reclamation system in NC from which wastewater was collected.

In this study, three 110 cm x 10 cm stainless steel columns were packed with 3/8", 5/16", and 'sand-sized' Stalite (Gold Hill, NC) sizes according to Figure 4.2. Each was equipped with ports at the bottom of the column to drain effluent and three ports along the sides of the column from which to collect core samples. To prevent biofilm growth in the column effluent during the experiments, the columns were wrapped with heat tape and insulation to maintain a temperature of 18-20 °C, then kept in a cold room at 5 °C. The cooled constant temperature room allowed for the collection of effluent from the columns directly into flasks that were kept cold during the period of the experiments and maintained the integrity of the sample without promoting further bacterial growth or chemical degradation in the water held outside the columns.

The wastewater was periodically changed by draining the columns and refilling with new wastewater over the course of more than four months to allow for the formation of a biofilm before the preliminary round of experiments (study 1) and for another 6 months between the first

and second round (study 2) of experiments. The column order for study 1 is presented in Appendix C, Figure C.1. For study 2, the order of the columns were run as presented in Figure 4.2, with the first and third columns aerobic and the second hypoxic.

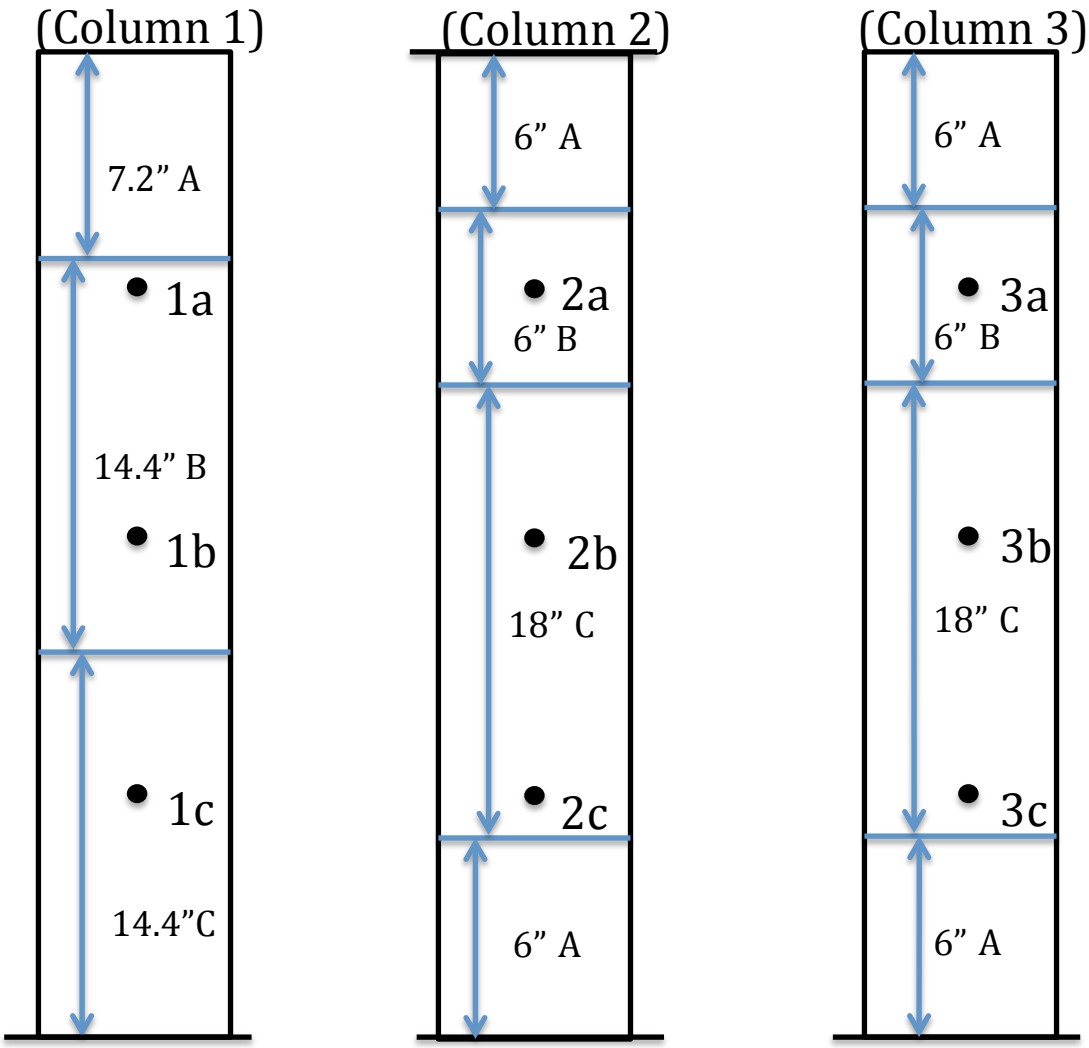


Figure 4.2. Diagram of the columns for study 1 packed with layers of (A) 3/8", (B) 5/16", and (C) sand-sized Stalite® material for the dose interval studies showing sampling ports 1a-c, 2a-c, and 3a-c. Columns 1 and 3 are kept aerobic and open to the air, while column 2 is kept hypoxic and capped.

After inoculation of the columns with periodically changed wastewater, bacterial presence on the Stalite was tested by taking sterile swabs of the tops and mid-points of each of the columns and inoculating them in LB broth overnight at 35 °C. Cloudiness, which indicated

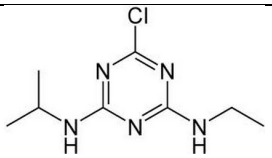
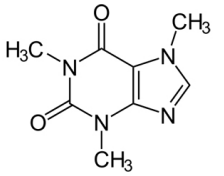
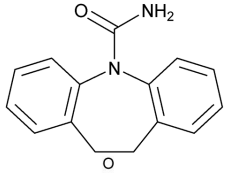
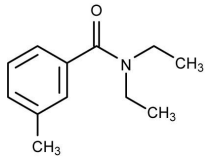
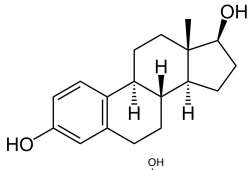
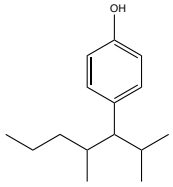
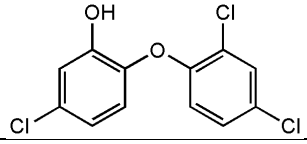
bacterial growth, was observed in all samples and, therefore, study 1 was started. After inoculation with wastewater following study 1, it was necessary to confirm whether there was still a biofilm present on the Stalite in the columns. Core samples of about 25 g were removed from ports 1a-c, 2a-c, and 3a-c with a stainless steel coring device that was wiped with ethanol between each sample and the core samples frozen overnight at -80 °C. The samples were allowed to thaw before DNA was extracted using the MP Bio FastDNA Spin Kit for Soil according to the protocol provided by the manufacturer and included in Appendix E. The extracts were then run on a 1% agarose gel and compared to a DNA ladder to determine whether any genomic DNA was present.

In a preliminary set of dose interval experiments, referred to as study 1, the columns were set to mimic an operating constructed wetlands field site in Chatham County, NC that alternates between zones in the order hypoxic-aerobic-hypoxic. The dose intervals in study 1 were each run for 14 days to allow for the establishment of a period in which the columns gave consistent removal of the target compounds, which were analyzed as previously described in section 4.2. This study was done in preparation for later work that would utilize the column study data. However, due to a change in the field site a second set of dose interval experiments, study 2, was undertaken. The primary difference between the two studies can be described as follows. In study 1, the wastewater at the field site was dosed to the wetland midway through the bed material in a hypoxic zone and then recirculated to dose a vegetated vertical flow filter before passing through the hypoxic zone for a second time, allowing for better removal of nitrogen species. In study 2, the wastewater was introduced to the vegetated vertical flow filter first, which is aerobic, before passing through a hypoxic zone and then recirculated.

4.2.6.2. Dose Intervals

The target chemicals were prepared as 1 g/L stock solutions in either HPLC grade methanol or LGW, depending on solubility, and then stored in amber vials at 5 °C for up to 5 months. The relevant properties of these chemicals are presented in Table 4.1, and they were spiked into the wastewater at 100 μg/L and mixed for each dose immediately prior to adding the water to column 1 for each time point during each dose interval. To ensure low oxygen levels in the hypoxic column 2, the effluent from column 1 was flushed with high purity argon (Airgas, Durham, NC) through a stainless steel transfer line for 30 min prior to addition to column 2, while the doses to be added to columns 1 and 3 were flushed with industrial grade air connected to a stainless steel transfer line (Airgas, Durham, NC) to increase the oxygen content. This procedure was done exactly the same for study 1, except that the doses to be added to columns 1 and 3 were flushed with high purity argon (Airgas, Durham, NC), while the dose to be added to column 2 was flushed with industrial grade air (Airgas, Durham, NC).

Table 4.1. Test chemicals used in the column and batch studies.

Name (Abbreviation)	Solubility (Solvent Used)	Use	MW (g/mol)	Structure
Atrazine (ATZ)	18 g/L[a] (Ethanol)	Herbicide	215.69	
Caffeine (CAF)	2.16E04 mg/L[b] (LGW)	Stimulant	194.19	
Carbamazepine (CBZ)	18.0 mg/L[c] (LGW)	Anti-epileptic	236.27	
N,N-Diethyl-meta-toluamide (DEET)	Very Soluble[d] (Ethanol)	Insect Repellent	191.27	
17β-estradiol (E2)	Very Soluble[e] (Ethanol)	Steroid	272.38	
4-Nonylphenol (4-NP)	Soluble[f] (Ethanol)	Surfactant Degradation Product	220.35	
Triclosan (TCS)	Readily Soluble[g] (Ethanol)	Antimicrobial	289.54	

[a] Handbook of Data on Organic Compounds, 1994. (30)
[b] Handbook of Aqueous Solubility Data Second Edition, 2010. (31)
[c] United States Environmental Protection Agency, 2009. (32)
[d] CRC Handbook of Chemistry and Physics 95th Edition, 2015. (33)
[e] CRC Handbook of Chemistry and Physics 88th Edition, 2008. (34)
[f] Hawley's Condensed Chemical Dictionary 15th Edition, 2007. (35)
[g] The Merck Index – An Encyclopedia of Chemicals, Drugs, and Biologicals. 2006. (36)

A summary of the dose interval experiments is shown in Table 4.2. For all intervals except the 8h saturated, the columns were allowed to drain freely from the port on the bottom of

the column into 2 L collection flasks after dosing. For the 8h-saturated interval, which represented a flooded wetland, the column ports were closed for the duration of each dose period and then allowed to drain before the addition of the next 8h dose. The intervals maintained a 1.61 L addition per 24 h period, which is the equivalent of 2 gal/ft^2/day for dose interval studies 8, 12, and 24 h but 1.61 L for the 36 or 48 h dose intervals.

Table 4.2. The batch loading intervals and volumes used for each of the column studies.

Dose Interval (h)	24 h Volume Added (L)	Study1 (Preliminary)	Study 2	Spike Concentration (µg/L)	Volume Per Dose (L)
8 Saturated	1.61		✓	100	0.537
8	1.61	✓	✓	100	0.537
12	1.61		✓	100	0.805
24	1.61	✓	✓	100	1.610
36	1.61[a]	✓	✓	100	1.610
48	1.61[a]	✓		100	1.610

[a]Volume added at the period of each interval, every 36h or 48h, respectively.

The doses were added in sequence, with the collected effluent from column 1 being added to column 2 and effluent from column 2 being added to column 3 at each dose time point. For study 1, the first and third columns were hypoxic, while column 2 was aerobic, as shown in Appendix C. This preliminary study was run for 14 days to measure the efficacy of the columns for removing the target chemicals. For study 2, the length of the study was shortened to 9 days based on the time period observed in study 1 to reach consistent measurements of the target compounds. Study 2, which mimics the JLBC constructed wetlands and the field site ultimately used for other parts of a larger study, was run with columns 1 and 3 as aerobic while column 2 was hypoxic. Sample doses were added slowly by funnel to the center of the column over 3-4 min in order to minimize potential boundary flow effects. Due to the size of the Stalite gravel compared to the column diameter and the slow rate of dose addition it is expected that there was minimal impact from boundary effects.

The entire sample volume from column 3 was collected while 50 mL aliquots were collected from columns 1 and 2 for each day and from all three columns in full for the final day of each dose interval. The effluent from column 3 for each time point was collected as an aggregate of all doses that combined for a full 1.61 L dose; for example, three 8 h dose points or two 12 h dose points. Due to the difference in time intervals and the lag time from collecting a full 1.61 L from column 3, the collected samples are labeled as time point (T) and column (C); for example, T2C3 is time point 2 column 3 effluent. The effluents that correspond to the final time points are labeled as T_{Final} because of the difference, again, in the time for the first full dose to exit column 3. These effluent samples were filtered using two Whatman GF/D (2.7 μm pore size, 4.7 cm diameter) and 934-AH (1.5 μm pore size, 5.5 cm diameter) glass fiber filters (Little Chalfont, UK) placed together, in line, in a vacuum filtration apparatus, and the filtrate subsequently filtered using a 0.45 μm pore size, 47 mm diameter nylon filter (Fisher, St. Louis, MO). Filtered samples were then used for their dissolved organic carbon (DOC), dissolved nitrogen (DN), ammonia, nitrate, nitrite analyses as well as for solid phase extraction (SPE) of the for target analytes and estrogenic activity. Unfiltered samples were used for the total organic carbon (TOC) and total nitrogen (TN) analyses. Raw unspiked wastewater was treated the same as samples for each interval for comparison. Samples were stored at 5 °C in amber bottles until analyses, which occurred during the week following the conclusion of each dose interval experiment.

4.2.6.3. Stalite Extractions and Estrogenic Activity

Core samples of an estimated 30 g Stalite were removed from column ports 1a-c, 2a-c, and 3a-c after the final dose interval of study 2 was completed. The Stalite was allowed to dry overnight at room temperature and then 20 g of each sample was weighed and crushed to a

coarse powder with a mortar and pestle. The loose powder was then transferred to a beaker, 10 mL of HPLC grade methanol was mixed in and the contents sonicated (Fisher Scientific, St. Louis, MO) for 45 min. The sample was allowed to settle and the supernatant liquid was transferred by pipette to a new flask. The process was repeated on the solid with another 10 mL of methanol. The methanol aliquots were combined and blown down to dryness under a gentle flow of high purity N_2 (Airgas, Radnor, PA) using a Pierce (Rockford, IL, USA) Reacti-Vap Model 18780 while on a heating block at 40 °C. The samples were then reconstituted in 1.5 mL of absolute ethanol and used for the yeast estrogen screen assay as previously described. A control sample of unadulterated Stalite (20 g) was extracted in the same manner to measure any background estrogenic activity that could be attributed to the solid itself.

4.2.7. Batch Study

To evaluate the contribution of sorption to the bed material as compared to biodegradation from the biofilm for the removal of the target chemicals, a batch study was undertaken. Stalite was first inoculated with JLBC wastewater and and aerated using Top Fin Aquarium air pumps (Petsmart, Phoenix, AZ) for 3 months in a large glass canister kept inside an incubator at 25 °C (Fisher Scientific, St. Louis, MO) with periodic recharges of the wastewater in order to allow for a biofilm growth. Two pairs of 1L acid-washed amber bottles were then filled with 120 g of Stalite, then with 600 mL of JLBC wastewater, to which 100 µg/L of the 7 target chemicals were added and mixed. One pair of bottles was then flushed with industrial grade air (Airgas, Radnor, PA) for 30 min to achieve aerobic conditions while the other was flushed with high purity argon (Airgas, Radnor, PA) for 30 min to decrease the dissolved oxygen to approach a hypoxic environment. The hypoxic bottles were then capped and sealed with Teflon tape. To determine adsorption effects only, another pair of amber bottles with

autoclaved wastewater (600 mL) was first autoclaved then prepared with autoclaved Stalite (120 g) from the same inoculation with wastewater as the aerobic and hypoxic bottles batch to determine sorption to the biofilm. These were then spiked at the same 100 µg/L concentrations as the previous bottles. A final pair of bottles contained autoclaved Stalite (120 g) and LGW (600 mL) along with 100 µg/L of each of the 7 target compounds for comparison of removal by sorption to colloidal carbon in the wastewater. All bottles were stored in an incubator at 25 °C on a slow shaker at ~60 rpm (Lab-Line Instruments, Melrose Park, IL) for the duration of their time points. At 1h, a bottle from each pair was sacrificed and another set after 30 h. The samples were immediately filtered and then processed with SPE for chemical analyses as described in sections 4.3.4 and 4.3.5, respectively. A second batch study was run with 600 g of Stalite in contact with 600 mL JLBC wastewater, containing 100 µg/L spikes of the target chemicals with sampling time points at 1h and 24 h for the hypoxic and aerobic wastewater samples and 24h for the LGW and autoclaved wastewater samples. The 1h point was to determine whether there was any rapid degradation or sorption to the Stalite, while the 24h time point represented the full contact time of the 8h saturated dose interval, which was the longest contact time in the column study.

4.3. Results and Discussion

4.3.1. Column Study

Prior to running the dose intervals for the column studies, a biofilm was confirmed on the Stalite packing material from DNA extractions of inoculated Stalite using the FastDNA Spin Kit for Soil. The first extraction showed genomic DNA present in the samples collected from ports 3b and 3c, while a second extraction after further wastewater inoculation showed genomic DNA in all of the remaining ports except 3a. At this point, the columns were considered conditioned and ready for the dose experiments. One notable difference between the column systems and the

field sites in studies 1 and 2 is that the bed material in the columns is not vegetated, which is due to the limitations of running a laboratory-scale column system in a cold room. The cold temperature (5 °C) of the room was necessary to prevent further bacterial growth in the column effluent collection vessels, but the lack of fresh air, sunlight, and warm temperatures along with the limited surface area of the top of the column (10 cm) prevented planting.

4.3.1.1. Study 1

Briefly, the results from study 1, which are presented in Appendix C, show consistently high removal of the target compounds from the wastewater effluent for caffeine, 17β-estradiol, 4-nonylphenol, and triclosan often to below their limit of quantitation (10 µg/L). The total percent removal of all chemicals for the final time points of each dose interval after columns 1, 2, and 3 treatment along with the average of the percent removals after column 3 for all of the time points are presented in Table C.1. The overall removals observed for each time point of each compound are presented in Figures C.2- C.8. In these figures, the consistency of the removal of the target compounds in each study is shown for all of the target compounds in the effluent samples except for atrazine and carbamazepine, which appear to be recalcitrant. In the literature, reports for the behavior of these compounds in wastewater in relation to sorption, biodegradation, and photolysis indicate that these results are consistent with what has previously been observed (37, 38) and are discussed in more detail for study 2. The results for the compounds atrazine and carbamazepine, which showed inconsistent removal in the effluent after column 3 throughout each dose interval, are presented in Appendix C, as well. The consistency between the target chemical removal and TOC (Figures C.9 – C.11) removal of each of the time points in all of the dose intervals indicated that these experiments could be shortened from 14 days for study 2. The results for TN, DN, and DOC, indicated that the length of each dose

interval could be changed to 9 days, which is the point at which the measurements of these parameters had become stable. The 9 day dose interval is also similar to the length of other studies measuring target contaminant removal (37). The poor consistency between samples and low overall removal for atrazine, carbamazepine, and DEET for the 48 h dose interval combined with the high removal observed for the shorter dose intervals resulted in this experiment being replaced in study 2. To determine whether saturated conditions in the columns instead of free flowing from them would result in better removal, the best dose interval from study 1 was selected as both an 8 h as before and as an 8 h saturated interval.

In vertical flow filters as well as wetlands, organic contaminants can be removed or treated through four primary mechanisms: volatilization, sorption, biodegradation, and photolysis (38). In this study, the columns are made of stainless steel and kept in a dark room while running, which limits the potential contribution of photolysis, and the compounds tested for removal are non-volatile which is one reason they persist in wastewater. This leaves biodegradation and sorption as the primary potential removal mechanisms in the column studies and the results presented show that both of these mechanisms contribute to the observed removal in the dose interval experiments.

4.3.1.2. Study 2

Due to the results of the stabilization period observed for study 1, which was run for 14 days, study 2 was shortened to 9 days, which is similar to that used for previous studies of this nature (37). In study 2, the analytical limit of detection for triclosan increased during analyses, which precluded calculations of its removal during treatment. The results of the chemical analyses for study 2 are summarized in Table 4.3, below. The source wastewaters used for each dose interval had concentrations of the target analytes that were below the limit of quantitation (5

μg/L). From this method, the percent recovery for the compounds, calculated by comparing the concentrated standard extracts to the standards spiked into solvent at the same concentration factor, are 53% for atrazine, 49% for caffeine, 77% for carbamazepine, 30% for DEET, 10% for 17β-estradiol, and 81% for 4-nonylphenol.

Table 4.3. Cumulative removal of target chemicals after each column in study 2 measured for each dose interval from columns 1 (n=2) and 2 (n=2), and column 3 (n as indicated) presented as mean ± standard deviation.

Dose Interval (h)	Column	ATZ (%)	CAF (%)	CBZ (%)	DEET (%)	E2 (%)	4-NP (%)
8 Saturated	$T_{Final}C1$	38	33	18	55	> 95[#]	> 95
	$T_{Final}C2$	-1.6	> 95	0.6	> 95	> 95	92
	$T_{Final}C3$	-2.4	> 95	4.1	> 95	> 95	> 95
	C3 (Avg) n=16	3.3 ± 9.5	> 95	5.9 ± 5.8	> 95	> 95	> 95
8	$T_{Final}C1$	20	42	-11	55	84	> 95
	$T_{Final}C2$	27	> 95	0.2	> 95	> 95	> 95
	$T_{Final}C3$	27	> 95	3.8	> 95	> 95	> 95
	C3 (Avg) n=16	27 ± 6.0	> 95	1.9 ± 3.7	> 95	> 95	89 ± 13
12	$T_{Final}C1$	4.1	51	-40	53	73	> 95
	$T_{Final}C2$	13	> 95	-33	91	> 95	> 95
	$T_{Final}C3$	5	> 95	-34	> 95	> 95	> 95
	C3 (Avg) n=14	10 ± 5.4	> 95	-24 ± 11	> 95	> 95	> 95
24	$T_{Final}C1$	11	14	-15	26	66	74
	$T_{Final}C2$	24	66	-15	75	> 95	94
	$T_{Final}C3$	56	91	-4.1	> 95	> 95	> 95
	C3 (Avg) n=12	46 ± 18	82 ± 5.7	17 ± 25	79 ± 9.5	> 95	95 ± 3.8
36	$T_{Final}C1$	-14	11	-20	41	48	94
	$T_{Final}C2$	19	77	18	62	> 95	> 95
	$T_{Final}C3$	18	85	20	78	> 95	> 95
	C3 (Avg) n=8	30 ± 10	94 ± 7.1	31 ± 8.2	91 ± 9.4	> 95	> 95

[#] LOQ = limit of quantitation for analytes is 5 μg/L. Percent removals reported as >95% for concentrations below that limit.

These results show that 17β-estradiol, 4-nonylphenol, DEET, and caffeine are consistently well removed (≥ 95%) by the time they exited the final column for the 8 h saturated, 8 h, and 12 h studies. 4-nonylphenol, the product of the environmental degradation of

nonylphenol ethoxylate surfactants, was known to readily sorb to the biofilm of streambeds (39). It was also observed as somewhat recalcitrant for anaerobic degradation but that could be enhanced with increased temperature or shaking, or decreased in the presence of metals, with the overall half-life measured to be between 23.9 to 69.3 days (40), compared to 0.9 to 13.2 days for aerobic conditions (41). The observation that 4-nonylphenol was more likely to biodegrade under aerobic settings corresponds well with the observed removal from column 1 with little enhancement coming from column 2 in this study (42). For comparison, wastewater treatment plants have shown 4-nonylphenol to primarily be removed through biodegradation, with physical treatment having less of a contribution to the overall removal (43). It should be noted, however, that this column study used a mixture of 4-nonylphenol isomers, which was similar to what would exist in a wastewater treatment plant but has also been observed to have isomer-specific biodegradation, particularly under aerated conditions, with chain length and degree of branching both considered contributing factors (41).

17β-estradiol, the potent estrogen and known endocrine disruptor, was well removed in this study. Chemical analyses showed linearly improved removal from the longest dose interval to the shortest for column 1, with the 8h saturated interval showing a removal of ≥ 95%. The consistently high removal of 17β-estradiol for the overall effluent from column 3 at each time point was in line with the literature assertion that 17β-estradiol removal in wastewater treatment was primarily accomplished through biodegradation and sorption (44). The saturated interval, which had the highest removal from C1, will have the longest residence time with the biofilm compared to the other dose intervals (45). Loss of these compounds from volatilization was unlikely, as steroid estrogens including 17β-estradiol and its metabolites have low vapor pressures, ranging from 2.3 E-10 to 6.7 E-15 mmHg (46). Consistently high removal of 17β-

estradiol from these systems mirrors previous studies that observed that 17β-estradiol and its metabolite, estrone, could biodegrade under aerobic, anoxic and anaerobic conditions, although with enhanced degradation under aerobic conditions (45). Furthermore, the $\log K_{ow}$ of 17β-estradiol is 4.01, which suggests it was likely to be removed through sorption to Stalite, would also enhance removal (45, 46).

The widely used stimulant caffeine, which is frequently detected in wastewater and streams, has been established as an organic contaminant that is easily biodegraded, which is the dominant method of removal for this analyte in wastewater treatment (47-50). Additionally, in a similar unsaturated column test published in 2017 (51), caffeine was not observed in any of the column effluent, which further supports the assertion that it was easily removed from wastewater and, therefore, less of an environmental concern. Sorption also contributed to immediate removal, which was observed as a fast initial sorption velocity (2055 $\mu g\ kg^{-1}\ h^{-1}$) in one recent study (51). The efficiency of caffeine removal in these previous studies corresponds well to the observed results here, which showed high overall removals for the shorter dose intervals. Drying out of the column bed material, which might occur during the longer dose intervals, could contribute to the lower removal in those experiments in addition to the effect of the higher dose volume, which would result in a larger mass of caffeine added at one time that might overwhelm the microbial communities.

DEET, an insecticide, is widely used in the United States with domestic usage estimated to be 1800 tons annually (52), resulting in its frequent detection in streams, often at the low parts-per-billion levels. DEET was well removed overall, but showed the highest percentage removal in the 8 h and 8 h saturated intervals. Both aerobic and anaerobic removal was observed, as column 2 showed similar net percentage removals from its influent to column 1 effluent

relative to its influent levels. Previous studies have shown that some removal from anaerobic degradation was possible but that there was a slightly enhanced removal when in an aerobic system (52, 53).

The herbicide atrazine and anti-epileptic carbamazepine were mostly recalcitrant, with inconsistent removal observed for the time point effluent samples from each dose intervals and when the overall removals from each dose interval were compared. This inconsistency was attributed to a few factors, including sorption and desorption of the compounds throughout the course of the study because they did not easily biodegrade. Samples that showed a negative percent removal was likely to be the result of desorption of the chemical that had previously accumulated on the column substrate; this was specifically evident for carbamazepine and atrazine, which were not known to degrade well in the environment. For example, carbamazepine has been classified as "no removal" in activated sludge since the biodegradation rate is < 1 g L^{-1} h^{-1} (48). It was also observed at higher concentrations in wastewater treatment plant effluent compared to plant influents, which is attributed to either the release of the compound from sludge, as those particles are degraded by microbes and the compound is released (54), or reconversion of metabolites into the parent compounds by the biofilm (55). Another source of its removal was transformation into epoxy-carbamazepine, which was previously observed as a product of incomplete biodegradation and could be due to overwhelming the biomass at this dose concentration (47).

4.3.1.3. Yeast Estrogen Screen

The estrogenic activity of the estrogenic standards, and the column treated wastewater at early, mid and final time points from each of the dose intervals was measured using the yeast estrogen screen (YES) assay and portions of that data are part of another thesis (56). Due to the

different times required to collect samples at a full time point from column 3, the points were described as time point (T) and then column (C) instead of as days or hours. The full list of time points and their corresponding hours are listed in Appendix D. Examples of a dose-response curve for 17β-estradiol and for one of the sample extracts are presented in Figure 4.3.

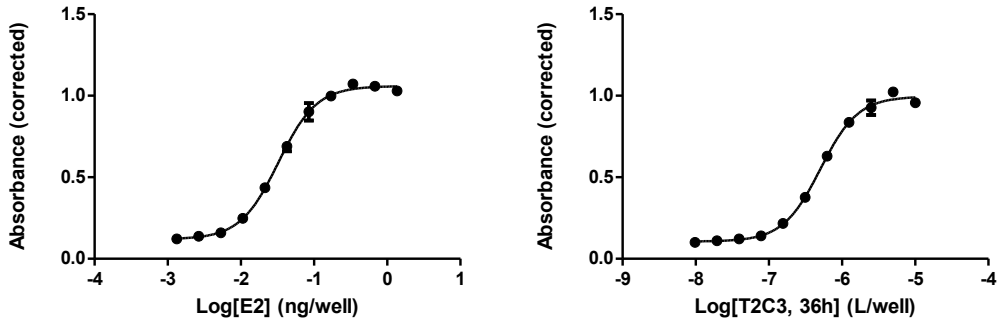


Figure 4.3. Example YES assay dose-response curves for E2 (ng/well) and a sample extract from T2C3 of the 36h dose interval (L/well).

Of the seven target chemicals monitored in this study only three (17β-estradiol, 4-nonylphenol, and atrazine) showed estrogenic activity within their aqueous solubility. The estrogenic activities measured by the YES assay are shown in Table 4.4 and represent the mean and standard deviation of the EC_{50} curves for each chemical, as well as the EEFs normalized by the EC_{50} of 17β-estradiol, which make the EEF of 17β-estradiol 1. The activity for the column effluent samples are presented in Table 4.5. These EC_{50} values each correspond well with the values from the literature for the YES assay of 0.2 – 2.5 nM for 17β-estradiol, and 0.35 – 210 μM for 4-nonylphenol (57). Although atrazine has not been tested individually with the YES assay, it has shown activity in other estrogen-dependent cell lines for gene expression including breast cancer MCF-7 and Ishikawa cancer cells (58).

Table 4.4. Summary of analysis of dose response curves for the most estrogenically active among the target chemicals.

	E2 (n=54)	4-NP (n=4)	ATZ (n=5)
Average EC_{50} (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	0.000013

The data for the column samples represent the concentrations after accounting for the concentration factor of 125, from the method described in section 4.2.3. In the spiked wastewater, the predicted EEQ should be 12.5 mg/L, based on 100 μg/L spikes of the target compounds and the concentration factor. The stronger potency of 17β-estradiol, as shown in Table 4.4, made it the dominant source of estrogenic activity in the wastewater, so the EEQ in the spiked and subsequent samples was primarily influenced by the concentration of 17β-estradiol or its potent transformation products. The EEQ values measured for the samples, presented in Table 4.5, indicate consistent removal trends compared to the chemical analyses, with the best removal observed for the shorter dose intervals. Notably, there was still estrogenic activity present in samples where the more potent active target compounds in this study, 17β-estradiol and 4-nonylphenol, were at or below their detection limit. Furthermore, even when measurable quantities of atrazine are still present, the contribution from this least potent target chemical is not significant enough to account for the observed estrogenic activity as shown by the predicted EEQs. This implies that these compounds have either been transformed into other estrogenic compounds, such as estrone or estriol from 17β-estradiol, or that other estrogenic compounds were present in the wastewater that were not targeted in the chemical analysis. The difference between the measured estrogenic activity and the activity predicted by the LC-MS measurements is presented as a parity plot in Figure 4.4, which clearly shows that there is not a linear association between the measured and predicted values.

Table 4.5. Experimental, predicted and percent accounted for of EEQs (ng/L) calculated for samples from the column study,*denotes a statistically significant difference between predicted and observed. n= 3 except as noted.

Dose Interval (h)	Sample	EEQ Predicted by LC-MS	Average Experimental EEQ by YES	% Experimental Activity Accounted for by LC-MS
8 Saturated	Source	0.336 µg/L	0.861 µg/L[a]	39
	Spiked*	1.26 mg/L	128 ± 14.9 µg/L	>100
	T1C3	1.10 µg/L	5.07 ± 7.07 µg/L	22
	T3C3*	0.224 µg/L	13.2 ± 1.56 µg/L	1.7
	T8C3*	0.146 µg/L	2.40 ± 0.344 µg/L	6.1
	T8C1*	1.31 mg/L	1.67 ± 0.494 mg/L	79
	T8C2*	0.529 µg/L	4.82 ± 3.17 µg/L	11
8	Source*	0.00	2.63 ± 0.437 µg/L	<1.0
	Spiked*	1.26 mg/L	6.20 ± 1.92 mg/L	20
	T1C3	0.058 µg/L	3.77 ± 5.83 µg/L	1.5
	T3C3	5.35 µg/L	7.12 ± 4.14 µg/L	75
	T6C3	0.772 µg/L	1.09 ± 1.06 µg/L	71
	T8C3	0.0696 µg/L	4.19 ± 4.10 µg/L	1.7
	T8C1*	217 µg/L	2.40 ± 0.443 mg/L	9.0
	T8C2*	0.0689 µg/L	7.13 ± 2.34 µg/L	<1.0
12	Source*	0.683 µg/L	1.99 ± 0.755 µg/L	3.4
	Spiked*	1.26 mg/L	693 ± 86.6 µg/L	>100
	T1C3	0.080 µg/L	4.43 ± 6.70 µg/L	1.8
	T3C3	0.0941 µg/L	0.684 ± 0.234 µg/L	14
	T5C3	0.0955 µg/L	1.81 ± 2.17 µg/L	5.3
	T7C3*	0.0109 µg/L	13.0 ± 2.52 µg/L	<1.0
	T7C1*	319 µg/L	3.99 ± 2.53 mg/L	8.0
	T7C2*	0.0832 µg/L	243 ± 4.88 µg/L	<1.0
24	Source*	0.23 µg/L	2.2 ±0.8 µg/L	11
	Spiked*	1.26 mg/L	8.11 ± 4.37 mg/L	16
	T3C3	104 µg/L	185 ± 86 µg/L	56
	T5C3*	10.6 µg/L	185 ± 86.1 µg/L	5.7
	T7C3	1.04 µg/L	92.9 ± 119 µg/L	1.1
	T9C3*	12.1 µg/L	210 ± 110µg/L	5.8
	T9C1*	430 µg/L	3.59 ± 0.4 mg/L	12
	T9C2*	693 µg/L	1.12 ± 0.181 mg/L	62
36	Source*	0.00155 µg/L	2.16 ± 0.801 µg/L	<1.0
	Spiked*	1.26 mg/L	6.20 ± 1.92 mg/L	20
	T1C3*	0.055 µg/L	10.5 ± 8.05 µg/L	<1.0
	T2C2*	0.046 µg/L	31.0 ± 22.9 µg/L	<1.0
	T3C3*	0.057 µg/L	2.29 ± 2.72 µg/L	2.5
	T4C3*	0.0347 µg/L	309 ± 102 µg/L	<1.0
	T4C1	6.79 mg/L	5.29 ± 0.608 mg/L	13
	T4C2	0.0437 µg/L	1.25 ± 0.283 mg/L	<1.0

[a]n = 2, no standard deviation

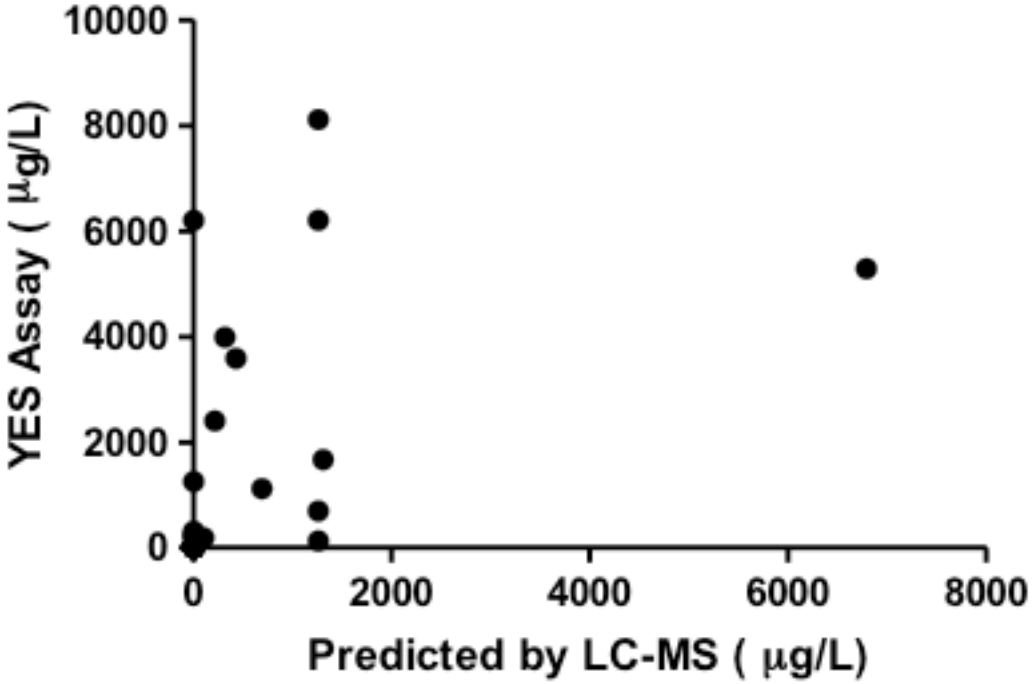


Figure 4.4. Parity plot of the relationship between YES assay results and LC-MS predicted EEQ values.

The predicted EEQs of the samples as derived by the chemical analysis data are also presented in Table 4.5, along with the percent difference between the experimental and predicted EEQ, part of which is part of another thesis (56). The results showed a statistically significant difference between the predicted EEQ value using the chemical analyses and the experimentally observed value from the YES assay for 26 of the 39 samples. The differences observed in the predicted and experimental wastewater extracts could be due to a number of factors, including metabolites of our target compounds, untargeted compounds already present in the wastewater, and colloidal carbon preferentially binding the hormones, as discussed below.

As mentioned previously, the predicted values were calculated based on the target chemical concentrations but do not take into account other chemicals that were present in the wastewater. The estrogenic activity measured for the source water, however, suggests that other chemicals were present that could influence the assay, including both agonists that increase

activity and antagonists that decrease it, because of the deviation between the predicted chemical activity and the measured activity from the bioassay. Similarly to the degradation of 4-nonylphenol isomers, the degradation of unknown chemicals in the mixture could bias the activities measured and these unknowns also might degrade differently in the columns. For example, antagonists would decrease the estrogenic activity in the spiked source water, which would result in a dampened spiked extract. These chemicals could, however, degrade preferentially in the columns over other agonists, thus resulting in an enhanced effluent activity as has been seen previously in a soil-aquifer treatment study using the YES assay (59). The same could also be observed for agonist degradation, which emphasizes the utility of using a bioassay to assess overall wastewater quality when chemical analyses might not capture all of the active components.

Another factor contributing to the difference could be the presence of colloidal organic matter, which has been previously observed to decrease the free aqueous concentrations of hormones and nonylphenols in batch studies (60-62). In that instance, the colloidal particles cannot be filtered from the wastewater but could sorb 17β-estradiol, of which even a small loss would cause significant impact on the activity due to its potency (5.39 E-10M). Furthermore, estrogens binding to colloids have been estimated to be in the range of 4 – 31% in wastewater (63), and up to 60% in one study (60), which could cause a significant impact on the results.

An additional factor was likely to be the formation of estrogenic metabolites through the biodegradation of any of the target compounds or others present in the source water, for example the formation of estrone and estriol from 17β-estradiol (45). 17β-estradiol was well removed in the columns, and often found below the quantitation limit, but incomplete biodegradation could result in activity from its metabolites. 4-Nonylphenol, the second most potent estrogen of our

target compounds, may also contribute to the poor correlation between the predictions and the measured EEQ, as different isomers have been shown to have different estrogenic activity (64) and different propensities for biodegradation (41), so the measured concentrations of total 4-nonylphenol would not necessarily correspond to the actual activity from that mix of isomers.

The removal efficiency, which compared the EEQ concentrations of the final column 3 samples to those of the 100 µg/L spiked source wastewater, allowed for comparison between the dose intervals. The efficiencies, shown in Figure 4.5 for the total removal, indicated that the shorter the interval the better the removal of estrogenic activity. The chemical analyses showed similarly high removals, as previously described.

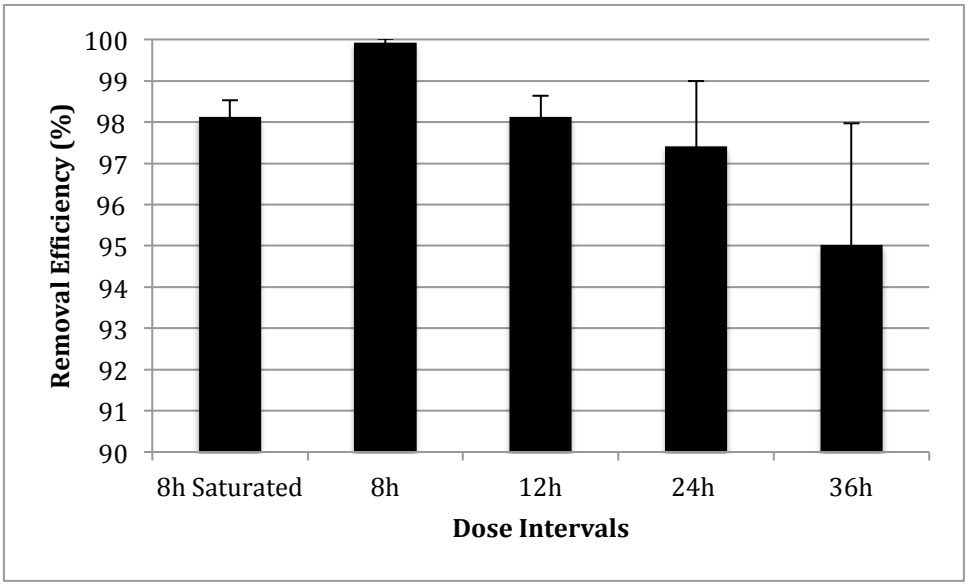


Figure 4.5. Overall removal efficiencies for the five different dosing intervals used in the study as calculated using YES assay derived EEQ values (56).

4.3.1.4. Other Analyses

The spiked chemicals were prepared in methanol, with the exception of caffeine, because of solubility limitations for making concentrated stocks. The addition of 0.6 mL of methanol/L of wastewater, equivalent to 178 mg of carbon, resulted in elevated organic carbon levels in the column 1 influent. The standards could not be weighed directly into the wastewater for each dose

because of the potential for even very small differences in 17β-estradiol concentrations to considerably impact the estrogen activity measurements. However, because the wastewater collected from JLBC for this study was already partially treated before use, the additional TOC from the methanol in the spike solutions brings the concentration to levels within a normal untreated wastewater range (65). The source wastewater TOC, DOC, TN, and DN concentrations and the corresponding concentrations with the chemical spikes are presented in Table 4.6, along with the DOC/DN ratio. While the mean TOC of the spiked wastewater was greater than the DOC, the standard deviation qualifies this as not a statistically significant difference. TOC, DOC, TN, and DN as the percent removal for the final time points and as an average of the percent removal of each time point for each column are presented in Table 4.7. Full graphs of individual points for TOC, DOC, TN, and DN are presented in Appendix C for study 1 and Appendix D for study 2. Although Hach kit readings were taken, and are presented in Table 4.8, it was not possible to do a mass balance for nitrogen due to limitations for the instrumental method. Similarly to the chemical analyses, shorter intervals showed better removal for these target indicators. These results suggest that the drying period that exists between dose intervals might contribute to this trend, either through physical disruption of the biofilm and greater sloughing off of the biofilm containing both organic material and sorbed compounds, or an oversaturation of the biofilm as the longer dose intervals also correspond to a higher dose volume and effective concentration of the target compounds.

Table 4.6. Wastewater carbon and nitrogen concentrations before and after adding the target chemical spikes. (n=2).

Source Wastewater			Spiked Wastewater		
DOC (mg/L)	DN (mg/L)	DOC/DN	DOC (mg/L)	DN (mg/L)	DOC/DN
2.3 ± 0.71	35 ± 14	0.07	245 ± 4.3	34 ± 13	7.2
TOC (mg/L	TN (mg/L)	TOC/TN	TOC (mg/L)	TN (mg/L)	TOC/TN
38 ± 13	36 ± 9.9	1.1	210 ± 50	36 ± 8.5	5.9

Table 4.7. The total percent removal results for the TOC, DOC, TN and DN measurements of effluent after each of the columns, relative to the spiked wastewater, at the final time point and as the average ± standard deviation of all of the samples from that column during each study.

Dose Interval (h)	Column	TOC (%)		DOC (%)		TN (%)		DN (%)	
		Final	Avg	Final	Avg	Final	Avg.	Final	Avg.
8 Saturated	C1	81	82 ± 1.8	87	88 ± 1.3	>98.5	98 ± 0.6	95	97 ± 1.3
	C2	97	95 ± 3.7	98	98 ± 0.2	98	95 ± 4.9	97	94 ± 6.1
	C3	97	97 ± 0.1	98	98 ± 0.1	97	83 ± 23	96	80 ± 26
8	C1	98	97 ± 2.4	98	96 ± 2.7	95	89 ± 5.4	77	77 ± 8.1
	C2	>98.5	98 ± 1.5	>98.5	98 ± 0.4	80	77 ± 2.7	95	90 ± 5.2
	C3	>98.5	99 ± 0.4	>98.5	>98.5	76	74 ± 13.5	97	81 ± 6.9
12	C1	75	84 ± 6.6	83	84 ± 6.6	75	71 ± 34	68	63 ± 40
	C2	97	98 ± 0.1	98	98 ± 0.1	25	25 ± 6.5	-4.1	1.4 ± 12
	C3	98	98 ± 0.2	98	98 ± 0.2	37	37 ± 23	-4.0	22 ± 29
24	C1	22	18 ± 3.0	28	21 ± 5.6	27	25 ± 1.4	85	41 ± 29
	C2	79	75 ± 4.8	68	72 ± 5.9	42	40 ± 11	43	41 ± 13
	C3	97	94 ± 5.0	96	89 ± 7.3	48	24 ± 33	47	24 ± 30
36	C1	40	67 ± 19	58	77 ± 14	53	87 ± 23	52	86 ± 22
	C2	96	96 ± 1.1	97	97 ± 0.9	86	72 ± 11	86	82 ± 16
	C3	96	97 ± 0.7	94	97 ± 2.1	89	83 ± 18	89	69 ± 9.2

Concentrations of ammonia, nitrate and nitrite (mg/L) and pH for each of the final time points for each column and as averages from those column effluent for the period of the study are presented in Table 4.8. The influent nitrate values, however, were likely elevated because of nitrate residual on the acid-washed glassware because one reading was particularly high, 112 mg/L, which indicated contamination of the sample. This nitrate reading was well above the total nitrogen measured for the same source water. These results show the measured value for the effluent from each of the three columns as well as the average for the influent from each column over the course of each dose interval. The pH of the effluent from each column remained steady throughout the course of each study. The removal of the other wastewater indicators presented in Table 4.8 again show the best removal for the shorter dose intervals, but a slightly enhanced removal for the 8h free flowing interval over the 8h saturated interval.

Table 4.8. Analysis of inorganic nitrogen and pH for each of the final time points and the average for all time points from each column compared to column 1 influent levels. N/A = not applicable as the effluent samples are the final readings.

Dose Interval (h)	Column	Ammonia as N (mg/L)		Nitrate as N (mg/L)		Nitrite as N (mg/L)		pH	
		Final	Avg.	Final	Avg.	Final	Avg.	Final	Avg.
C1 Influent		N/A	3.4 ± 0.39	N/A	28[a]	N/A	0.16 ± 0.21	N/A	6.8 ± 0.1
8 Saturated	C1	0.1	0.1 ± 0.0	8.0	10 ± 2.8	0.1	0.1 ± 0.1	7.16	7.1 ± 0.0
	C2	0.0	0.1 ±0.0	8.0	13 ± 6.7	0.0	0.1 ± 0.1	7.81	7.6 ± 0.2
	C3	0.0	0.1 ±0.0	8.0	30 ± 21	0.1	0.1 ± 0.0	7.84	7.7 ± 0.1
8	C1	0.1	0.6 ± 0.8	8.0	4.0 ± 2.6	11	1.7 ± 4.1	6.65	6.9 ± 0.2
	C2	0.1	0.1 ± 0.1	7.0	7.3 ± 1.8	0.1	0.4 ± 0.6	7.45	7.5 ± 0.1
	C3	0.1	0.1 ± 0.1	8.0	7.7 ± 1.9	0.1	0.2 ± 0.2	7.79	7.7 ± 0.1
12	C1	0.1	1.0 ± 1.1	5	15 ± 9.4	0.0	0.1 ± 0.1	6.9	6.8 ± 0.1
	C2	0.1	0.6 ± 0.1	41	62 ± 19	0.1	0.1 ± 0.1	6.8	6.9 ± 0.1
	C3	3.1	0.1 ± 0.1	23	48 ± 19	0.2	0.1 ± 0.1	7.5	7.5 ± 0.1
24	C1	2.7	1.6 ± 1.8	3.0	1.8 ± 1.3	0.1	0.1 ± 0.1	6.4	6.6 ± 0.3
	C2	1.3	1.0 ± 0.3	5.0	4.8 ± 1.0	0.0	1.0 ± 1.1	6.6	6.8 ± 0.3
	C3	0.6	0.5 ± 0.5	7.0	7.8 ± 1.0	2.0	0.6 ± 0.9	7.0	7.0 ± 0.2
36	C1	0.1	0.1 ± 0.1	0.0	0.5 ± 1.0	0.1	0.0 ± 0.0	6.8	7.1 ± 0.4
	C2	1.1	0.7 ± 0.5	4.0	9.5 ± 8.2	0.1	0.3 ± 0.4	7.3	7.2 ± 0.2
	C3	0.3	0.2 ± 0.2	3.0	8.3 ± 8.5	0.0	0.4 ± 0.8	7.1	7.2 ± 0.2

[a] Experimental contamination resulted in n=1 as a reference but is comparable to study 1 readings.

Removal of total coliform and *E. coli* as measured by IDEXX Colilert Quanti-trays, indicated nearly complete removal for each of the dose intervals. No total coliform was detected

in either the 8h or 8h saturated tests, while the 12h had 63 colony forming units per 100 mL, the 24h sample had 41 colony forming units per 100 mL, and the 36h samples had 10 colony forming units per 100 mL, compared to 750 colony forming units per 100 mL for the source wastewater. It was not unexpected that these levels were low compared to a raw wastewater, as the source water used in this study was already partially treated through a constructed wetland and total coliform levels vary considerably in wastewater, with one study showing variations of 0 to 10, 000 CFUs over the course of a year (66). The log removal values (LRV) of total coliform was calculated using Equation 4.3, below.

$$LRV = log_{10}[\frac{Influent\ Concentration}{Effluent\ Concentration}] \quad (4.3)$$

Table 4.9. Log removal values of total coliform from the dose intervals compared to the source water.

	Dose Interval				
	8h	8h Saturated	12h	24h	36h
LRV	>10	>10	1.0	1.3	1.9

4.3.1.5. Stalite Extraction

To approximate the loss of estrogenic compounds to sorption on the Stalite an extraction was done on 20 g core samples removed from each of the column ports at the end of the study, on source Stalite that had not been in contact with wastewater, and on Stalite onto which 2 µl of the 1 g/L stocks was spiked. The results for the extract of target chemicals from Stalite core samples and subsequent YES analysis are shown in Table 4.10, below. Due to the low measured activity, further analyses of core samples taken in between the dose interval experiments were not evaluated as these consisted of 10x less Stalite by mass and were unlikely to show a response. An extraction of the source Stalite, done in the same manner as the core samples, that had not been in contact with wastewater did not result in a measurable EC_{50} so the material itself is not expected to leach estrogenic compounds. An extraction of the chemicals spiked directly

onto the Stalite showed a low recovery of 3%, compared to its predicted EEQ, which was likely due to strong sorption of the estrogenic compounds to the Stalite (63). This was a similar value to some of the observed percentages from the predicted EEQs and the experimental YES extract values.

Table 4.10. Estrogenic activity of extracted core samples from column 1, 2, and 3.

		EEQ ± Std Dev (ng/L)	
Source Stalite		8.10 ± 4.99	
Spiked Stalite		3.1E4 ± 5.1E4	
Port	Column 1 (n=2)	Column 2 (n=3)	Column 3 (n=3)
a	4.3 ± 6.1	0.6 ± 0.9	12 ± 2.3
b	0.5 ± 0.6	1.0 ± 0.9	4.0 ± 5.5
c	0.6 ± 0.9	1.3 ± 0.9	3.9 ± 1.7

Extraction of the column core samples showed low-level (ng/L) estrogenic activity, shown in Table 4.10, and did not reach full dose response curves, and were also well below the values observed for the effluent extracts. However, the low recovery from this extraction contributed to the low readings from the column extracts, indicating that these numbers underestimated the actual loss to sorption. Column 3 showed a statistically significant difference between sorption to the 3a core sample compared to all other core samples, but none of the other ports were statistically different from one another. These results indicated that sorption is a contributing factor to the removal of the estrogenic compounds in the mixture but because of conversion between 17β-estradiol and other estrogenic compounds, it was not possible to say whether the activity was coming from 17β-estradiol or an accumulation of its metabolites or other low-level wastewater contaminants. Furthermore, a limitation of this approach is that it only looks at the accumulation of 3 points within the columns but it was possible that there was sorption at the top layer which first came in contact with the spiked wastewater or at the bottom

of the columns by the exit port, which had a much more prolonged interaction with the wastewater.

4.3.2. Batch Study

The batch results from both studies are presented in Table 4.11. The 30h time point was chosen to allow for maximum chemical removal in the study so as to be able to measure a difference in effect between biological (microbial degradation) and physical (sorption) removal from the wastewater. This was also in a timeframe similar to that used in the column study, in which the wastewater had a maximum contact time of 24 h between the three columns, which was for the 8h-saturated dose interval. There was removal of each compound after 30h but they were not significantly different between the conditions in the first batch study, which used a lower Stalite mass (120 g for 600 mL JLBC wastewater (WW), 100 µg/L target compounds). The removal levels in the first study indicated that the substrate:solute ratio might have been too low resulting in a limited amount of removal from either biodegradation or soption to the bed material for a difference to be observed. The second batch study, which used 600 g of Stalite for 600 mL (100 µg/L added target compounds) (samples 3 and 4) showed enhanced removal by biodegradation for 17β-estradiol, which was below the detection limit in the 24h samples for both the aerobic and hypoxic trials. Atrazine showed slightly elevated removals for the aerobic (33% in sample 3) and hypoxic (34% in sample 4) trials compared to the sorption in the autoclaved wastewater sample (25% in sample 2), which supported the column study results and previous literature findings (38). The higher ratio of Stalite to wastewater in batch study 2 also enhanced removal of atrazine, supporting the column study data and previous literature that sorption is an important removal mechanism. Surprisingly, caffeine had a low removal observed through biodegradation, which could be attributed to its fast sorption velocity mentioned

previously. Similarly, carbamazepine, which had a logK_{ow} of 2.25, was also expected to adsorb to the biofilm on the bed material but was unlikely to be biodegraded at that point, which could explain the inconsistency between the time points and condition results (67). DEET showed enhanced removal in the second batch study compared to the first, with higher removal in the hypoxic system than the aerobic, and removal efficiencies similar to what has been observed at treatment plants (68). 4-nonylphenol was well removed in both batch studies but showed an enhanced removal in batch study 1, which could be in part due to the presence of colloidal carbon as previously described. The generally enhanced removals observed for the higher Stalite to solute ratio indicated the importance of chemical access to the packing material and on limiting the amount of liquid added in order to allow for efficient removal. In a column or field system, this would equate to a shorter dose interval where smaller volumes of wastewater are put into contact with the bed material.

Table 4.11. Batch study results for percent removal of target compounds with two substrate:solute ratios and four conditions, including 1) LGW with autoclaved Stalite, 2)Autoclaved JLBC wastewater with autoclaved Stalite, 3) Aerobic wastewater with an active biofilm, and 4) Hypoxic wastewater with an active biofilm. NM = not measured.

		Stalite : Wastewater (g : mL)			
		120:600		600: 600	
Target Compound	Sample	1h Time Point Removal (%)	30h Time Point Removal (%)	1h Time Point Removal (%)	24h Time Point Removal (%)
ATZ	1	-19	-1	NM	29
	2	-2.2	-4.9	NM	25
	3	-0.7	-1.1	22	33
	4	-12	4.3	35	34
CAF	1	1.7	17	NM	25
	2	22	14	NM	24
	3	15	13	21	29
	4	5.8	18	25	23
CBZ	1	-8.5	-8.8	NM	-2.5
	2	-0.8	3.7	NM	-3.5
	3	-3.8	-5.4	-1.3	5.8
	4	-6.8	1.3	7.9	-6.8
DEET	1	-18	-33	NM	62
	2	-36	-36	NM	55
	3	-53	-13	39	39
	4	-4.4	-34	45	57
E2	1	12	41	NM	17
	2	28	26	NM	35
	3	23	47	-5.9	> 90[a]
	4	27	40	77	>90[a]
4-NP	1	33	54	NM	28
	2	52	65	NM	37
	3	48	84	38	30
	4	53	59	38	28

[a] >90% removal or below 10 µg/L.

4.4. Conclusions

The removal of emerging contaminants and their estrogenic activity from wastewater was not considered in the design and operation of conventional treatment plants, leading to their presence in receiving water bodies (69). In a recent nationwide study it was shown that estrogenic activity is measured consistently in river waters, with 34 of 35 test sites across the

U.S. showing agonistic responses, which means an increase in gene transcription targets from the estrogen receptor (70). Furthermore, the results of that study indicated a linear correlation between the concentrations of individual steroidal estrogens and the observed *in vitro* estrogenic activity, after correcting for the potency of each compound (70). Beyond endocrine disruption, the contamination from mixed organic contaminants of anthropogenic origins coming from runoff and wastewater is well documented in surface waters (70-73). Clearly, reuse of wastewater also needs to consider the fate of these chemicals if the technology is to be expanded into areas that currently use drinking water and where human and ecosystem exposure could occur (74).

The operational parameters of alternating between aerobic, hypoxic, and aerobic zones while utilizing different dosing intervals tested in this study with Stalite, clearly indicated that shorter periods between doses and smaller volumes were preferred for the removal of estrogenic compounds. The removal of recalcitrant chemicals was inconsistent and suggests that only partial removal can be achieved under any of these conditions but that sorption is likely to dominate. The removal of estrogenic activity for the tested dose intervals followed the same trends as the removal of the target chemicals. A notably dampened effect, however, was observed when the predicted EEQs calculated from the most potent estrogenic chemicals in the treated water were compared to the observed estrogenic activity. This is likely due to antagonists in the mixture, the formation of untargeted potent estrogenic transformation products, or the increased sensitivity for the bioassay compared to chemical analyses. The source wastewater itself also had measurable estrogenic activity, a common occurrence, and supports the need for further optimization of on-site treatment systems to address endocrine activity (69, 70). In each of the dose interval studies, the removal was above 90% by column 3 but the activity that

remained was at a similar level to the background originally present in the raw wastewater. Importantly, the YES assay results were able to bridge the gap between the detection limitations of chemical analyses and the actual estrogenic activity as they measured reproducible activity in samples that were below the detection limit for the test chemicals in the instrumental analysis. However, further work could be undertaken to develop an extraction process to capture a wider array of compounds and, therefore, potentially recover more of the estrogenic chemical contaminants for comparison to the bioassay.

Target chemical removal in "green" engineered, on-site treatment systems is an active area of study as environmental professionals look to wastewater reuse to accommodate water shortages (75). The benefits of being able to reuse wastewater puts a decreased burden on the drinking water system, but requires high quality treated wastewater effluent. Of concern is how to remove common organic contaminants from wastewater to a point where the effluent does not pose an environmental risk if reused. The target chemicals in this study have varied degradation pathways that have been studied extensively, under abiotic and biotic conditions (6, 76-79). The most efficient way to enhance removal in rural wastewater treatment settings is to utilize these results when designing a passive treatment system, like a vertical flow filter or constructed wetland. In the results presented in this study, clear trends in chemical removal show enhanced removal for shorter dose intervals for 17β-estradiol, 4-nonylphenol, caffeine, and DEET. These results correspond well to both the removal of organic carbon and organic nitrogen, as well as to other nitrogen species. The enhanced removal at shorter intervals could likely be due to lower ratio of target compound to biofilm, which would prevent over-saturating the bacterial community with target compounds to degrade. If this were the case, it would also support the results from the batch study, which showed little difference in the removal of the target

compounds between the active biofilm and inactive (autoclaved) samples. The spike concentration of the compounds, which was chosen to mimic the concentration spiked into the column wastewater samples to allow for other measurements, might have already saturated the bed material in the system. The compounds carbamazepine and atrazine, which were not expected to biodegrade, showed varied removal that was likely due to sorption and desorption to the Stalite, observations that are seen in streambeds (6, 80, 81).

Due to the elevated levels at which these compounds were spiked, it is not possible to say how efficient this system would be at removing compounds in the low microgram to nanogram per liter levels at which they are typically found in the environment. The trends in removal, however, would indicate that shorter dose intervals should still be applied when the primary concern is treatment of estrogenic activity or biodegradable compounds. For recalcitrant compounds the dose interval seems to be less important, as there is not a direct correlation between this operational parameter and their overall removal.

A limitation of this study is that the doses were studied at such elevated chemical levels. Therefore, future work should evaluate source wastewater with the 8h and 8h saturated intervals to determine the removal efficiency of emerging contaminants and estrogenic activity when at lower levels. The YES assay has been demonstrated to be suitable for evaluating wastewater treatment as it relates to endocrine activity and would provide useful insight into those low-level studies because of its sensitivity. Furthermore, the ability of the YES assay to detect estrogenic activity when the analytical method for individual chemicals did not detect any target compounds supports the assertion that future studies on the treatment of estrogenic activity in wastewater would be better served by bioassays to first identify activity and treatment effectiveness on its removal and then chemical analysis to identify the specific micropollutants.

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CHAPTER 5: CONCLUSIONS

5.1. Summary of Motivation

Water treatment, whether for waste- or drinking water, is concerned with the mitigation of contaminants that can be harmful to the consumer or the receiving water body. Specifically, the primary concern is the inactivation of pathogens in order to produce a safe effluent. Generally, conventional drinking water treatment plants employ steps including sedimentation, flocculation, filtration and disinfection to remove biological and chemical contaminants of concern. It has been well established, however, that the conventional treatment of drinking and wastewater has unintended consequences for consumers and the environment. Low-level contaminants produced in the process of treating drinking water, and contaminants that accumulate through household chemical use and agricultural runoff in wastewater, are of particular concern because of their far-reaching health implications. In particular, it is unclear how habitual exposure to these chemicals can impact the health of drinking water consumers. It is, however, clear that some of the contaminants that can be found in treated wastewater are of toxicological concern. As drinking water sources become scarcer, wastewater reuse is being expanded for purposes such as golf courses, landscaping, agriculture, and toilet flushing (1). Furthermore, if future reuse is to be extended to replace the more demanding potable water consumption of crop irrigation, then it is necessary to evaluate the efficacy of treatment in removing a wide range of pollutants and their biochemical activity. The

focus of this dissertation was to evaluate both drinking water and wastewater treatment in terms of the mitigation of emerging contaminants and endocrine disruption.

While drinking water disinfection is necessary to inactivate harmful pathogens, these strong oxidants react with organic matter and produce disinfection by-products (DBPs) of which over 700 unique chemicals have been identified (2). The toxicological behavior of these low-level contaminants in humans is not well understood, but epidemiological and lab-based studies have indicated an association between exposure and the development of several negative health outcomes, including bladder cancer and infertility (3, 4). Among the pathways of concern that have been implicated from those results are the ones that make up the endocrine system, because of their involvement with female and male hormone signaling. Although many environmental pollutants have been identified as endocrine active substances, including pesticides, plasticizers, and surfactants, it remains unclear whether DBPs have endocrine activity (5-7). To date, the structure-activity relationships and the overall toxicological activities of the vast majority of DBPs for the estrogen and androgen signaling pathways are unknown. Despite evidence to suggest that chronic low-level exposure to these chemicals may be harmful to human and ecosystem health, the specific areas of concern have yet to be identified and the compound classes of interest remain unclear.

Unlike drinking water, the estrogenic activity of wastewater and the identification of some chemicals that contribute to it are already well established (8-11). The removal of this biological activity is vital if wastewater reuse is to be applied in certain settings such as agricultural use, because of the potential for these contaminants to accumulate in the environment, including crops, or cause disruption to other species (12-15). The issue

of mitigating endocrine disruption, however, is an ongoing challenge for both conventional wastewater treatment systems and onsite treatment systems alike (10, 16, 17). Add-on processes to septic systems for on-site wastewater treatment include sand filters and constructed wetlands that may assist towards this goal and their application in reuse is an active area of investigation (15, 17, 18). Furthermore, optimization of these systems to directly address estrogenic activity is an important but as yet underexplored area for rural wastewater treatment.

Specifically, the goals for this dissertation were to identify estrogenic and androgenic DBPs individually, in binary mixtures, and *in silico*, from a subset of compounds that have been previously identified in structure-activity studies as potentially disruptive to the endocrine system. The remediation of estrogenic activity in relation to target common wastewater pollutants was then addressed through a systematic column study of varying dose intervals to determine the optimal operational conditions that could ultimately be transferred to full scale constructed wetlands treatment.

5.2. Major Findings

The primary findings from these studies were the identification of estrogenic and androgenic DBPs using competitive binding assays and the determination of the best dose interval for wastewater addition to a vertical flow filter for estrogenic activity removal.

Specifically, the results from Aim 1 found that 14 of 21 tested DBPs were able to bind to the androgen receptor in a competitive binding assay over the range 13.5 μM for 3,4,5,6-tetrachloro-benzoquinone to 1.86 mM for 2,3-dichloropropionamide. Seven of these active compounds were found to follow concentration addition when in binary mixtures with a known, weak endocrine disruptor, 4-*n*-nonylphenol. Steric hindrance

showed some influence on binding, with dichloroacetonitrile able to bind when trichloroacetonitrile could not, and iodoacetic acid binding more strongly than diiodoacetic acid. Planar molecules showed the reversed trend, with more halogenated structures showing stronger binding affinities for the receptor. Using *in silico* predictions, it was determined that all but one of these compounds was likely to bind as an antagonist, therefore potentially decreasing androgen-related gene transcription. These results indicate that DBPs are a potentially important chemical class of newly identified androgen disruptors and that they could contribute to some of the negative health outcomes already associated with exposure to disinfected water. Furthermore, the identified compounds represent a wide range of structural groups, which indicates the importance of performing further structure-activity studies into the activity of other potentially androgen-active DBPs.

The results from Aim 2 identified 9 of the selected 21 DBPs as estrogen-binding compounds when in an estrogen receptor competitive binding assay individually, with IC_{50} values found in the concentration range of 148 μM for bromopicrin to 6.46 mM for 2-chlorophenol. In binary mixtures with the known weak endocrine disruptor, 4-*n*-nonylphenol, the compounds again followed the concentration addition model, suggesting that their effects would be additive in mixtures like those found in drinking water. Despite having IC_{50} values that are above occurrence levels in drinking water, the ability of DBPs to bind to the estrogen receptor and the chronic low-level exposures to which humans are exposed, cannot be ignored because of the potential for cumulative effects. The variety of active compounds in this study indicate that DBPs are indeed a

contributing factor to the observed biological activity of disinfected waters and that estrogen disruption is a pathway of interest.

In Aim 3, a reporter gene assay was used to measure the estrogenic activity of column-treated wastewater samples, in terms of their estradiol equivalents (EEQs). The findings determined that for the dose intervals of 8 h, 8 h saturated, 12 h, 24 h, and 36 h, the removal of both estrogenic activity and anthropogenic chemicals was enhanced as the dose interval became shorter and contact time became longer. For recalcitrant target compounds, such as atrazine and carbamazepine, variability measured for the removal within the dose interval time points and between the dose interval experiments suggested that these compounds are likely sorbing and desorbing to the bed material throughout the course of the studies. It was observed that the removal of 17β-estradiol and 4-nonylphenol responded well to shorter intervals, as well as to the increased contact time of the saturated 8h interval. The shorter dose intervals also correlated well to the removal of organic carbon and nitrogen measured in the column effluent. While the target compounds and overall estrogenic activity were well removed, the chemical analysis did not account for a significant portion of the observed estrogenic activity in some of the samples. This discrepancy could be attributed either to the possible formation of estrogen active metabolites from 17β-estradiol or other compounds present in the wastewater, or to the preferential degradation of antagonists in the columns such as some 4-nonylphenol isomers. Overall, the vertical flow filter column design in this study was effective for the removal of the estrogenic activity that is attributed to surfactant degradation products and hormones, and was best attained through shorter dose intervals and longer contact times with the filter bed material (Stalite) that was used. The yeast estrogen screen assay was

determined to be an effective tool to measure the estrogenic activity of complex mixtures and a complementary technique to targeted chemical analyses, which is particularly important for the analysis of complex environmental samples.

5.3. Future Work

The results from these studies provoke several interesting research questions that could be explored in future work. While it was determined that DBPs were capable of binding to both the estrogen and androgen receptors, and the nature of that binding was predicted by *in silico* means, a more complete analysis of the agonist or antagonist nature of their activity could be undertaken using spectroscopic or polymerase chain reaction (PCR) array analyses. Furthermore, the impact of long-term, low-level exposure to these compounds could begin to be addressed using controlled exposures to model organisms such as Zebrafish or Medaka fish. Finally, while the results from Aim 3 suggest shorter dose intervals are best for the remediation of estrogenic activity and the degradation of these target compounds, a more complete study could include isotope-labeled standards and subsequent microbial analyses to determine the microbial communities responsible for the degradation in these columns. These results are also specific to a column study that uses equal dose addition periods for each of the column stages, but altering the retention time or the order of the columns should be evaluated to determine whether all of these stages are necessary for estrogen activity remediation. The results were also specific to Stalite, which may have a higher adsorption capacity, and in turn could cause the system to fail by clogging. Additionally, a non-targeted analysis of dose intervals with raw, unspiked, wastewater that is already inherently estrogenic could be used to determine the remediation of estrogenic compounds and activity at environmentally

relevant levels. Other studies could include an analysis of the other endocrine endpoints of concern, including the androgen and thyroid signaling pathways, or a comparison between wastewater types, such as those from agricultural runoff, large scale animal operations, or hydraulic fracturing effluent. These other types of wastewater may have attributes that alter the ability of the columns to remove estrogenic activity, such as their salinity, metal content, or competing organic contaminants.

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APPENDIX A: DETAILED METHODS FOR CHAPTER 2

A.1. Solution preparations

TEDG Binding Buffer Preparation:

To make 100 ml of TEDG ARBA binding buffer, the following are added in order.

40 ml ddH_2O
40 ml of 2 M NaCl
5 ml of 1 M Tris-HCl
10 ml of glycerol
31.2 mg DTT
1.0 g human γ-globulin

Mix components and adjust the solution to pH 7.4 using 1N NaOH. The final concentrations will be: (50 mM Tris-HCl, pH 7.4; 2 mM DTT; 10% glycerol; 10 mg/mL human γ-globulin; 0.8 M NaCl)

Unlabeled R1881 preparation:

The unlabeled R1881 is supplied as a powder. To 20 mg add 5 mls 100% ethanol; this gives you 14.08 mM stock. Measure 14.2 µl and add to 20 ml 100% ethanol; this gives you 10 µM or 10^{-5} M stock. Store at -20°C until ready to use.

0.1 µM [^{3}H]-R1881 solution preparation:

Prepare a 0.1 µM or 10^{-7}M stock by adding by pipette 1 µl of the original 1.0 mCi/ml stock solution for every specific activity unit (Ci/mmol) and diluting this to 10.0 mls with ethanol. Thus, when the [^{3}H]-R1881 stock is 85.1 Ci/mmol specific activity, you should pipette 85.1 µL into a vial containing 9.914 ml of ethanol. Invert this to mix. This is the 0.1 µM stock, store at -20°C.

Chemical Stock Preparations:

The stock solutions of chemicals to be tested are made by first preparing a 500 mM stock. This is diluted to varying concentrations, depending on the chemical tested.

The unlabeled-R1881 stocks were made by first preparing a 1 mM stock, then diluting to the following concentrations.

R1881 Standard Curve

Stock [ ]	Final [1x] in experiment
30 μM	100 nM
10 μM	33 nM
3 μM	10 nM
1 μM	3.3 nM
300 nM	1 nM
100 nM	0.33 nM
30 nM	0.1 nM
3 nM	0.01 nM

Androgen Receptor Solution:

To make an adequate solution of androgen receptor, based on the results from the range finding study, 1342 μL of the thawed original 1:50 dilution from 1,000,000 cell equivalents/mL (20,000 cell eq/mL stock) were diluted in 3358 μL of TEDG ARBA buffer and gently mixed by pipette. This solution is made the day of use and kept on ice.

A.2.Chimp androgen receptor range finding assay procedure

Day 1:

The specific activity of the [^{3}H]-R1881 ligand was 85.1 Ci/mmol. In a 20 ml scintillation vial add 4.5 ml of opti-fluor scintillation cocktail and 10 μl of 0.1 μM [^{3}H]-R1881 solution. Read on scintillation counter and complete the following calculation to determine volume needed for assay:

$2.22 * 10^{12}$ dpm/Ci * specific activity = dpm/pmole
2220 * *85.1 Ci/mmol* = 188922 dpm/pmole (insert your spec. activity from supplier)
188922 * 0.004 pM = 755.7 dpm/ul

For a 1.3 ml sample: 755.7 dpm/μl * 1300 μl = 1616160 dpm's needed to give 1 nM

in the well.

1616160 dpm in 1.3ml/ your dpm reading from scint. Counter = volume in μl needed for each sample.

Example: 1616160 dpm in 1.3 ml/22,808 dpm/μl = 70.9 μl of [^{3}H]-R1881 used

The desired dpm reading in the well for total added was 30,000 – 40,000 dpm; both of these considerations were taken into account in the androgen range finding calculations.

In a 20 ml scintillation vial, add 4.5 ml of opti-fluor scintillation cocktail and 10 μl of 0.1 μM [^{3}H]-R1881 solution. Read on scintillation counter and complete the AR range finding calculation. Adjust the desired dpm reading to 30,000- 40,000 dpm in order to give a more consistent reading with lower noise on the scintillation counter.

To two borosilicate glass culture tubes:

For total binding, add 369 μl of [^{3}H]-R1881 (10^{-7} M solution)

For NSB, add 369 μl of [^{3}H]-R1881 (10^{-7} M solution) and 369 μl of unlabeled R1881 (10^{-5} M solution).

Dry down on the speed vac. for 20 min at 45°C, setting 5.1. Once dry, reconstitute in 4.7 ml of HSB + BSA. Vortex briefly (about 30 seconds) to mix.

Into a 96-well, round-bottom plate, add:

50 μl of HSB + BSA (TEDG buffer) to all wells

25 μl of [^{3}H]-R1881 to total binding wells

25 μl of unlabeled E2 + [^{3}H]-R1881 mix to NSB wells

Shake for 5 min at 4°C.

To three 20 ml scintillation vials, add 25 μl of [^{3}H]-E2 and store at 4 °C overnight. These are for the 'total added' measurement.

Make receptor dilutions once chAR receptor is fully thawed on ice and gently mixed (lightly by pipette or slow inversion):

Stock (1:50 of 1,000,000) cell equivalents/ml →

(A) 1:10 dilution by adding 44 μl stock to 400 μl TEDG →
(B) 1:20 dilution by adding 220 μl to 220 μl TEDG →
(C) 1:40 dilution by adding 220 μl to 220 μl TEDG →
(D) 1:80 dilution by adding 220 μl to 220 μl TEDG →
(E) 1:160 dilution by adding 220 μl to 220 μl TEDG →
(F) 1:320 dilution by adding 220 μl to 220 μl TEDG

Add 25 μl of each concentration to corresponding row, cover with plate-sealing film, and shake overnight at 4°C.

Weigh 0.25 g dextran coated charcoal and transfer to a 50 ml falcon tube containing 5 ml of TEDG buffer. Flip at 4°C overnight.

Day 2:

Remove 96-well plate and charcoal slurry from shaker. Add 50 μl of charcoal surry to all wells using a repeat pipette. Shake plate, sealed with plate-sealing film, at 4°C for 10 min. Centrifuge plate at 1000 rpm, 4°C, for 5 min.

Add 4.5 ml scintillation cocktail to labeled 20 ml scintillation vials. Tilt plate to 45 degrees on ice and pipette 25 μl of sample from well into the correspondingly labeled scintillation vial. Swirl to mix the vial. Read all vials on scintillation counter for ^{3}H, with 2 min run times and 2 runs per sample (protocol 13).

3.2. Androgen Receptor Binding Assay (ARBA)

Day1:

[^{3}H]-R1881 Solution Preparation:

Using the calculation from the range finding assay, 369 µl of 10^{-7}M [^{3}H]-R1881 stock solution is added by pipette to a borosilicate glass tube. This is dried down on the speed vac. for 20 min at 45°C, setting 5.1. To this, 4.7 ml of ARBA TEDG binding buffer is then added and the solution is vortexed then kept on ice during use. This will result in a final concentration of approximately 1 nM in the well.

Unlabeled R1881 Solution for Non-Specific Binding (NSB) Preparation:

From the 1 x 10^{-5} M stock in ethanol, 4 µl are added by pipette to a borosilicate glass tube and dried down on the speed vac. for 20 min at 45°C, setting 5.1. This is reconstituted with 200 µl of ARBA TEDG binding buffer and 1.3 µl of 100% ethanol. This solution is vortexed to mix and kept on ice during use.

Chemical Stock Preparations:

The stock solutions of chemicals to be tested are made by first preparing a 500 mM stock and then making a total of 10 dilutions, all in 100% ethanol, based on the solubility of the chemical. The stocks were stored at -20°C in sealed amber vials until further use.

From each of these stock solutions in the assay, 1.1 µl was added by pipette to a borosilicate glass tube containing 166 µl of ARBA TEDG binding buffer, on ice. These working stocks were then covered with parafilm and briefly mixed by vortex, then kept on ice until use.

R1881 Standard Curve Preparation:

From a 1mM stock of R1881 solution in 100% ethanol, the following dilutions are made. Each concentration is diluted in 100% ethanol and then briefly mixed by vortex.

Following the stock preparation, 1.1 µl of each stock is added by pipette into a corresponding borosilicate glass tube containing 166 µl of TEDG binding buffer. This is then briefly mixed by vortex and kept on ice until use. All stocks in ethanol are stored in amber vials at -20 °C until further use.

R1881 Standard Curve

Stock [ ]	Final [1x] in experiment
30 µM	100 nM
10 µM	33 nM
3 µM	10 nM
1 µM	3.3 nM
300 nM	1 nM
100 nM	0.33 nM
30 nM	0.1 nM
3 nM	0.01 nM

Androgen Receptor Solution:

The range finding assay showed that a receptor dilution of 1342 µl of 1:50 diluted chAR stock mixed into 3358 µl ARBA TEDG binding buffer would give around 10% binding. The chAR was thawed on ice and then gently mixed by pipette before use. This working solution was kept on ice during use, as well.

Plate Preparation Procedure:

While on ice, the following solutions are added to a 96-well, round bottom plate.

For the total binding wells, add 50 µl of ethanol and TEDG binding buffer (made by added 1.3 µl of 100% ethanol to 200 µl TEDG binding buffer and mixing) to each of three wells.

For the non-specific binding wells, add 50 µl of unlabeled R1881 solution to each of the three wells. For all chemical dilution wells, add 50 µl of the working stock solution to each of three wells per dose.

Using a repeat pipette, add 25 µl of the 4x [^{3}H]-R1881 stock solution to each well. Add 25 µl to each of three scintillation vials as well, for the ;total added to each well' calculation. Keep this at 4°C until use on day 2.

Shake the plate lightly on speed 2.5 for 10 minutes at 4 °C.

Using a repeat pipette, gently add 25 µl of the diluted 4x receptor stock solution to each well. Cover the plate with plate sealing film and lightly shake the plate on setting 2.5 overnight, at 4°C.

Solutions are added in the following scheme to the 96-well plate.

Table A.1. Diagram of the competitive binding 96-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	R1881 100 nM			Total Counts [^{3}H]-R1881			DCAN 0.33 nM			MA 10 nM		
B	R1881 33 nM			NSB R1881			DCAN 0.1 nM			MA 3.3 nM		
C	R1881 10 nM			DCAN 330 nM			DCAN 0.033 nM			MA 1 nM		
D	R1881 3.3 nM			DCAN 100 nM			DCAN 0.01 nM			MA 0.33 nM		
E	R1881 1 nM			DCAN 33 nM			BLANK			MA 0.1 nM		
F	R1881 0.33 nM			DCAN 10 nM			MA 330 nM			MA 0.033 nM		
G	R1881 0.1 nM			DCAN 3.3 nM			MA 100 nM			MA 0.01 nM		
H	R1881 0.01 nM			DCAN 1 nM			MA 33 nM			BLANK		

Example DBPs: DCAN = dichloroacetonitrile; MC = mucochloric acid

5% Dextran Coated Charcoal Solution Preparation:

The dextran coated charcoal solution is prepared by suspending 0.325 g of dextran-coated charcoal in 6.5 mls of TEDG binding buffer in a 50 ml falcon tube. This is capped and then placed on a rotary mixer overnight at 4 °C.

Day 2:

To harvest the receptors, the plate and charcoal slurry are removed from the cold room. To each well, 50 μl of the charcoal solution is added using a repeat pipette. The plate is then returned to the shaker for 30 minutes at 4 °C. During this time, 4.5 mls of opti-fluor scintillation cocktail is added to each scintillation vial labeled to correspond with each well on the plate, as well as to the three 'total added' vials prepared on day 1. The plate is then removed and centrifuged at 1000 rcf, 4 °C, for 5 minutes. With the plate at a 45-degree angle, 25 μl of each sample is removed and added by pipette to the corresponding scintillation vial. These are briefly swirled to mix and the outside of the vials is wiped with methanol to prevent misreading on the scintillation counter.

The vials are then read on the scintillation counter for ^{3}H, with 2 min run times and 2 runs per sample (protocol 13), to determine the androgenic activity of each of the two test chemicals, as compared to the standard R1881 curve. The curves are plotted again using GraphPad prism 4 software.

APPENDIX B: DETAILED METHODS FOR CHAPTER 3

B.1. Solution preparations

TEDG ERBA Binding Buffer preparation:

To make 100 ml of TEDG binding buffer, the following are added in order.

77.15 ml ddH_2O
10 ml of 4 M KCl
1 ml of 1 M Tris-HCl
10 ml of glycerol
0.1 ml of 1 M sodium molybdate
0.75 ml of 200 mM EDTA
1.0 ml 100 mM PMSF
15.4 mg DTT
1 g Bovine Serum Albumin (BSA)

Mix and adjust the solution to pH 7.4 using 1N NaOH. The final concentrations will be: (10 mM Tris-HCl, pH 7.4; 1 mM DTT; 10% glycerol; 10 mg/mL BSA; 400 mM KCl; 1 mM PMSF; 1 mM sodium molybdate; 1.5 mM EDTA)

Unlabeled 17β-estradiol preparation:

The unlabeled E2 is supplied as a powder. Weigh out 2.72 mg of E2 and add to 1 ml 100% ethanol. This is a 10 mM stock solution. Dilute this 1:1000 to get 10 μM or 10^{-5} M stock. Store at -20°C until ready to use.

0.01 μM [^{3}H]-E2 solution preparation:

Prepare a 0.1 μM or 10^{-7}M stock by pipetting 1 μl of the original 1.0 mCi/ml stock solution for every specific activity unit (Ci/mmol) and diluting this to 10.0 mls with ethanol. Thus, when the ^{3}H-E2 stock is 140 Ci/mmol specific activity, you should pipette 140 μL into a vial containing 9.86 ml of ethanol. Invert this to mix. This is the 0.1 μM stock, store at -20°C.

B.2. Human Estrogen receptor range finding assay procedure

In a 20 ml scintillation vial, add 4.5 ml of opti-fluor scintillation cocktail and 10 μl of 0.1 μM [^{3}H]-E2 solution. Read on scintillation counter and complete the following calculation to determine volume needed for assay:

2.22 * 10^{12} dpm/Ci * specific activity = dpm/pmole
2220 * *140 Ci/mmol* = 310800 dpm/pmole (insert your spec. activity from supplier)
310800 * 0.004 pM = 1243.2 dpm/ul

For a 1.3 ml sample: 1243.2 dpm/μl * 1300 μl = 1616160 dpm's needed to give 1 nM in the well.

1616160 dpm in 1.3ml/ your dpm reading from scint. Counter = volume in μl needed for each sample

Example: 1616160 dpm in 1.3 ml/22,808 dpm/μl = 70.9 μl of [^{3}H]-E2 used

To two borosilicate glass culture tubes:

For total binding, add 70.9 μl of [^{3}H]-E2 (10^{-7} M solution)

For NSB, add 70.9 μl of [^{3}H]-E2 (10^{-7} M solution) and 70.9 μl of unlabeled E2 (10^{-5} M solution).

Dry down on speed vac. For 20 min at 45°C, setting 5.1. Once dry, reconstitute in 1.3 ml of HSB + BSA. Vortex briefly (about 30 seconds) to mix.

Into a 96-well, round-bottom plate, add:

50 μl of HSB + BSA (TEDG buffer) to all wells

25 μl of [^{3}H]-E2 to total binding wells

25 μl of unlabeled E2 + [^{3}H]-E2 mix to NSB wells

Shake for 5 min at 4°C.

To three 20 ml scintillation vials, add 25 µl of [^{3}H]-E2 and store at 4 °C overnight. These are for the 'total added' measurement.

Make receptor dilutions once receptor is fully thawed on ice and gently mixed (lightly by pipette or slow inversion):

Stock 1,000,000 cell equivalents/ml →

(A) 1:10 dilution by adding 44 µl stock to 400 µl TEDG (1,000,000 cell eq./ml)

2500 cell eq. in assay →

(B) 1:20 dilution by adding 220 µl to 220 µl TEDG (50,000 cell eq./ml) *1250 cell eq. in assay* →

(C) 1:40 dilution by adding 220 µl to 220 µl TEDG (25,000 cell eq./ml) *625 cell eq. in assay* →

(D) 1:80 dilution by adding 220 µl to 220 µl TEDG (12,500 cell eq./ml) *312 cell eq. in assay* →

(E) 1:160 dilution by adding 220 µl to 220 µl TEDG (6,250 cell eq./ml) *156 cell eq. in assay* →

(F) 1:320 dilution by adding 220 µl to 220 µl TEDG (3,125 cell eq./ml) *78 cell eq. in assay*

Add 25 µl of each concentration to corresponding row and shake overnight at 4°C.

Weigh 0.25 g dextran coated charcoal and transfer to a 50 ml falcon tube containing 5 ml of TEDG buffer. Flip at 4°C overnight.

Range Finding Day 2

Remove 96-well plate and charcoal slurry from shaker. Add 50 µl of charcoal surry to all wells. Shake plate, covered, at 4°C for 10 min. Centrifuge plate at 1000 rpm, 4°C, for 5 min.

Add 4.5 ml scintillation cocktail to labeled 20 ml scintillation vials. Tilt well to 45 degrees and pipette 25 μl of sample from well into the correspondingly labeled scintillation vial. Swirl to mix the vial. Read all vials on scintillation counter for ^{3}H, with 2 min run times and 2 runs per sample (protocol 13).

B.3. Estrogen Receptor Binding Assay (ERBA)

Day 1:

[^{3}H]-E2 Solution Preparation:

Using the calculation from the range finding assay, 114.1 μl of 10^{-7}M [^{3}H]-E2 stock solution is added by pipette to a borosilicate glass tube. This is dried down on the speed vac. for 20 min at 45°C, setting 5.1. To this, 2.7 ml of TEDG binding buffer is then added and the solution is vortexed then kept on ice during use. This will result in a final concentration of 1 nM in the well.

Unlabeled E2 Solution for Non-Specific Binding (NSB) Preparation:

From the 1 x 10^{-5} M stock in ethanol, 4 μl are added by pipette to a borosilicate glass tube and dried down on the speed vac. for 20 min at 45°C, setting 5.1. This is reconstituted with 200 μl of TEDG binding buffer and 1.3 μl of 100% ethanol. This solution is vortexed to mix and kept on ice during use.

Chemical Stock Preparations:

The stock solutions of chemicals to be tested are made by first preparing a 1 mM stock. This is then diluted into 100% ethanol to make the following concentrations.

Test Chemical

Stock []	Final [1x] in experiment
100 μM	330 nM
30 μM	100 nM
10 μM	33 nM
3 μM	10 nM
1 μM	3.3 nM
300 nM	1 nM
100 nM	0.33 nM
30 nM	0.1 nM
10 nM	0.033 nM
3 nM	0.01 nM

From each of these stock solutions, 1.1 μl added by pipette to a borosilicate glass tube containing 166 μl of TEDG binding buffer, on ice. These working stocks are then briefly mixed by vortex and kept on ice until use. The stock chemical solutions in ethanol are stored at -20 °C until further use.

E2 Standard Curve Preparation:

From a 1mM stock of E2 solution in 100% ethanol, the following dilutions are made. Each concentration is diluted in 100% ethanol and then briefly mixed by vortex. Following the stock preparation, 1.1 μl of each stock is added by pipette into a corresponding borosilicate glass tube containing 166 μl of TEDG binding buffer. This is then briefly mixed by vortex and kept on ice until use. All stocks in ethanol are stored at -20 °C until further use.

E2 Standard Curve

Stock [ ]	Final [1x] in experiment
30 μM	100 nM
10 μM	33 nM
3 μM	10 nM
1 μM	3.3 nM
300 nM	1 nM
100 nM	0.33 nM
30 nM	0.1 nM
3 nM	0.01 nM

Estrogen Receptor Solution:

The range finding assay showed that a receptor concentration of 312 cell equivalents/ml would result in 8.8% binding. To make this dilution, the original 1,000,000 cell equivalents/ml stock solution was thawed on ice. This was gently mixed by slow inversion or pipette before 50 μl was removed and added to 3,950 μl of TEDG binding solution in a borosilicate glass tube. This was gently mixed and kept on ice until use.

Plate Preparation Procedure:

While on ice, the following solutions are added to a 96-well, round bottom plate.

For the total binding wells, add 50 μl of ethanol and TEDG binding buffer (made by added 1.3 μl of 100% ethanol to 200 μl TEDG binding buffer and mixing) to each of three wells.

For the non-specific binding wells, add 50 μl of unlabeled E2 solution to each of the three wells.

For all chemical dilution wells, add 50 μl of the working stock solution to each of three wells per dose.

Using a repeat pipettor, add 25 µl of the 4x [^{3}H]-E2 stock solution to each well. Add 25 µl to each of three scintillation vials as well, for the total added to each well calculation. Keep this at 4°C until their use on day 2.

Shake the plate lightly on speed 2.5 for 10 minutes at 4 °C.

Using a repeat pipettor, gently add 25 µl of the diluted 4x receptor stock solution to each well.

Cover the plate with plate sealing film and lightly shake the plate on setting 2.5 overnight, at 4°C.

Solutions are added in the following scheme to the 96-well plate.

Table B.1. Diagram of the competitive binding 96-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	E2 100 nM			Total Counts [^{3}H]-E2			DCAN 0.33 nM			MA 10 nM		
B	E2 33 nM			NSB E2			DCAN 0.1 nM			MA 3.3 nM		
C	E2 10 nM			DCAN 330 nM			DCAN 0.033 nM			MA 1 nM		
D	E2 3.3 nM			DCAN 100 nM			DCAN 0.01 nM			MA 0.33 nM		
E	E2 1 nM			DCAN 33 nM			BLANK			MA 0.1 nM		
F	E2 0.33 nM			DCAN 10 nM			MA 330 nM			MA 0.033 nM		
G	E2 0.1 nM			DCAN 3.3 nM			MA 100 nM			MA 0.01 nM		
H	E2 0.01 nM			DCAN 1 nM			MA 33 nM			BLANK		

E2 = estradiol; DCAN = dichloroacetonitrile; MC = mucochloric acid

5% Dextran Coated Charcoal Solution Preparation:

The dextran coated charcoal solution is prepared by suspending 0.325 g of dextran-coated charcoal in 6.5 mls of TEDG binding buffer in a 50 ml falcon tube. This is caped and parafilmed, then placed on a rotary mixer overnight at 4 °C.

Day 2:

To harvest the receptors, the plate and charcoal slurry are removed from the cold room. To each well, 50 μl of the charcoal solution is added using a repeat pipettor. The plate is then returned to the shaker for 30 minutes at 4 °C. During this time, 4.5 mls of opti-fluor scintillation cocktail is added to each scintillation vial labeled to correspond with each well on the plate, as well as to the three total added vials prepared on day 1. The plate is then removed and centrifuged at 2200 rpm, 4 °C, for 5 minutes. With the plate at a 45-degree angle, 25 μl of each sample is removed and added by pipette to the corresponding scintillation vial. These are briefly swirled to mix and the outside of the vials is wiped with methanol to prevent misreading on the scintillation counter.

The vials are then read on the scintillation counter to determine the estrogenic activity of each of the two test chemicals, as compared to the standard E2 curve.

APPENDIX C: OPTIMIZATION STUDIES OF A VERTICAL FLOW STALITE FILTER STUDY 1 DATA

APPENDIX C.1. Study Design

An initial study was completed with columns run in the order of hypoxic-aerobic-hypoxic, based on the design of an operating wetlands system in Chatham County, NC. This study was undertaken to prepare for future work in our laboratory that was to utilize a field site with a similar operational design. The alternation between hypoxic and anaerobic zones is to enhance the nitrification/denitrification process for the removal of ammonia, nitrate, and nitrite, which can be harmful to receiving water bodies and are important aspects in wetland design (1-3). The use of hybrid systems such as this area also becoming increasingly popular, as they have been shown to have better removal for some target emerging organic contaminants (4). The study was run for 14 days for each dose interval, as described in section 4.3. The interval period was meant to allow for stabilization of the column effluent samples, with consistent results observed between sampling points. The consistent removal at each time point for the target compounds and TOC, however, indicated that a shorter experimental period could be used for each dose interval in study 2. The period required to reach consistent readings for the TN, DN, and DOC, indicated that a 9-day study would be suitable to accurately indicate the removal of the columns for each dose interval. The poor target chemical removal from the 48 h interval, however, along with the inconsistency in the water quality indicators measured for those samples over the course of that study, resulted in it being excluded from study 2 and replaced by the 8 h saturated interval. These results are described briefly in Appendix C, section C.2.

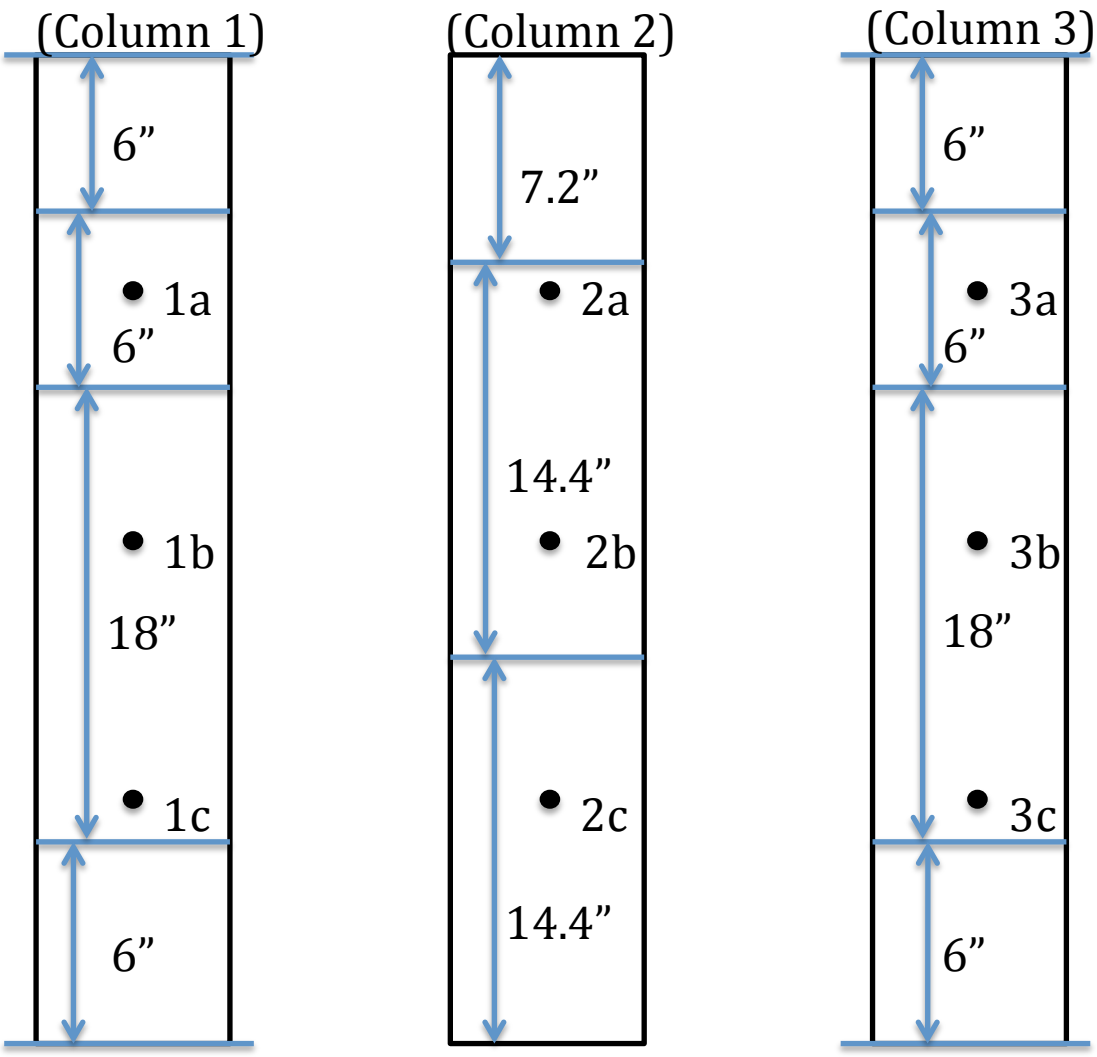

Figure C.1. Diagram of the columns for Trial 1 packed with layers of (A) 3/8”, (B) 5/16”, and (C) sand-sized Stalite® material for the column experiments showing sampling ports 1a-c, 2a-c, and 3a-c.

APPENDIX C.2. Preliminary Study 1 Results

The study analysis was carried out as previously described in section 4.3. Results are shown below for the chemical analysis, TOC, DOC, TN, DN, ammonia, nitrate, and nitrite measurements of these preliminary experiments. YES assays were not run on these samples. The behavior of these chemicals and water quality indicators are presented for each time point over the course of the study to demonstrate the equilibration period of the treatment of the column effluent, which informed the length of the intervals used for

study 2. The results from these 14-day dose intervals indicate that the removal of the target chemicals after column 3, which is the result of the cumulative exposure to all three columns and is presented for each time point as a 24 h composite sample of all effluent collected in that period, are consistently high for all compounds except CBZ and ATZ, which is expected to be due to the reasons described for study 2. The results for each of the samples representing the overall removal from the system at each time point in the interval are shown in Figures A. are shown in The percent of each compound removed, relative to the concentration spiked (100 μg/L) for the final time points from columns 1, 2 and 3 are presented in Table C.1. The average overall removal after column 3, which is again after treatment with all of the columns, is shown as well.

Table C.1. Target chemical percent removal for trail 1 experiments of 8, 24, 36, and 48 h dose intervals at the final time points for columns 1, 2, and 3 and the average daily removal for column 3. (LOQ= limit of quantitation, not detected).

Dose Interval (h)	Column	ATZ (%)	CAF (%)	CBZ (%)	DEET (%)	E2 (%)	4-NP (%)	TCS (%)
8	1	37	≥90	49	≥90	≥90	≥90	LOQ
	2	-10	≥90	13	≥90	n/d	≥90	LOQ
	3	-18	≥90	9	≥90	n/d	≥90	LOQ
	3 (Avg)	0.8	≥90	15	≥90	≥90	≥90	LOQ
24	1	14	≥90	37	49	≥90	≥90	≥90
	2	28	≥90	37	82	≥90	≥90	LOQ
	3	10	≥90	31	76	≥90	≥90	LOQ
	3 (Avg)	12	≥90	33	83	≥90	≥90	LOQ
36	1	7.3	64	16	67	88	≥90	≥90
	2	10	≥90	27	77	78	≥90	LOQ
	3	5.9	≥90	28	88	≥90	≥90	LOQ
	3 (Avg)	8.7	≥90	18	87	≥90	≥90	LOQ
48	1	28	≥90	37	65	89	≥90	LOQ
	2	23	≥90	38	57	≥90	≥90	LOQ
	3	12	≥90	43	-145	≥90	≥90	LOQ
	3 (Avg)	16	≥90	37	57	≥90	≥90	LOQ

These data show that the overall removal of E2, 4-NP, TCS, and CAF were high, while DEET showed consistently high removal from the final column but after columns 1

and 2 there was limited removal, which did not show trends between dose intervals. The removal of ATZ and CBZ, which are both known to be persistent water pollutants and recalcitrant, were minimal and varied. The daily percent removal results for each compound are presented in Figures C.1 – C.7 and the daily removal of TOC (Figures C.8 – C.10), DOC (Figures C.11 – C.13), TN (Figures C.14 – C.16), and DN (Figures C.17 – C.19) are presented below. These results indicate that, consistent with the chemical results, the shorter dose interval of 8h is the most effective at removal of both organic carbon and organic nitrogen.

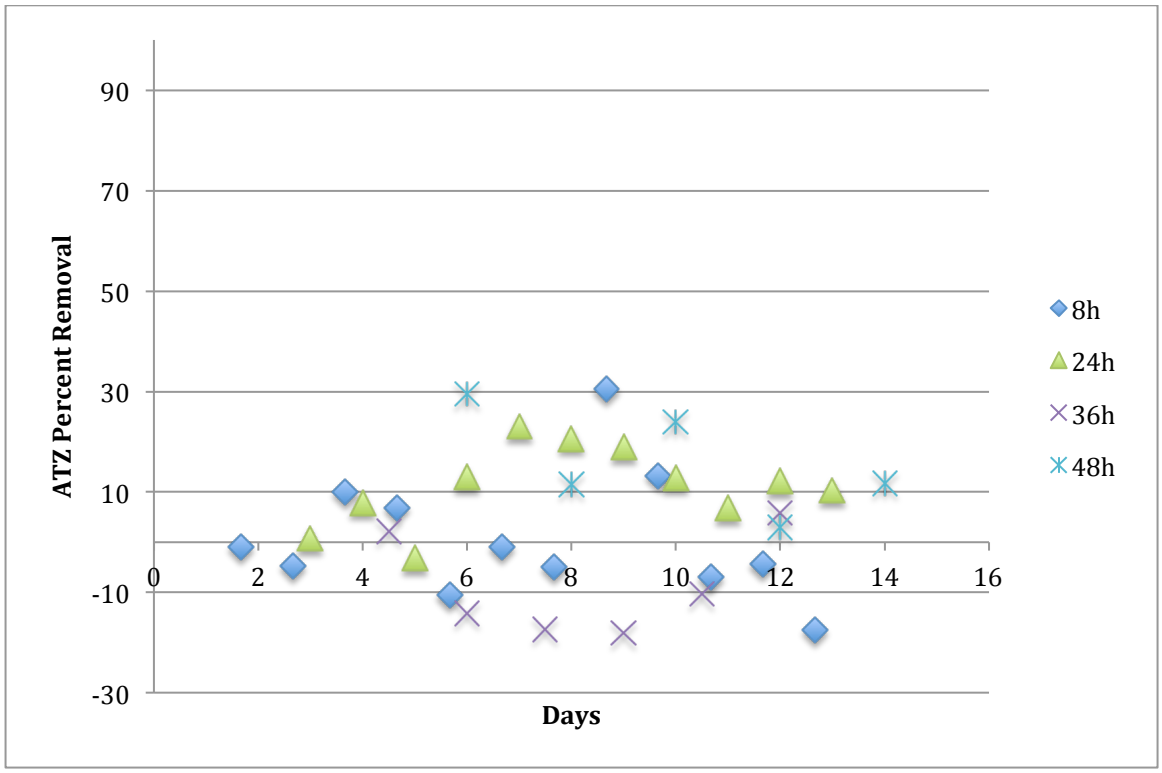

Figure C.2. Atrazine daily percent removal from column 3 for all dose intervals.

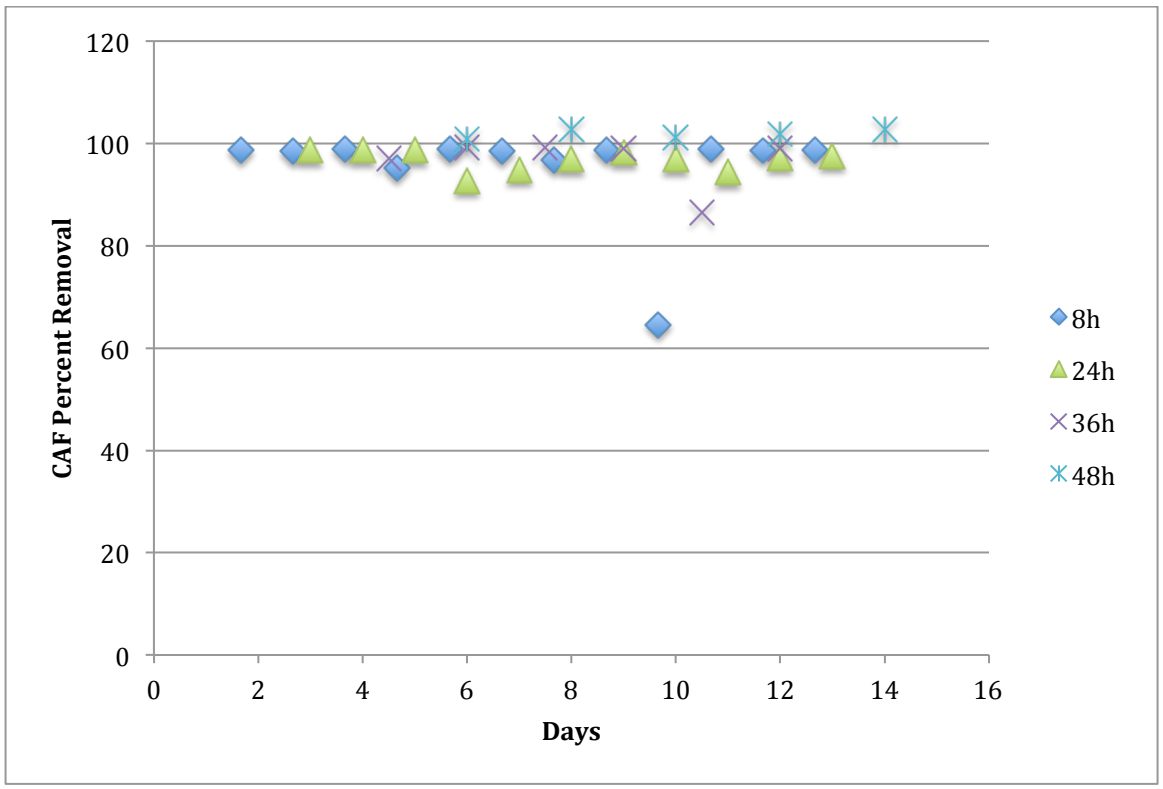

Figure C.3. Caffeine daily percent removal from column 3 for all dose intervals.

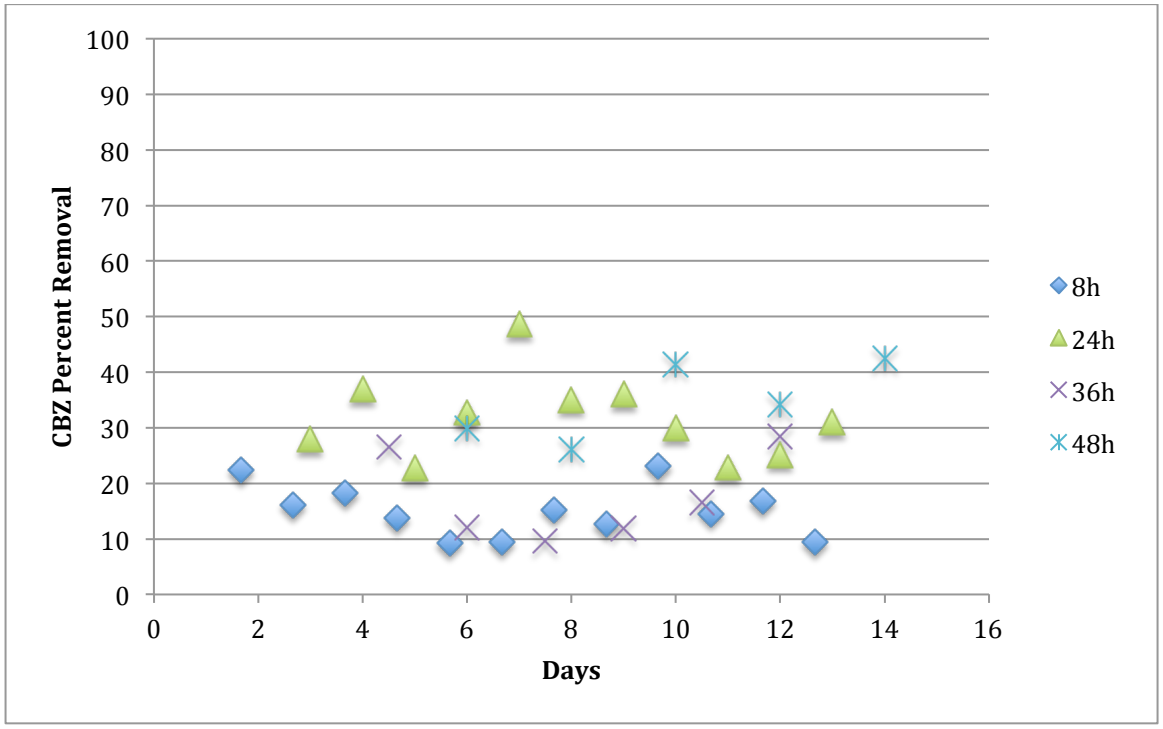

Figure C.4. Carbamazepine daily percent removal from column 3 for all dose intervals.

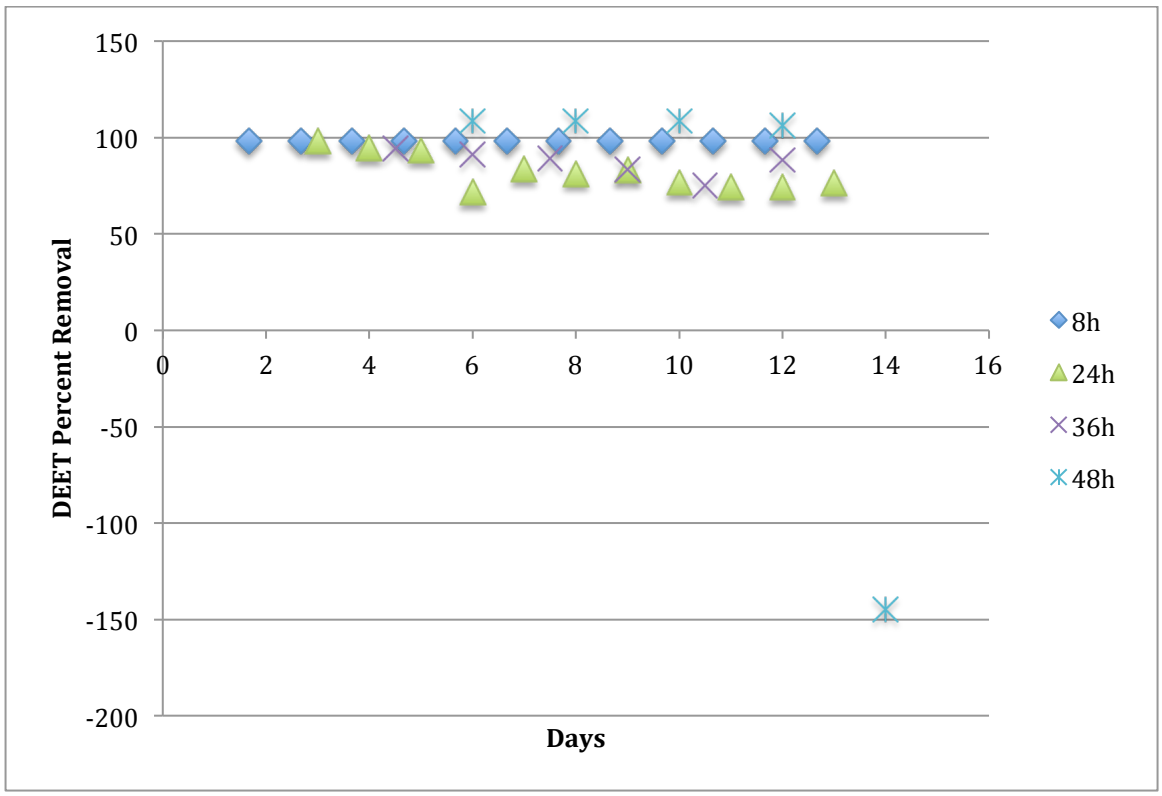

Figure C.5. DEET daily percent removal from column 3 for all dose intervals.

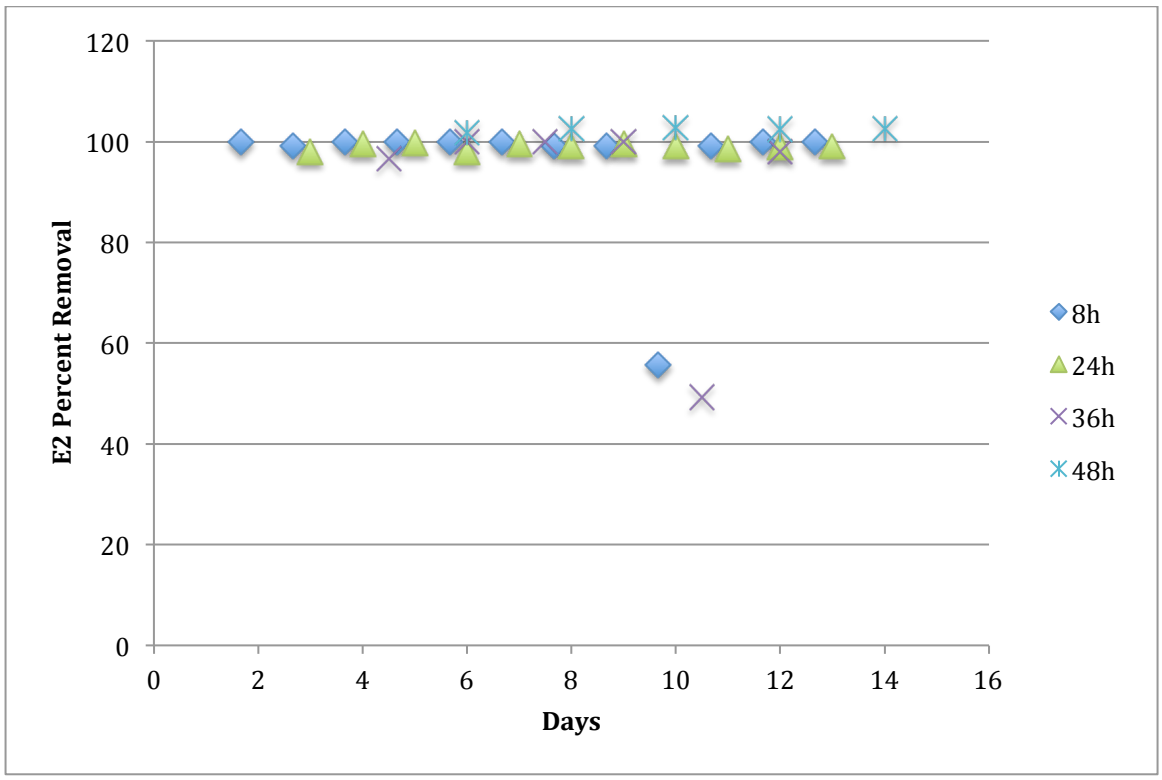

Figure C.6. 17β-estradiol daily percent removal from column 3 for all dose intervals.

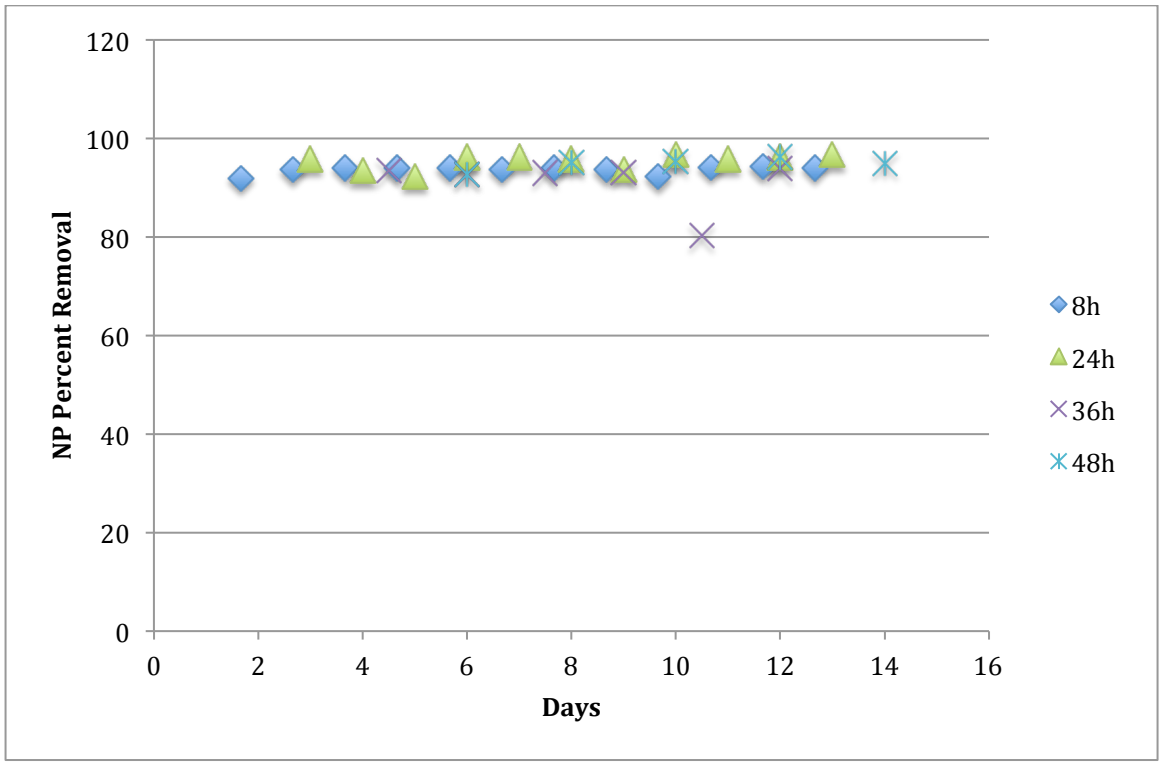

Figure C.7. Nonylphenol daily percent removal from column 3 for all dose intervals.

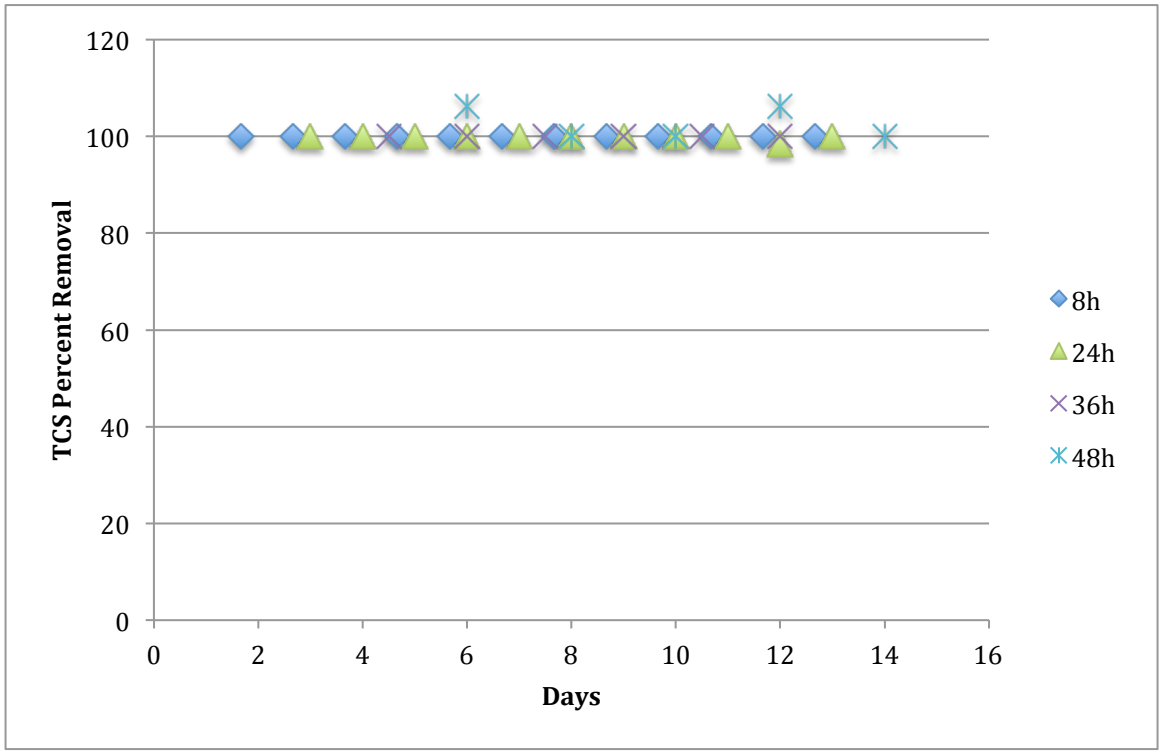

Figure C.8. Triclosan daily percent removal from column 3 for all dose intervals.

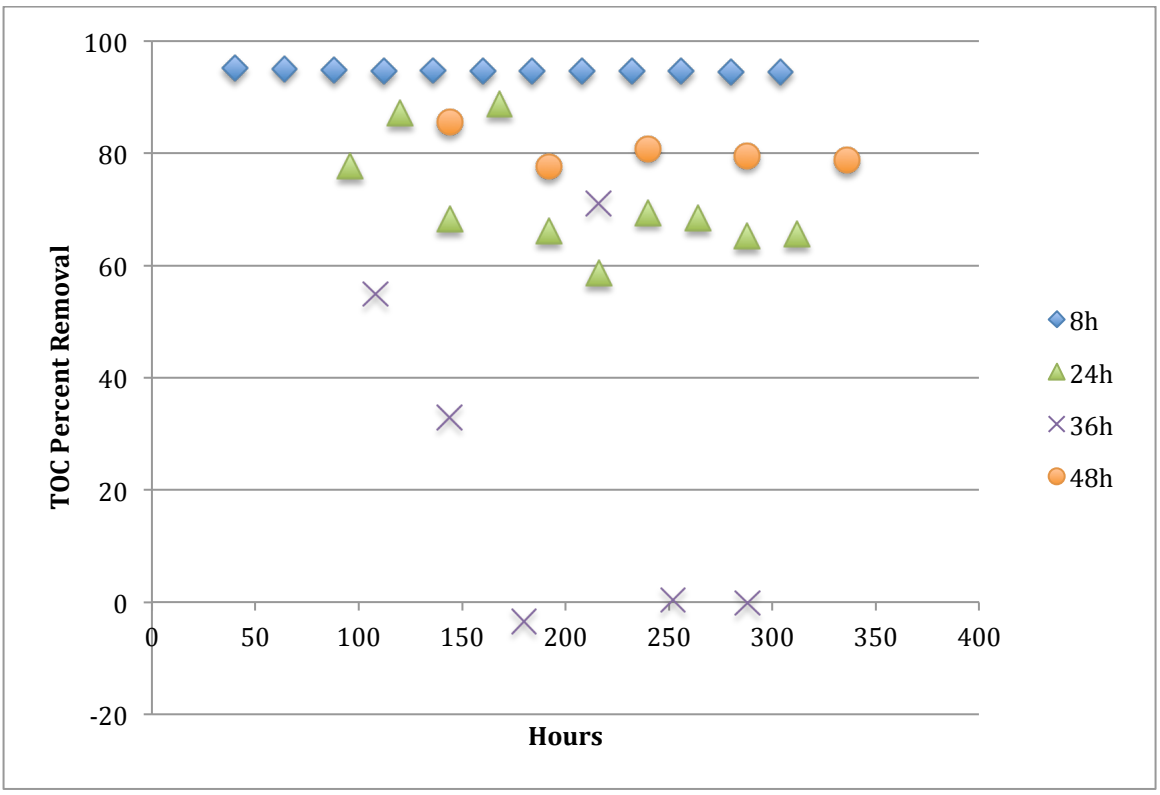

Figure C.9. The TOC percent removal for each time point after column 1 for all dose intervals.

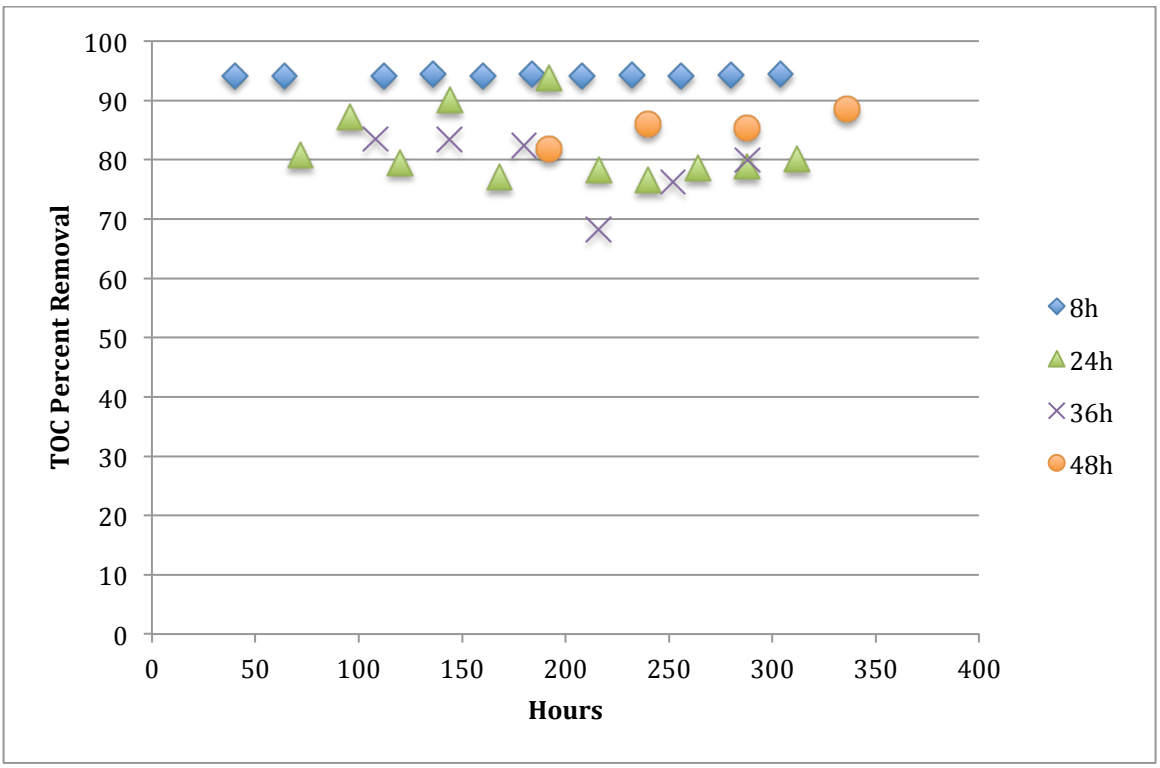

Figure C.10. The TOC percent removal for each time point after column 2 for all dose intervals.

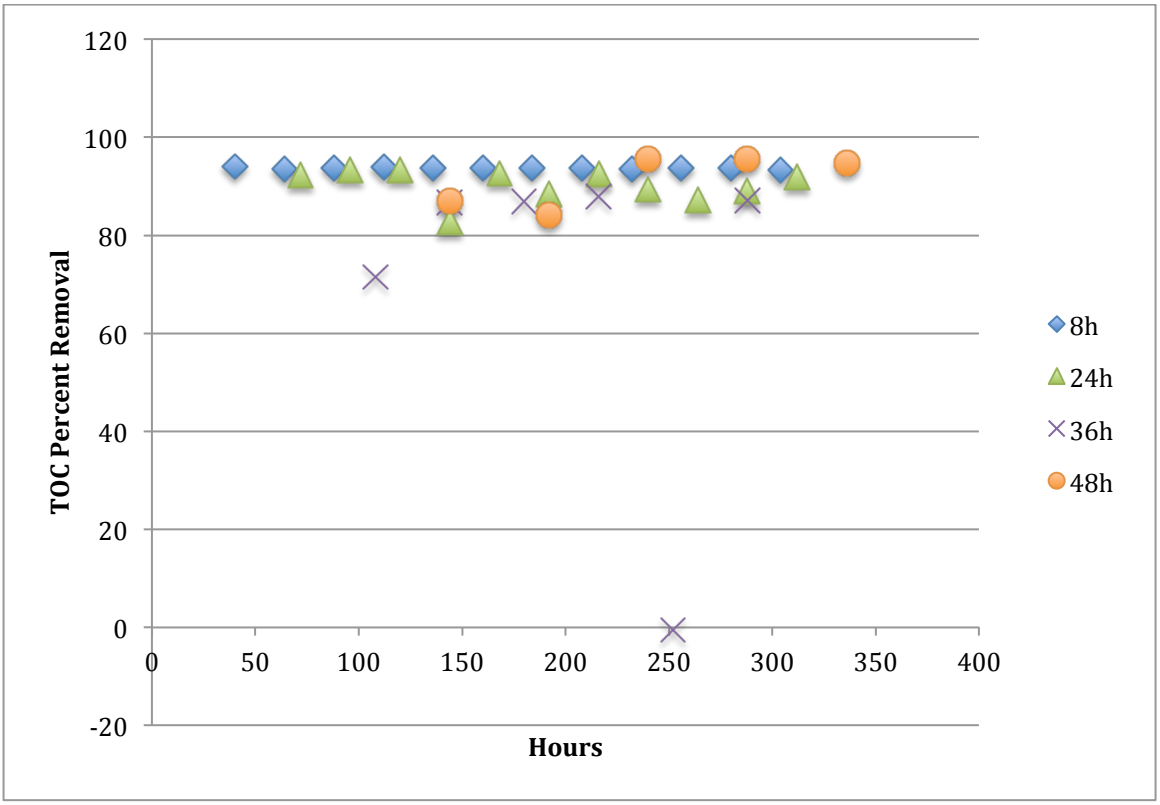

Figure C.11. The TOC percent removal for each time point after column 3 for all dose intervals.

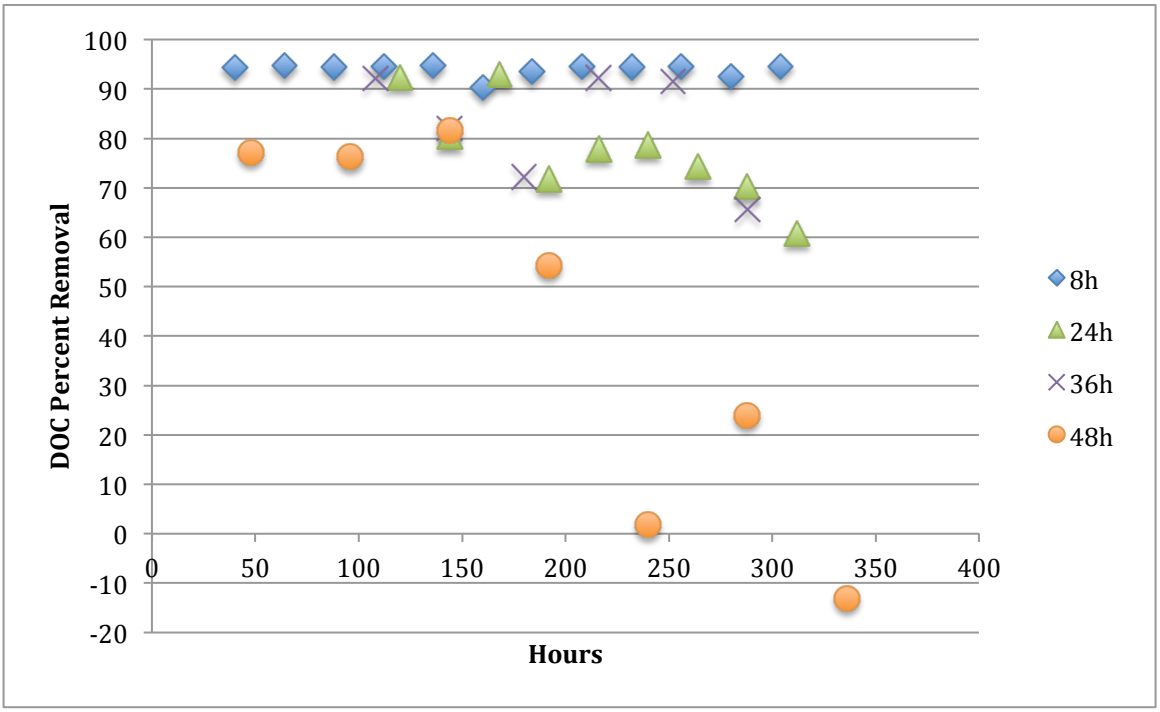

Figure C.12. The DOC percent removal for each time point after column 1 for all dose intervals.

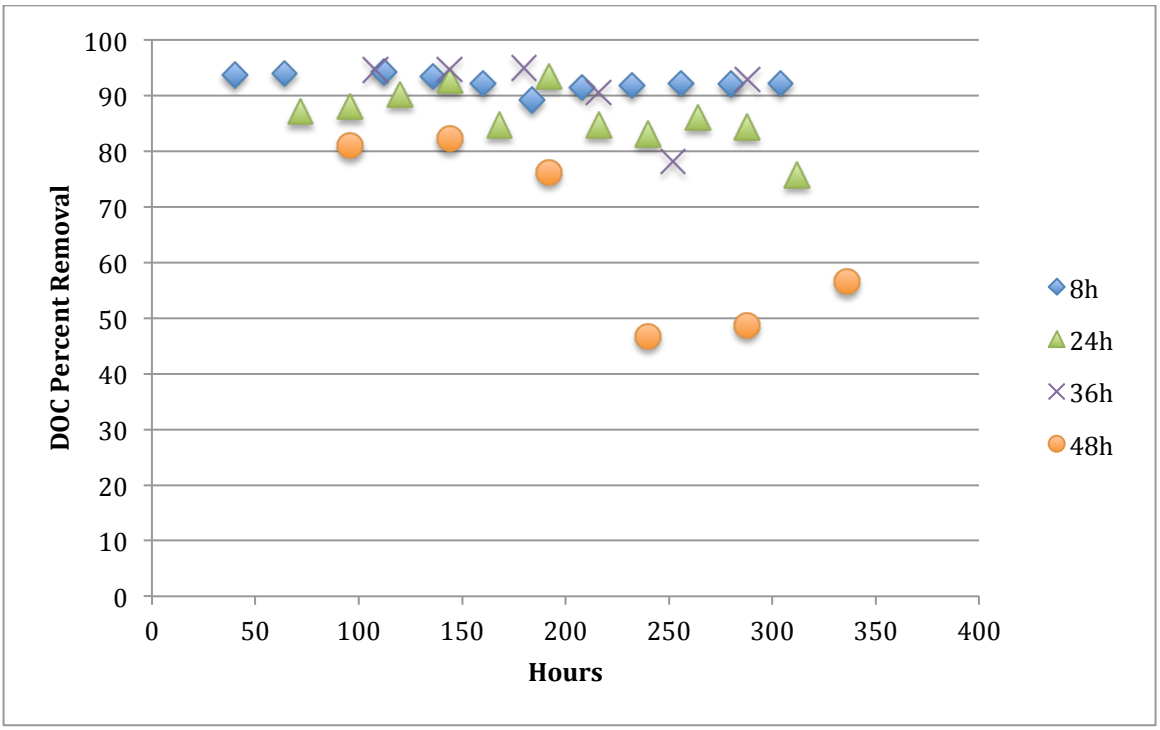

Figure C.13. The DOC percent removal for each time point after column 2 for all dose intervals.

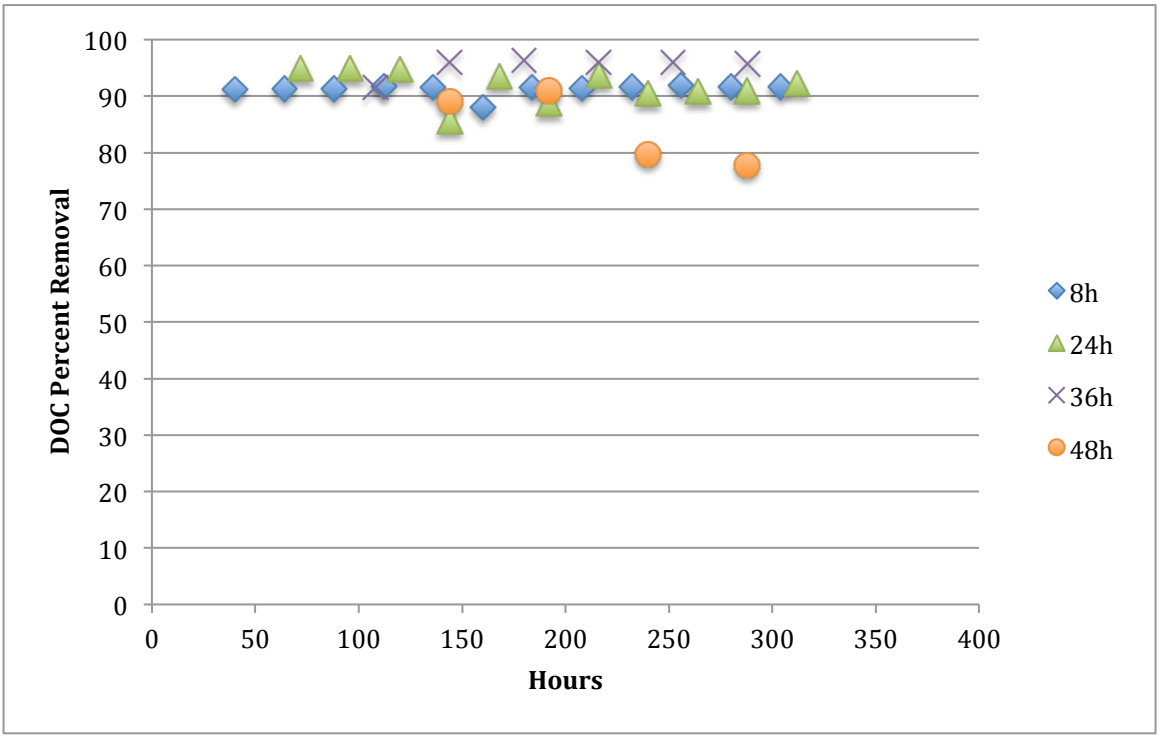

Figure C.14. The DOC percent removal for each time point after column 3 for all dose intervals.

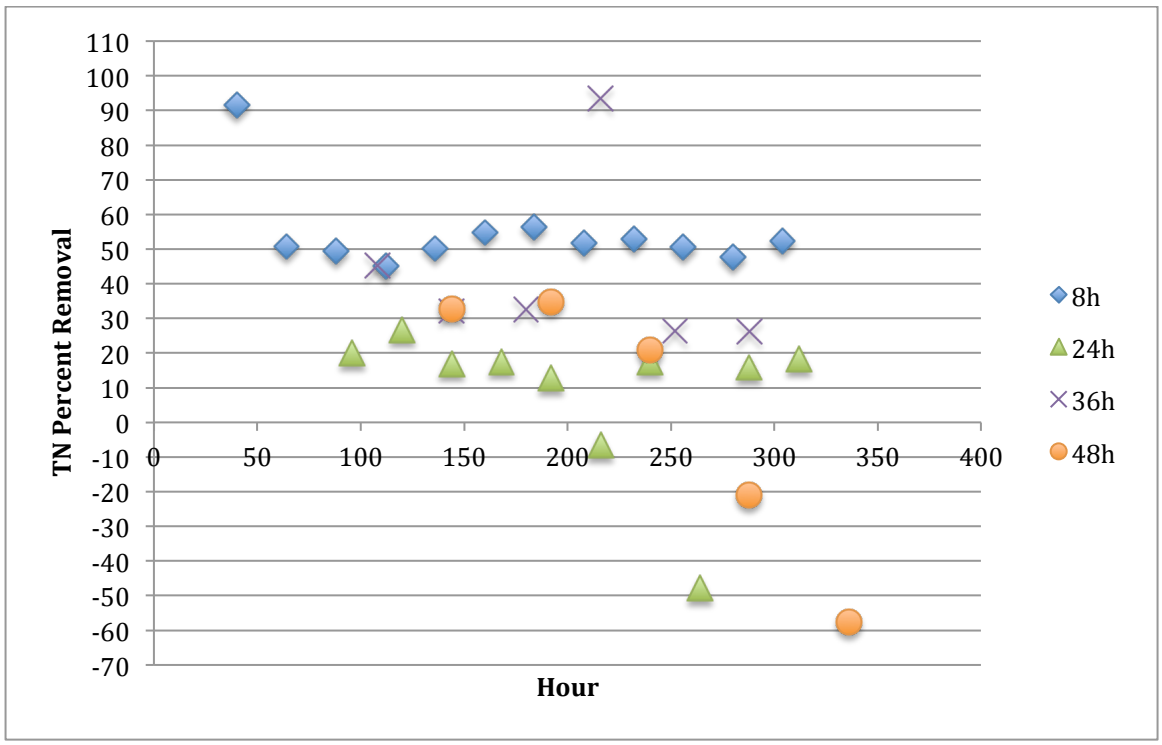

Figure C.15. The TN percent removal for each time point after column 1 for all dose intervals.

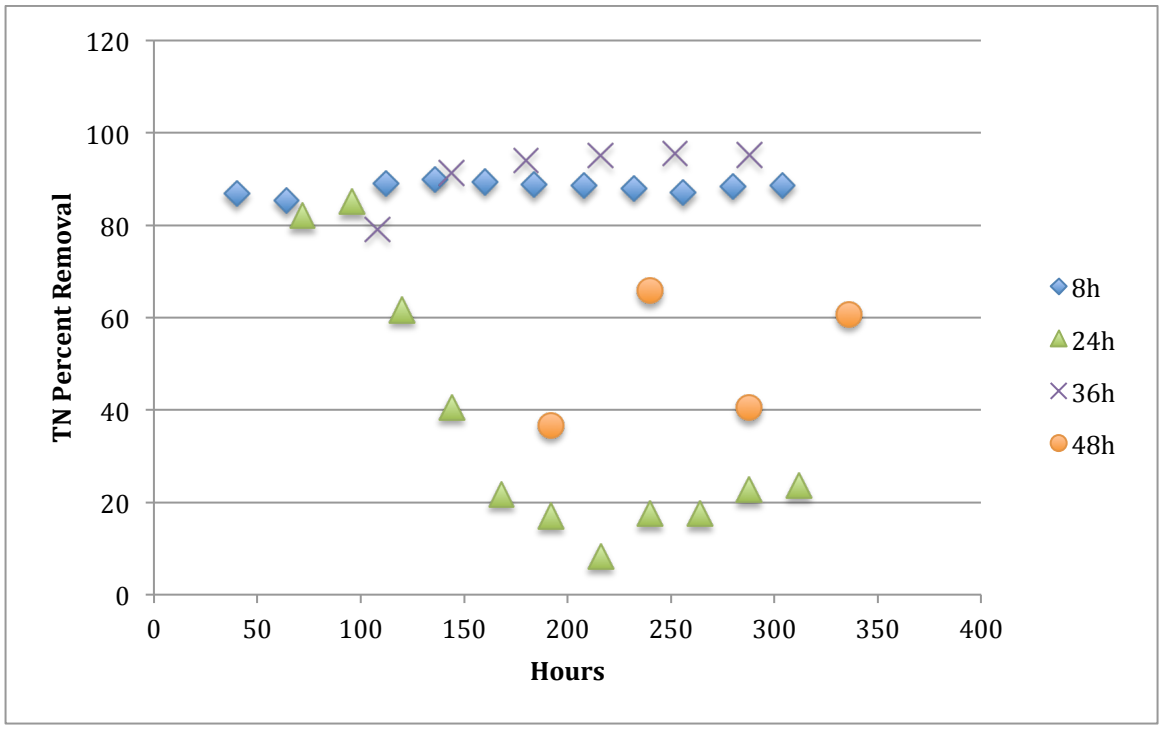

Figure C.16. The TN percent removal for each time point after column 2 for all dose intervals.

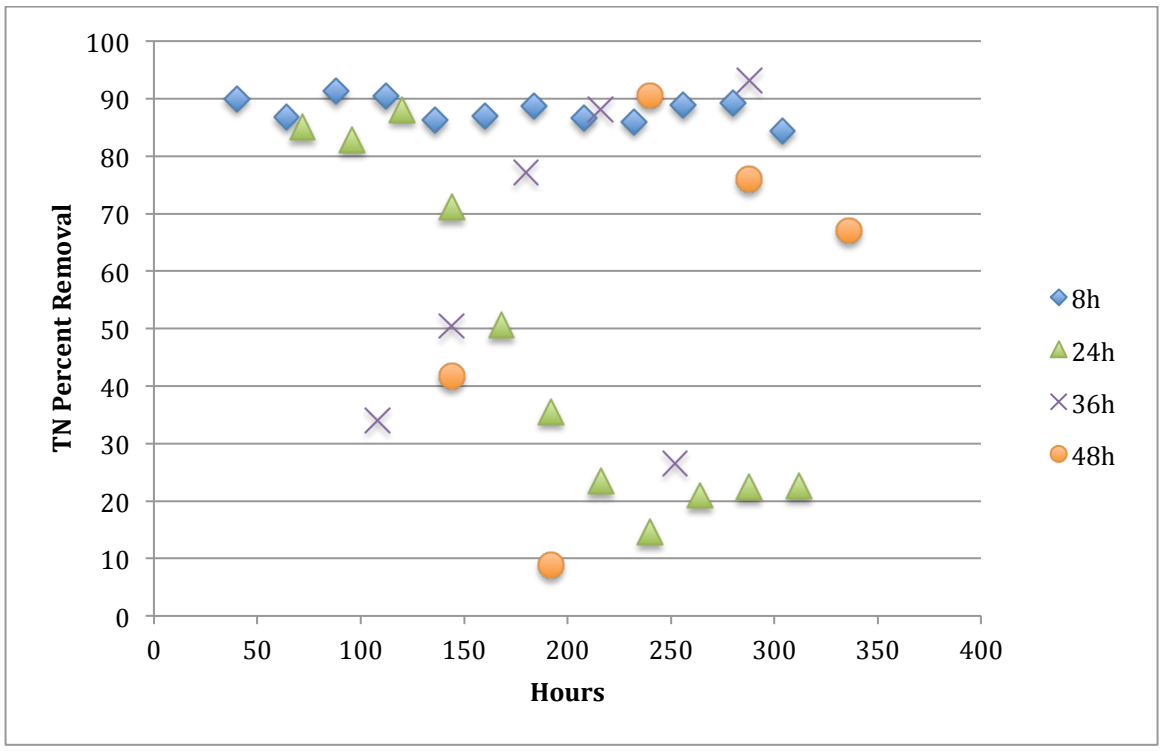

Figure C.17. The TN percent removal for each time point after column 3 for all dose intervals.

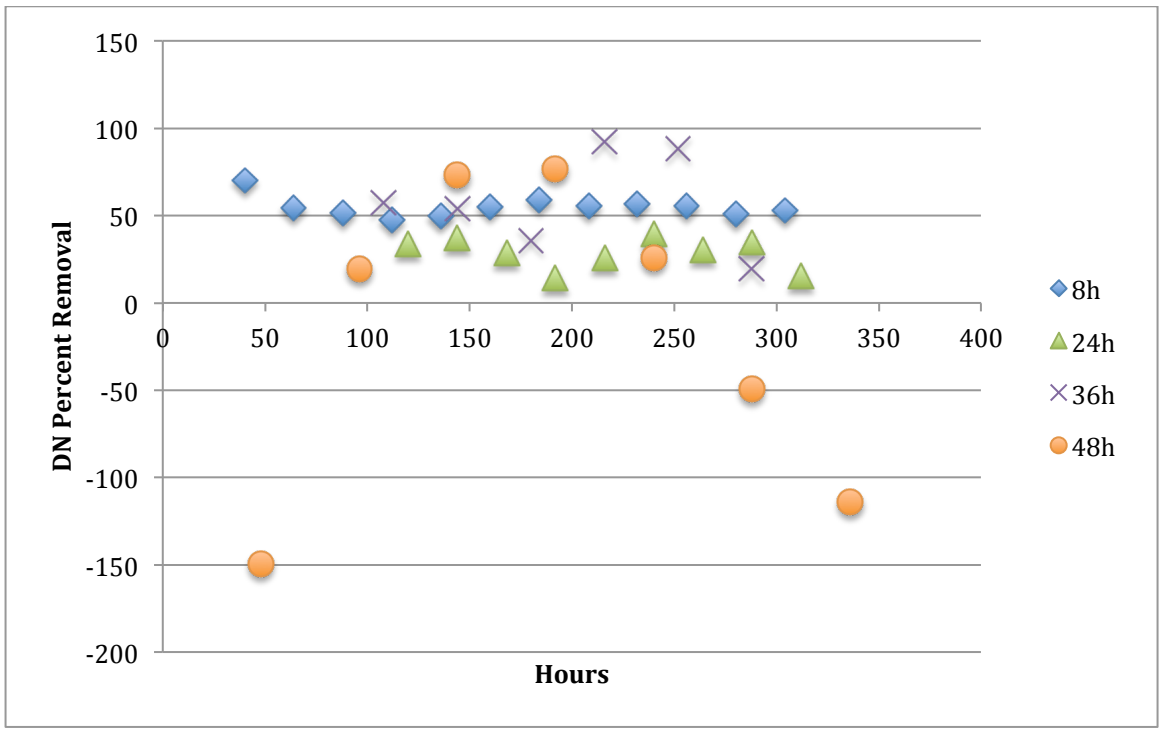

Figure C.18. The DN percent removal for each time point after column 1 for all dose intervals.

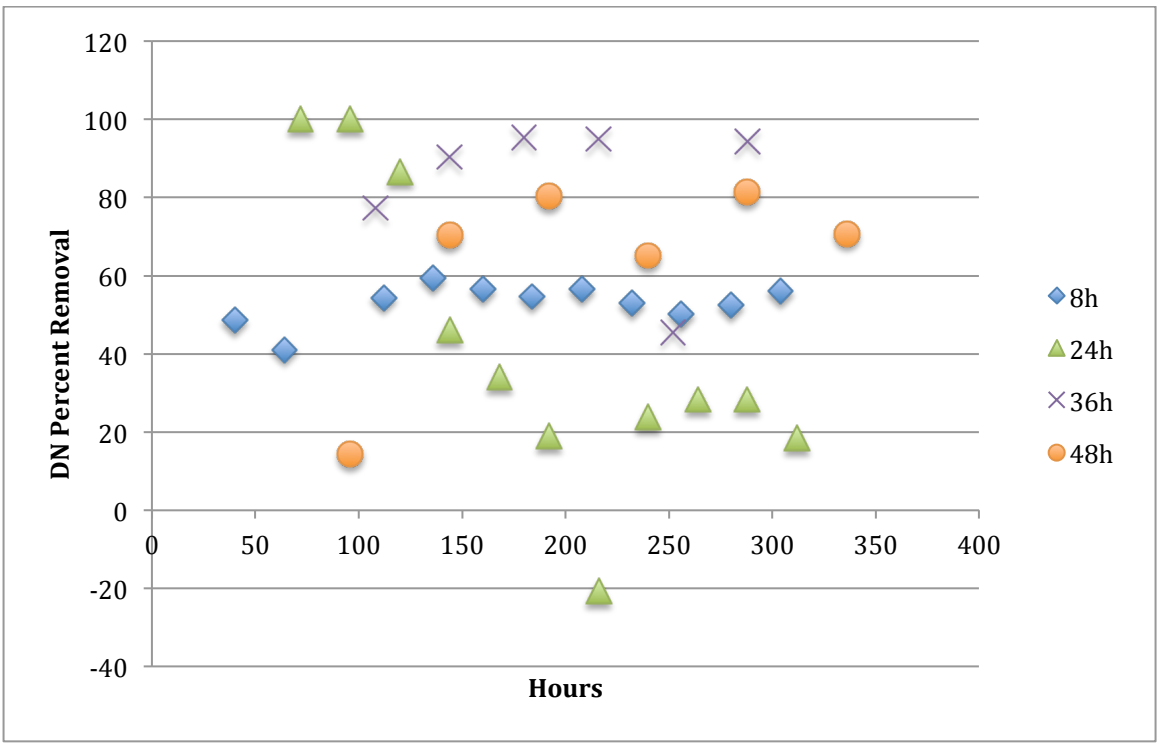

Figure C.19. The DN percent removal for each time point after column 2 for all dose intervals.

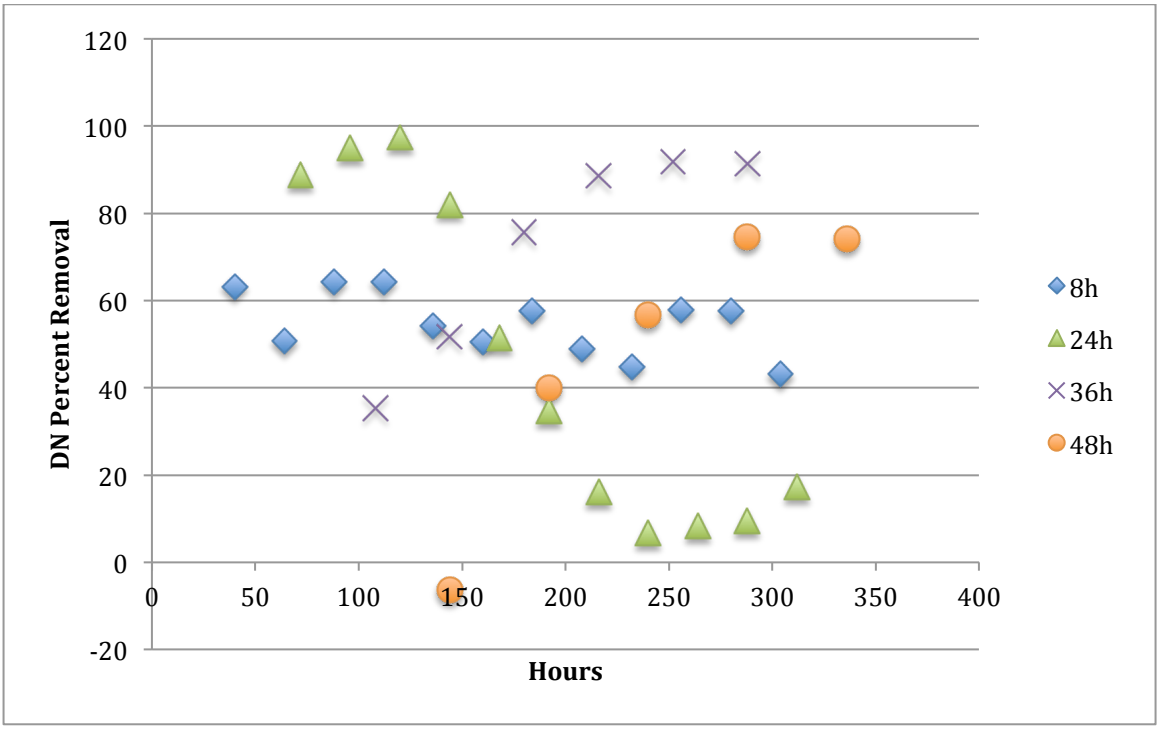

Figure C.20. The DN percent removal for each time point after column 3 for all dose intervals.

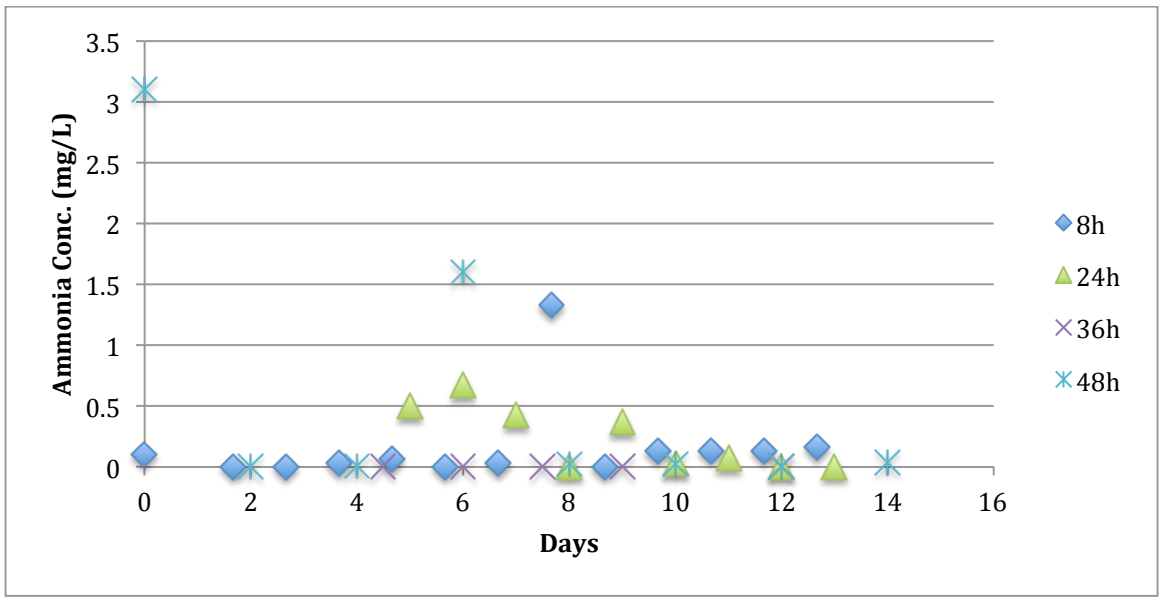

Figure C.21. The ammonia concentration in effluent samples from column 1 for each time point from each dose interval.

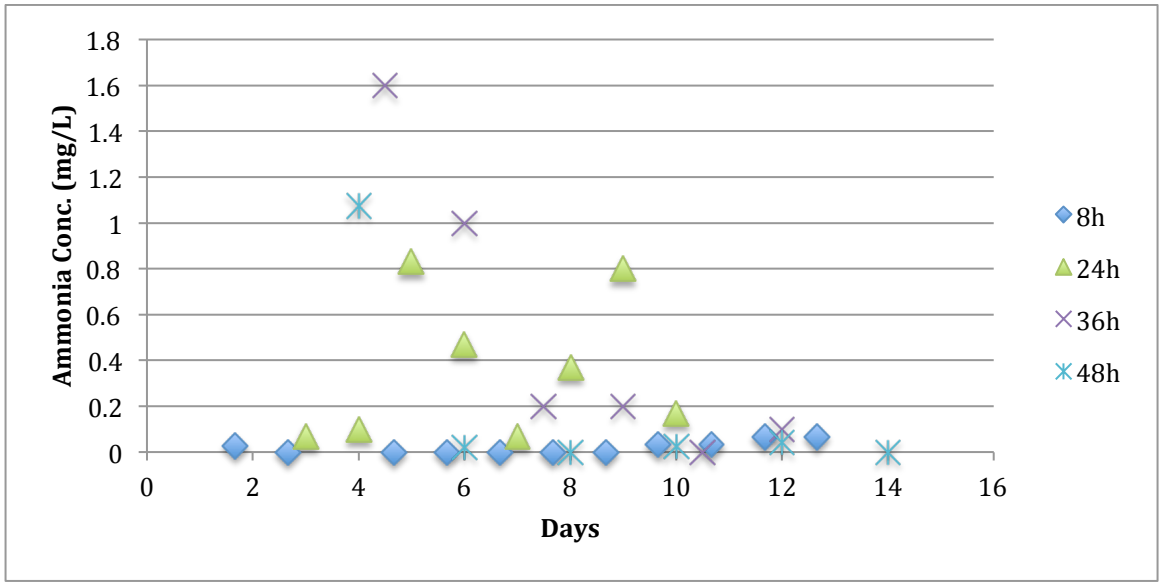

Figure C.22. The ammonia concentration in effluent samples from column 2 for each time point from each dose interval.

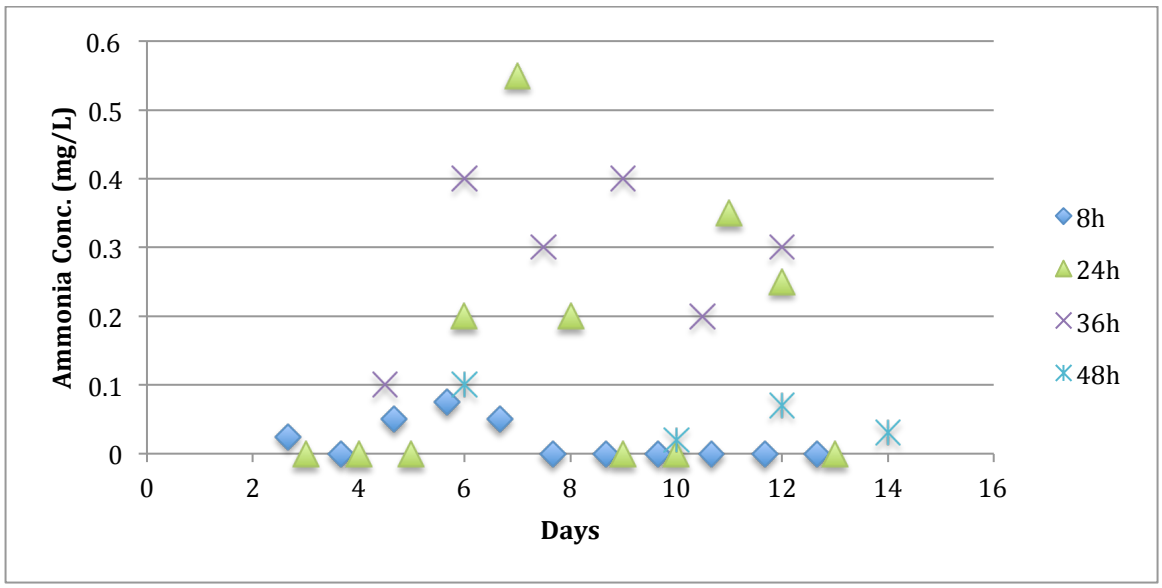

Figure C.23. The ammonia concentration in effluent samples from column 3 for each time point from each dose interval.

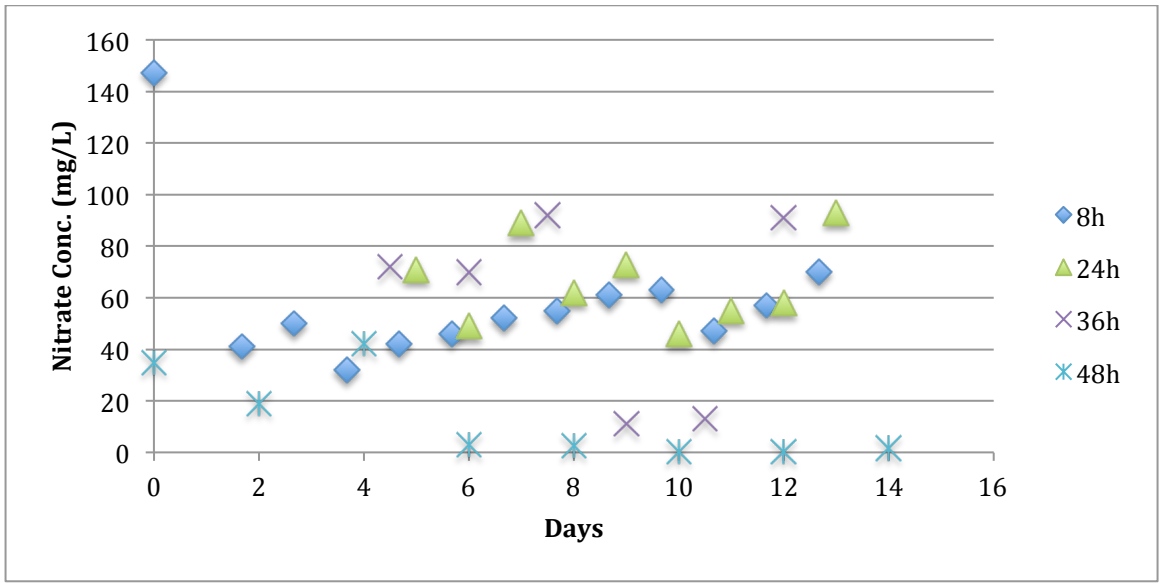

Figure C.24. The nitrate concentration in effluent samples from column 1 for each time point for each dose interval.

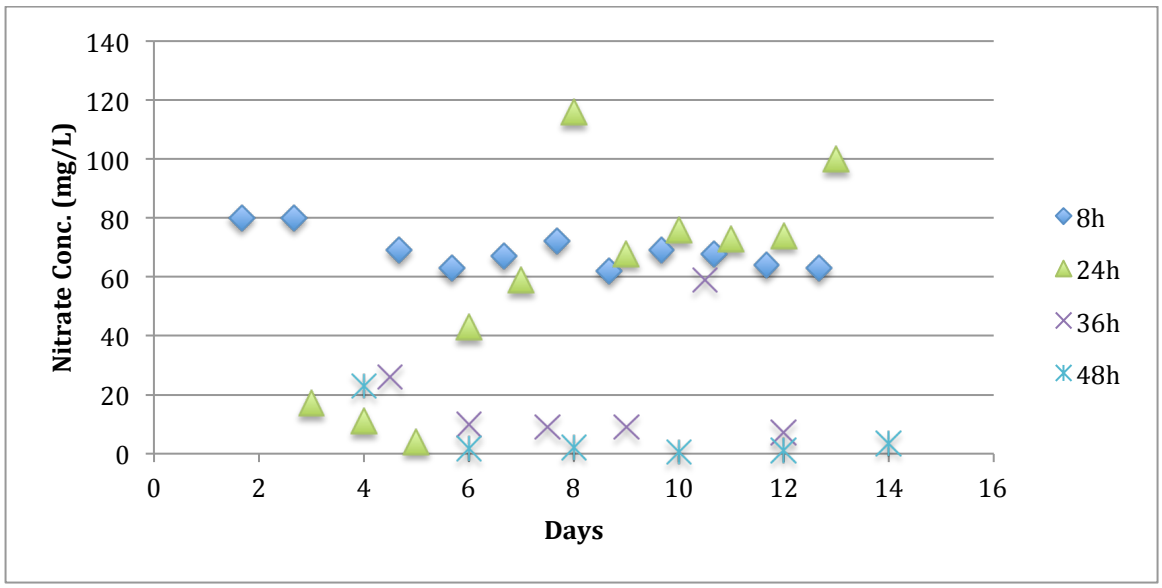

Figure C.25. The nitrate concentration in effluent samples from column 2 for each time point for each dose interval.

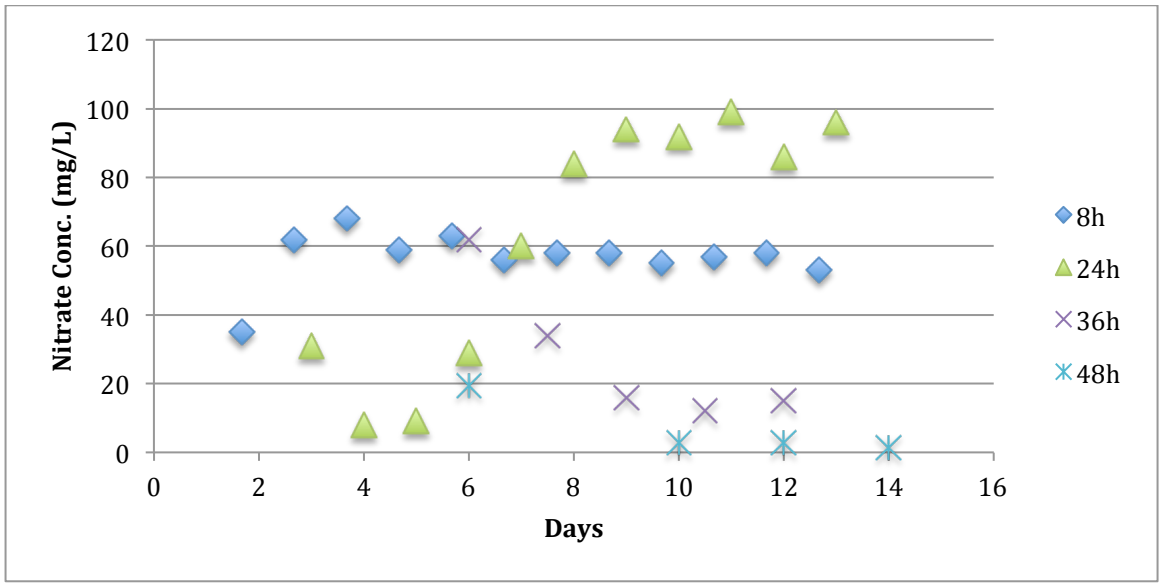

Figure C.26. The nitrate concentration in effluent samples from column 3 for each time point for each dose interval.

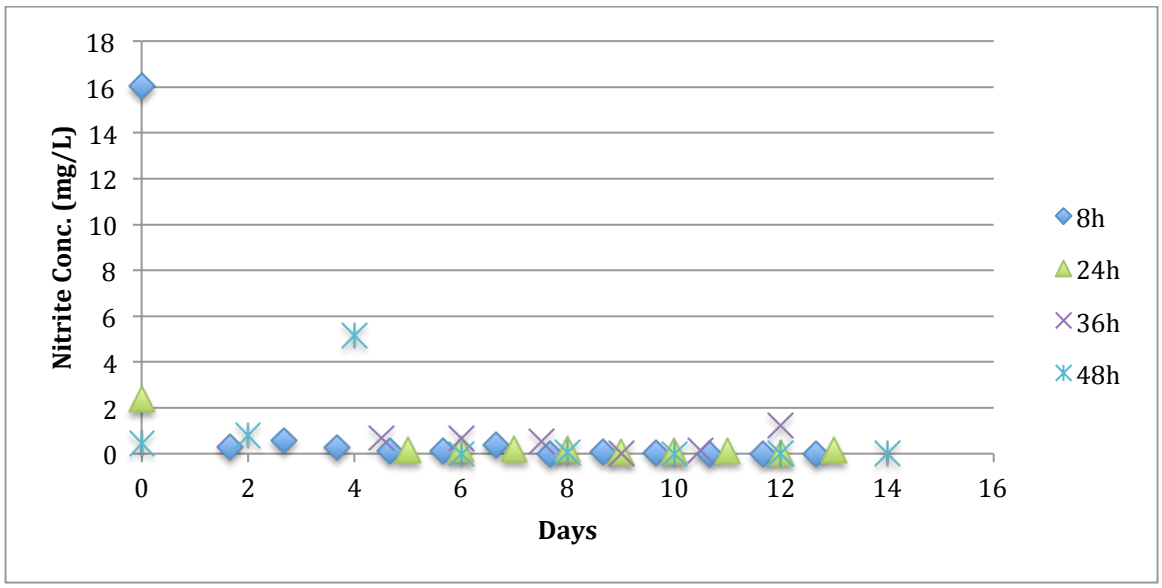

Figure C.27. The nitrite concentration in effluent samples from column 1 for each time point for each dose interval.

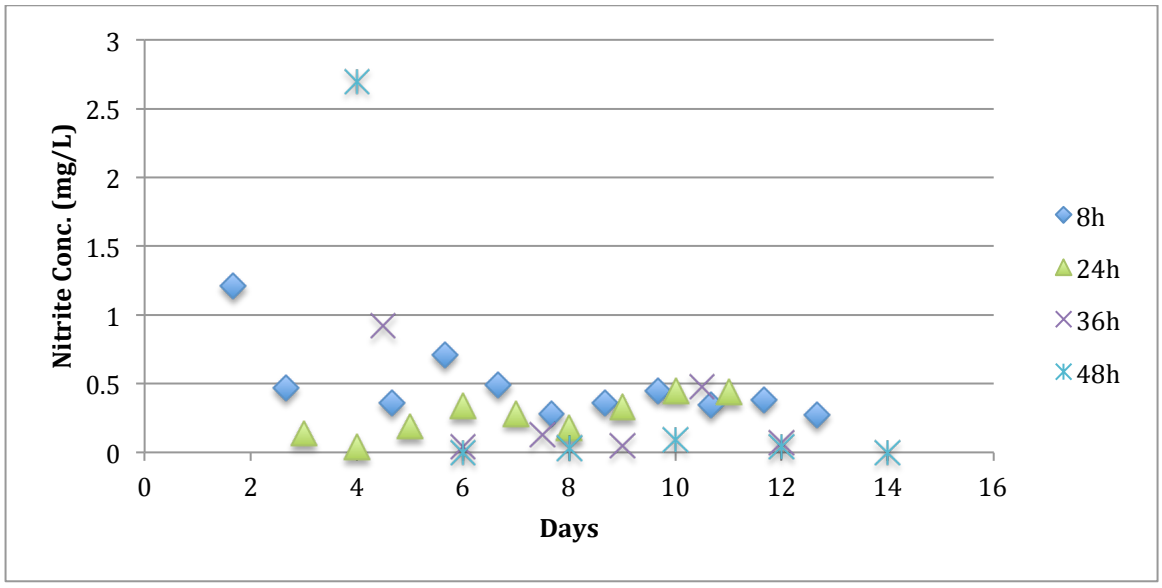

Figure C.28. The nitrite concentration in effluent samples from column 2 for each time point for each dose interval.

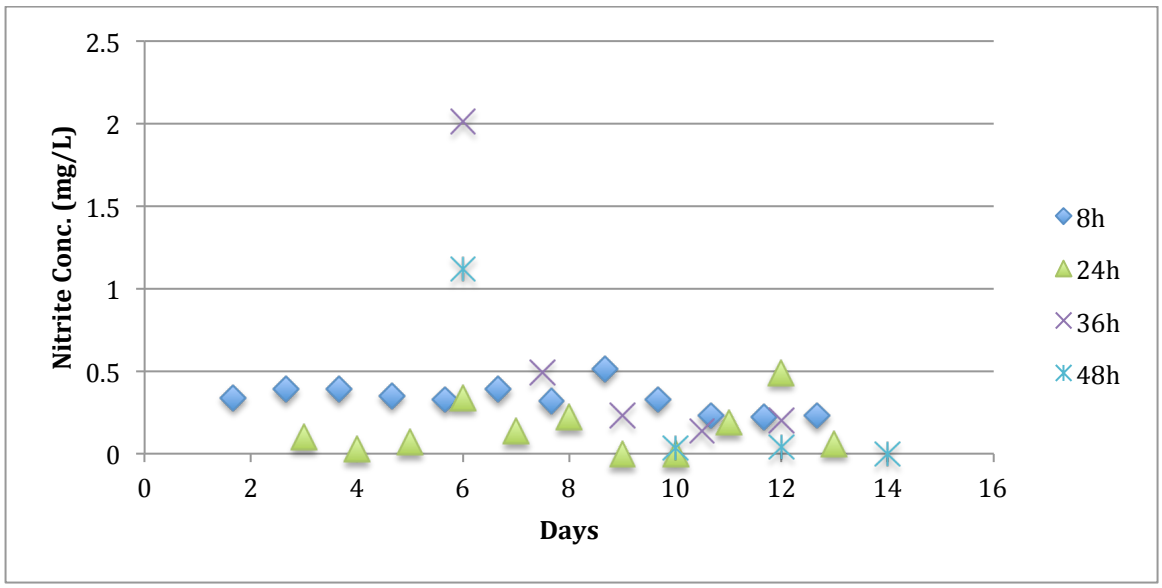

Figure C.29. The nitrite concentration in effluent samples from column 3 for each time point for each dose interval.

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1. Wu S, Kuschk P, Brix H, Vymazal J, Dong R. Development of constructed wetlands in performance intensifications for wastewater treatment: a nitrogen and organic matter targeted review. Water Research. 2014;57:40-55.

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3. Lu H, Chandran K, Stensel D. Microbial ecology of denitrification in biological wastewater treatment. Water Research. 2014;64:237-54.

4. Vymazal J. The use of hybrid constructed wetlands for wastewater treatment with special attention to nitrogen removal: a review of a recent development. Water Research. 2013;47(14):4795-811.

APPENDIX D: FULL DATA SETS FOR CHAPTER 4 STUDY 2 AND SUPPORTING YES DATA

APPENDIX D.1. Dose interval clarification.

A key to clarify the meaning of the sample codes and which specific hour in the study each point corresponds to is given below. Note, the last sample for each dose interval is listed as C3, but samples from all three columns were collected on that day.

Table D.1. Dose interval time points compared to hours and days for clarification.

Hours	Days	8h & 8h Saturated	12h	24h	36h
0	0.0				
4	0.2				
8	0.3				
12	0.5				
16	0.7				
20	0.8				
24	1.0				
28	1.2				
32	1.3				
36	1.5				
40	1.7	T1C3			
44	1.8				
48	2.0				
52	2.2				
56	2.3				
60	2.5				
64	2.7	T2C3			
68	2.8				
72	3.0		T1C3	T1C3	
76	3.2				
80	3.3				
84	3.5				
88	3.7	T3C3			
92	3.8				
96	4.0		T2C3	T2C3	
100	4.2				
104	4.3				
108	4.5				T1C3
112	4.7	T4C3			
116	4.8				

120	5.0		T3C3	T3C3	
124	5.2				
128	5.3				
132	5.5				
136	5.7	T5C3			
140	5.8				
144	6.0		T4C3	T4C3	T2C3
148	6.2				
152	6.3				
156	6.5				
160	6.7	T6C3			
164	6.8				
168	7.0		T5C3	T5C3	
172	7.2				
176	7.3				
180	7.5				T3C3
184	7.7	T7C3			
188	7.8				
192	8.0		T6C3	T6C3	
196	8.2				
200	8.3				
204	8.5				
208	8.7	Y8C3			
212	8.8				
216	9.0		T7C3	T7C3	T4C3

APPENDIX D.2. Additional data for the YES assay work.

The pertinent data from Kathleen McDermott for addition YES testing for the column study samples is provided below (1).

Table D.2. Average EEQs Mix of the Seven Targets Spiked in Solvent at 12.5mg/L.

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
12.16	10.64	8.59	10.5 ± 1.79	12.51	17.8%

Table D.3. 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	4.58 μg/L	8.43 μg/L	54.3	4.58 μg/L	8.43 μg/L	54.3
T0 + 100 μg/L	1.27 mg/L	11.2 mg/L	11.3	1.27 mg/L	11.2 mg/L	11.3
1 hour	24.3 mg/L	6.17 mg/L	394	0.902 mg/L	6.92 mg/L	13.0
8 hours	14.2 mg/L	7.01 mg/L	202	5.15 μg/L	5.80 mg/L	0.089
24 hours	6.77 μg/L	3.33 mg/L	0.203	6.98 μg/L	2.73 mg/L	0.256

Table D.4. Comparison of EEQs Calculated from LCMS and YES Assay Derived Data.

Dose Interval	EEQ of T0 + 100 μg/L	Predicted EEQ
8 hour	6.20 ± 1.92 mg/L	1.21 mg/L
8 hour flooding	128± 14.9 μg/L	
12 hour	693 ± 86.6 μg/L	
24 hour	8.11 ± 4.37 mg/L	
36 hour	8.11 ± 0.884 mg/L	

The average concentrations of the total organic carbon (TOC), dissolved organic carbon (DOC), total nitrogen (TN), and dissolved nitrogen (DN) data and the ammonia, nitrate and nitrite concentrations data for specific time points are presented below. The percent removals of the TOC, DOC, TN, and DN are graphed to make viewing trends over the course of the each dose interval easier. This data shows the behavior of these indicators over the course of Study 2. The average percent removals are presented in Chapter 4, section 4.3.1.2.

Table D.5. The results for the TOC, DOC, TN and DN measurements of effluent from each column presented for the final time points and as the average and standard deviation for that column for the duration of each study.

Dose Interval (h)	Column	TOC (mg/L)		DOC (mg/L)		TN (mg/L)		DN (mg/L)	
		Final	Avg	Final	Avg	Final	Avg.	Final	Avg.
8 Saturated	1	32	30 ± 3.3	34	32 ± 3.2	1.3	0.7 ± 0.3	0.4	0.6 ± 0.2
	2	5.7	5.9 ± 0.5	5.8	5.9 ± 0.5	0.9	1.5 ± 1.5	0.6	1.4 ± 1.5
	3	6.3	6.1 ± 0.2	5.5	5.1 ± 0.2	1.0	4.9 ± 6.3	0.8	4.7 ± 6.5
8	1	4.4	8.1 ± 6.0	3.0	9.1 ± 6.6	2.2	4.8 ± 2.3	3.1	4.6 ± 2.0
	2	2.2	4.0 ± 3.7	6.1	4.3 ± 1.0	8.4	9.6 ± 1.1	8.8	9.4 ± 1.2
	3	2.6	2.8 ± 1.0	5.7	3.3 ± 1.0	10	11 ± 5.7	9.7	12 ± 3.9
12	1	44	42 ± 17	42	39 ± 16	7.6	8.7 ± 10	7.8	9.2 ± 10
	2	5.2	5.7 ± 0.6	4.8	5.2 ± 0.3	24	23 ± 2.0	26	24 ± 2.9
	3	4.0	4.0 ± 0.4	5.2	4.5 ± 0.5	25	19 ± 7.0	26	19 ± 7.1
24	1	191	202 ± 7.3	175	190 ± 13	31	31 ± 0.6	6.4	25 ± 12
	2	52	61 ± 12	77	67 ± 14	24	25 ± 4.8	24	25 ± 5.3
	3	8.5	15 ± 12	10	26 ± 18	22	32 ± 14	23	32 ± 13
36	1	105	57 ± 33	103	56 ± 33	20	5.3 ± 9.5	20	6.1 ± 10
	2	7.2	7.1 ± 1.9	6.9	7.5 ± 2.1	5.7	7.0 ± 7.4	7.0	7.6 ± 6.8
	3	6.4	4.8 ± 1.2	15	7.1 ± 5.0	4.7	12 ± 4.8	7.2	13 ± 3.9

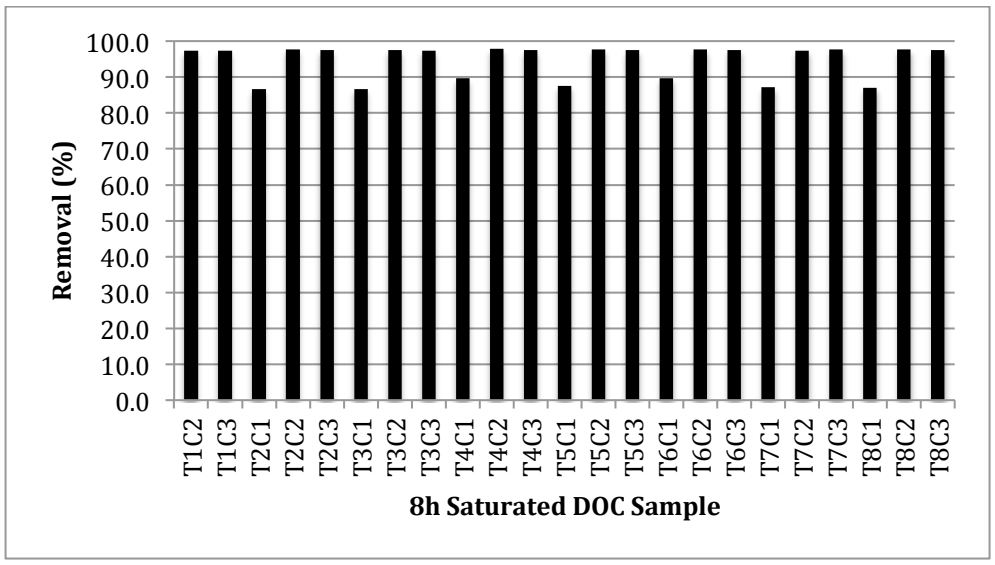

Figure D.1. Dissolved organic carbon (DOC) removal percentages measured at each time point (T) and for each column (C) for the wastewater through the 8h saturated dose interval, for example T3C3 is time point 2, column 3.

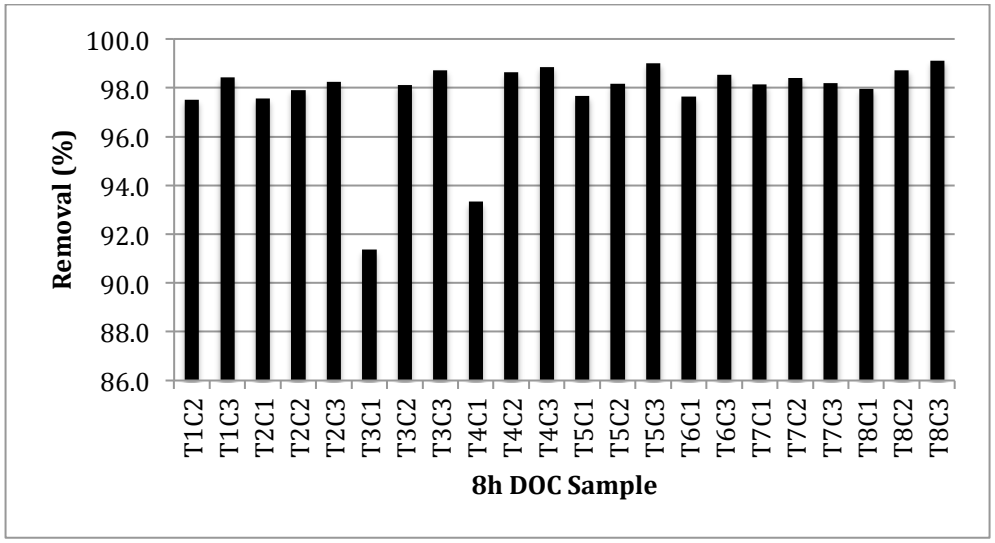

Figure D.2. Dissolved organic carbon (DOC) removal percentages measured at each time point (T) and for each column (C) for the wastewater through the 8h dose interval.

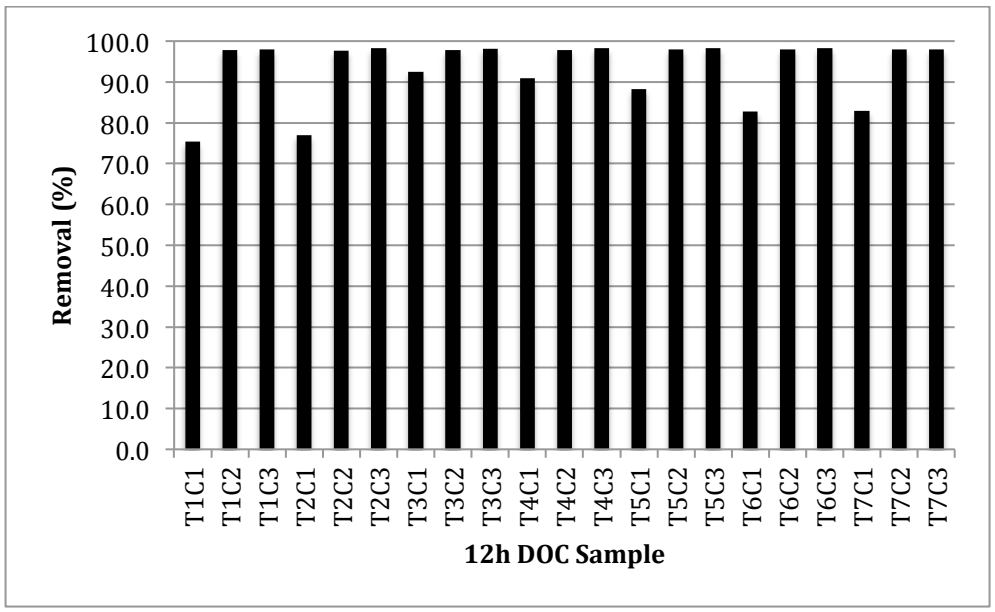

Figure D.3. Dissolved organic carbon (DOC) removal percentages measured at each time point (T) and for each column (C) for the wastewater through the 12h dose interval.

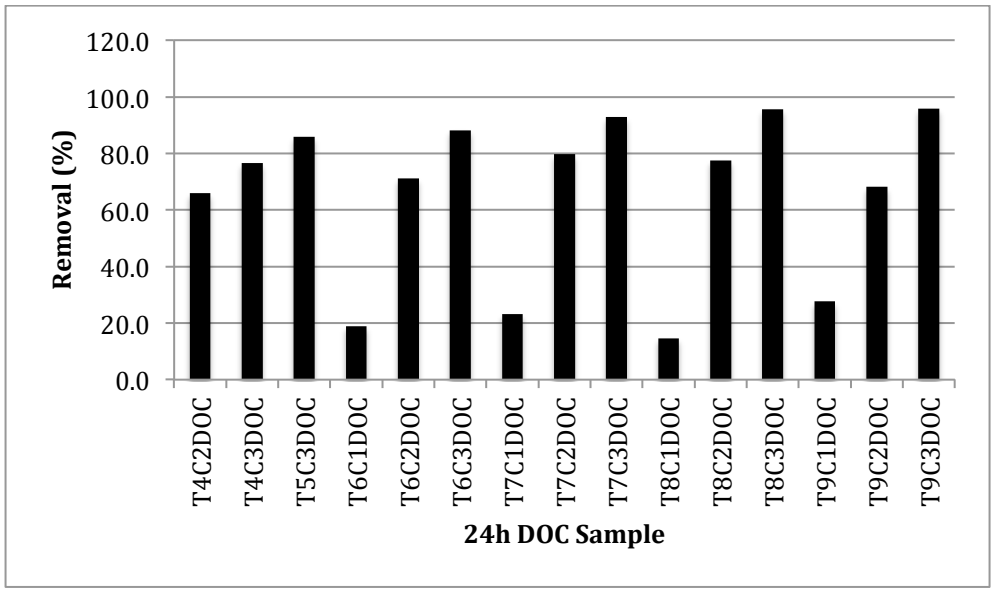

Figure D.4. Dissolved organic carbon (DOC) removal percentages measured at each time point (T) and for each column (C) for the wastewater through the 24h dose interval.

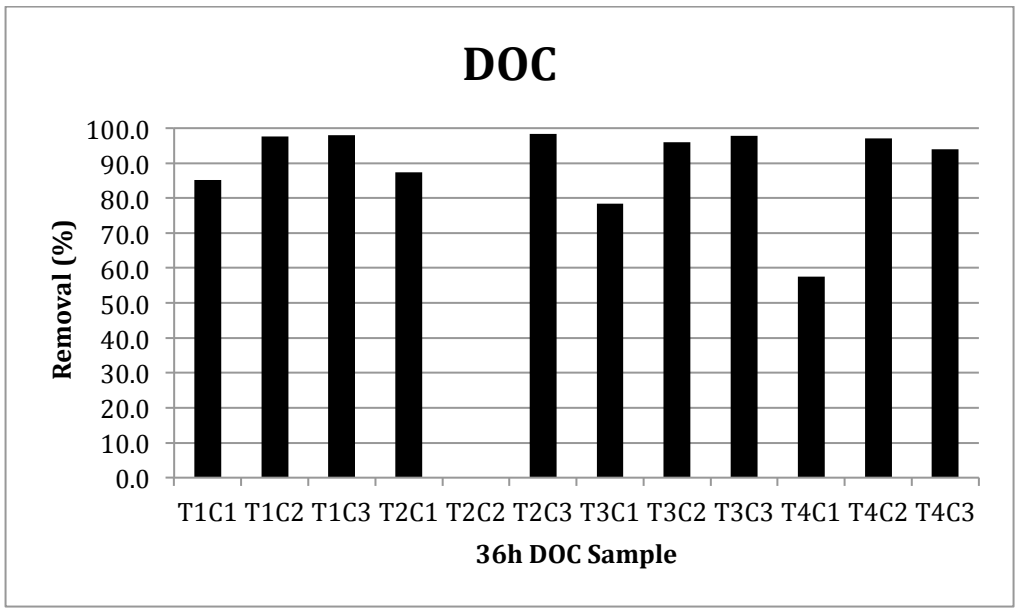

Figure D.5. Dissolved organic carbon (DOC) removal percentages measured at each time point (T) and for each column (C) for the wastewater through the 36h dose interval.

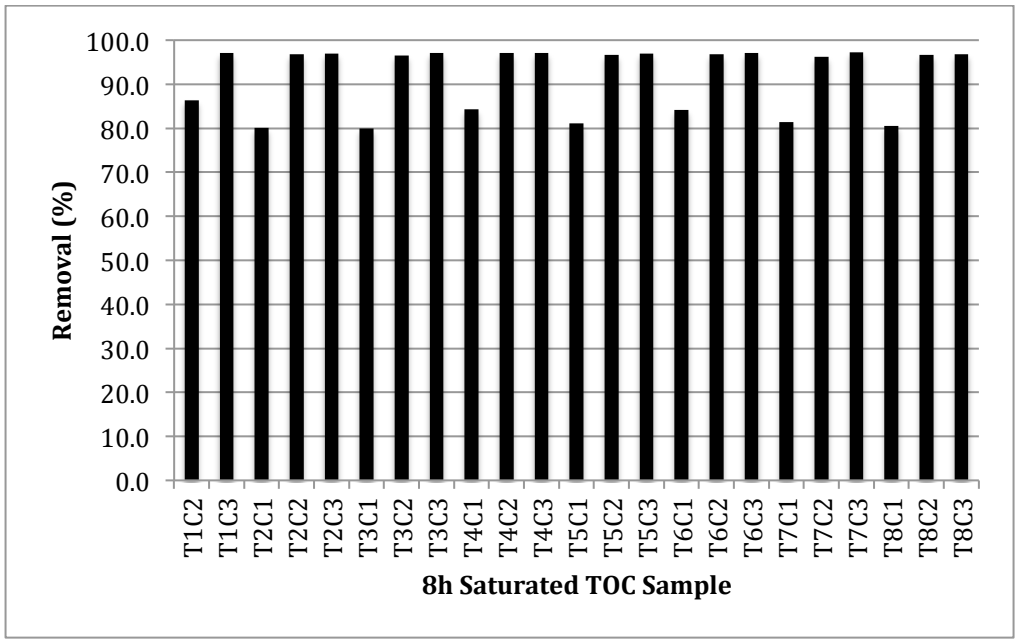

Figure D.6. Total organic carbon (TOC) removal percentages measured at each time point (T) and for each column (C) for the wastewater through the 8h saturated dose interval.

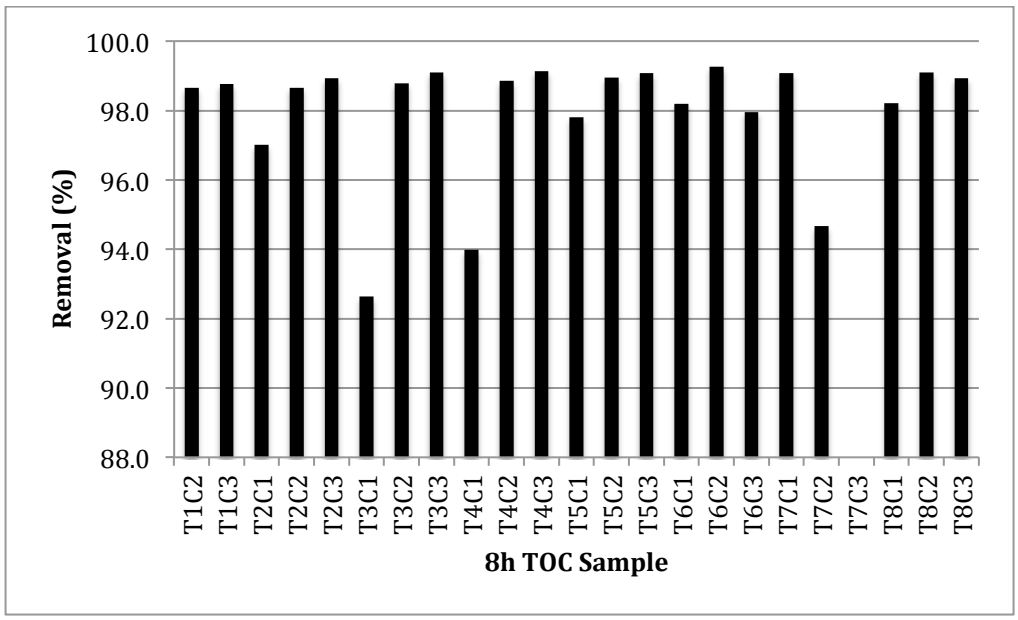

Figure D.7. Total organic carbon (TOC) removal percentages measured at each time point (T) and for each column (C) for the wastewater through the 8h dose interval.

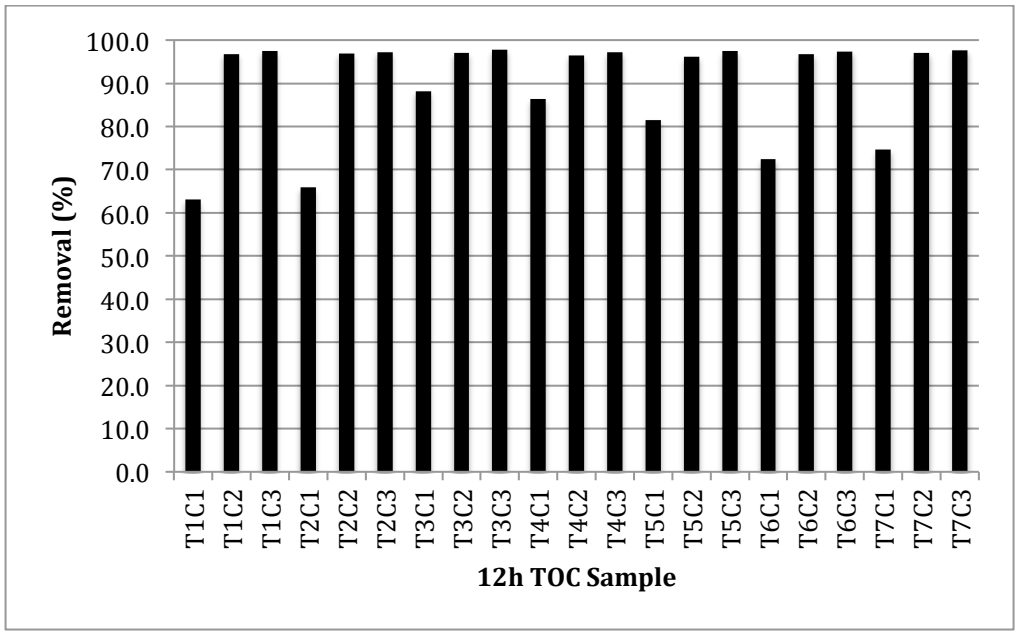

Figure D.8. Total organic carbon (TOC) removal percentages measured at each time point (T) and for each column (C) for the wastewater through the 12h dose interval.

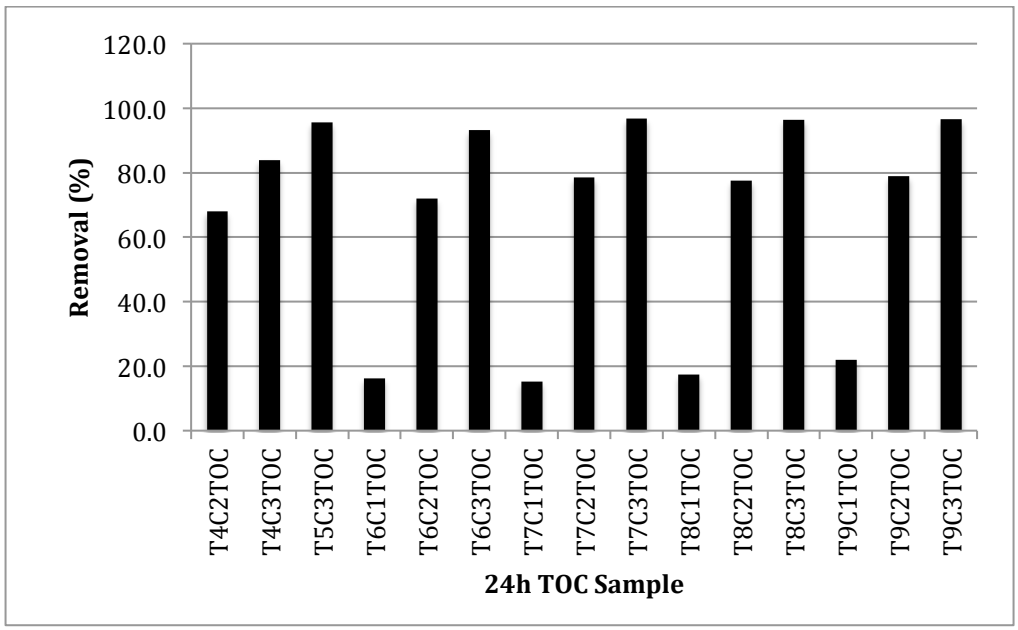

Figure D.9. Total organic carbon (TOC) removal percentages measured at each time point (T) and for each column (C) for the wastewater through the 24h dose interval.

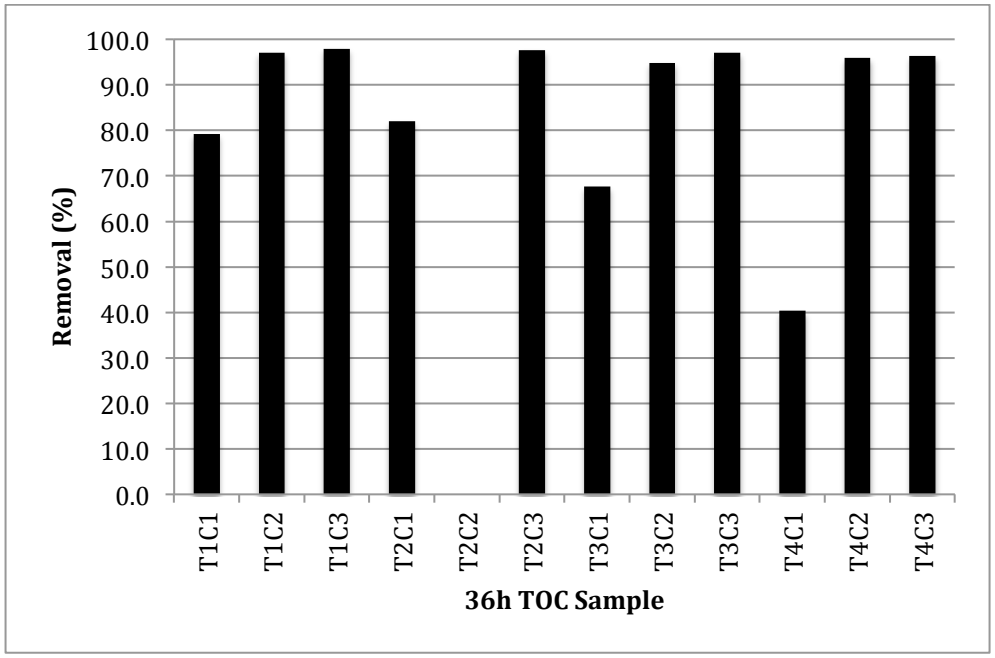

Figure D.10. Total organic carbon (TOC) removal percentages measured at each time point (T) and for each column (C) for the wastewater through the 36h dose interval.

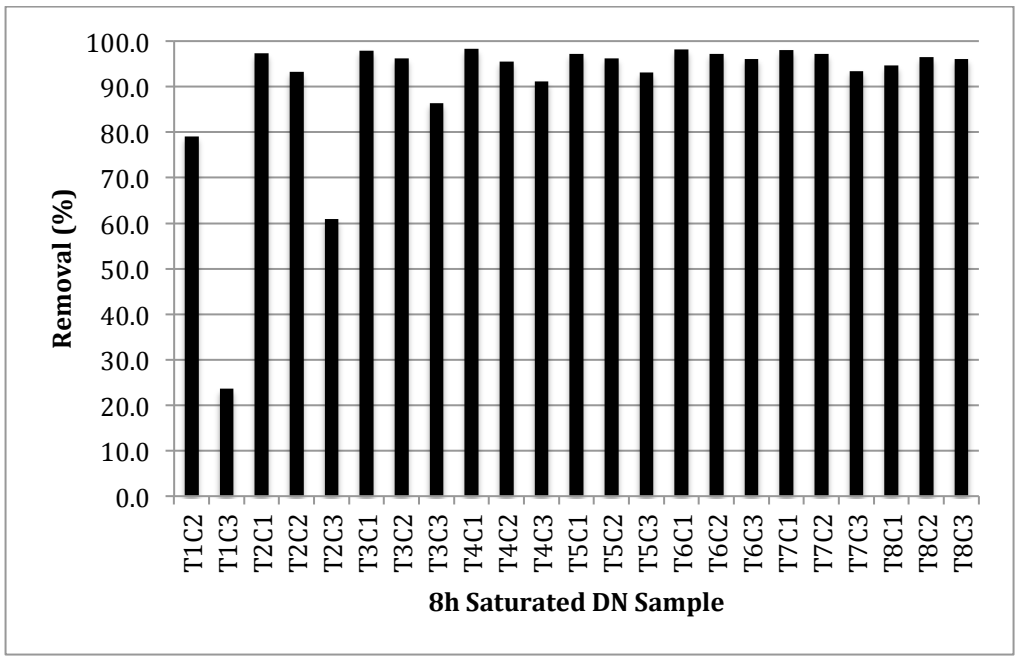

Figure D.11. Dissolved nitrogen (DN) removal percentages measured at each time point (T) for each column (C) for the wastewater effluent of the 8h saturated dose interval.

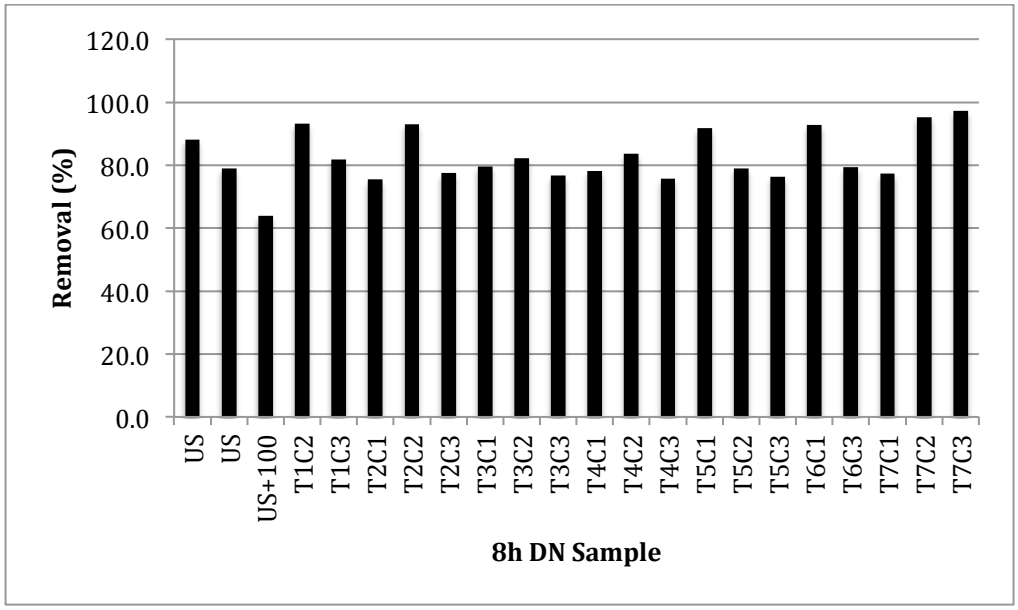

Figure D.12. Dissolved nitrogen (DN) removal percentages measured at each time point (T) for each column (C) for the wastewater effluent of the 8h dose interval.

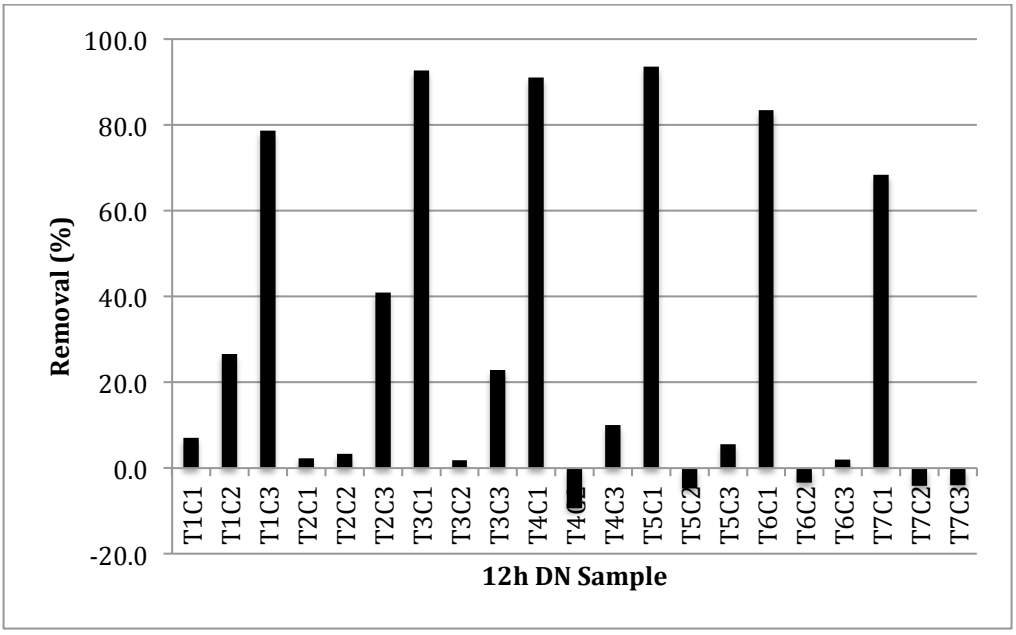

Figure D.13. Dissolved nitrogen (DN) removal percentages measured at each time point (T) for each column (C) for the wastewater effluent of the 12h dose interval.

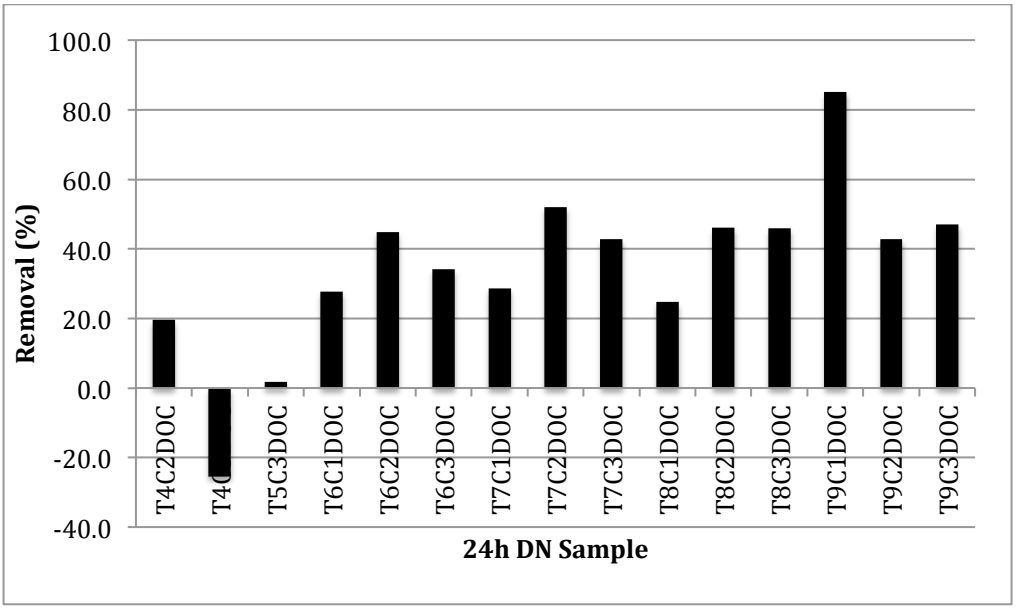

Figure D.14. Dissolved nitrogen (DN) removal percentages measured at each time point (T) for each column (C) for the wastewater effluent of the 24h dose interval.

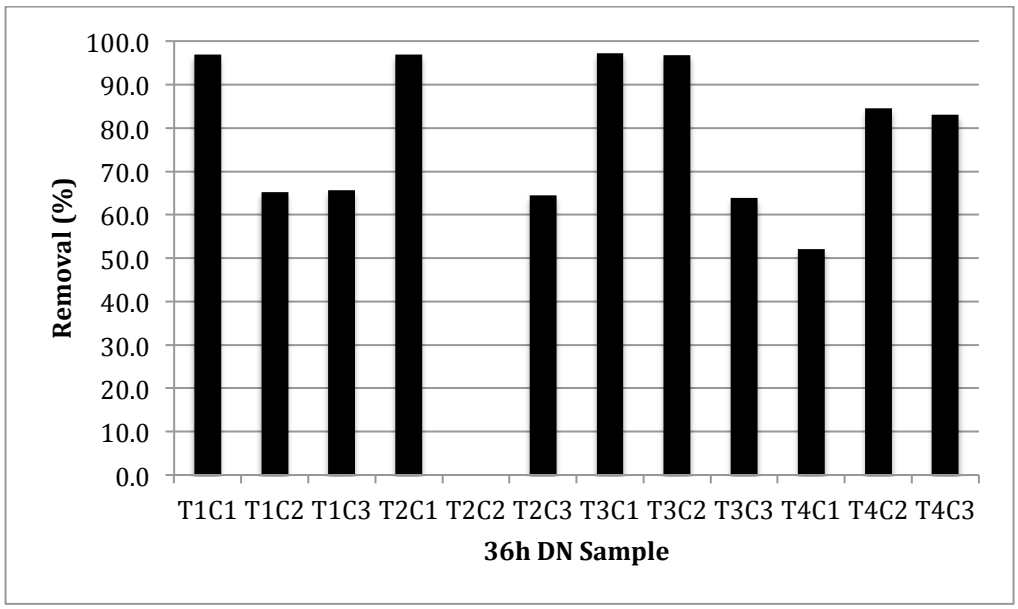

Figure D.15. Dissolved nitrogen (DN) removal percentages measured at each time point (T) for each column (C) for the wastewater effluent of the 36h dose interval.

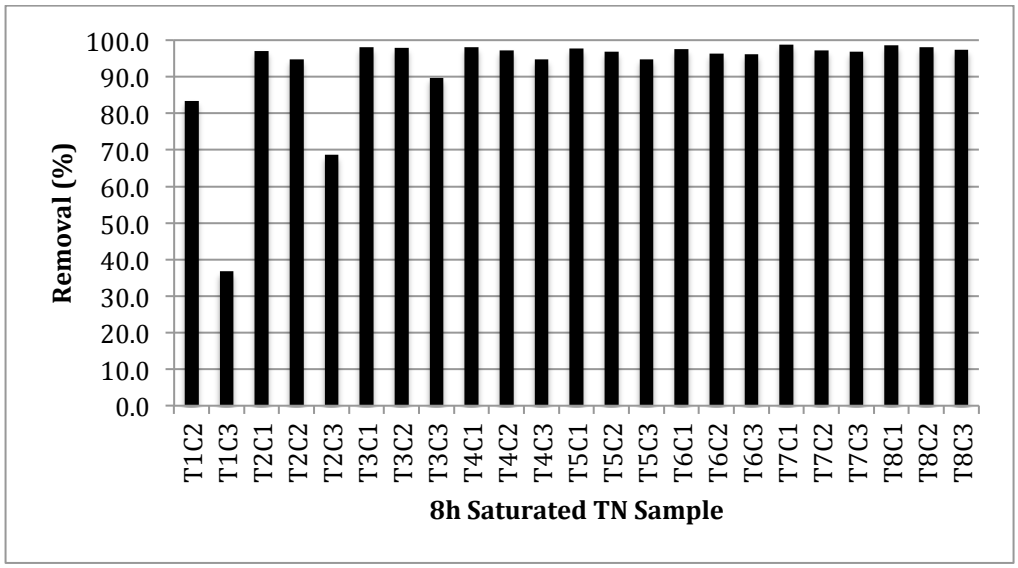

Figure D.16. Dissolved nitrogen (TN) removal percentages measured at each time point (T) for each column (C) for the wastewater effluent of the 8h saturated dose interval.

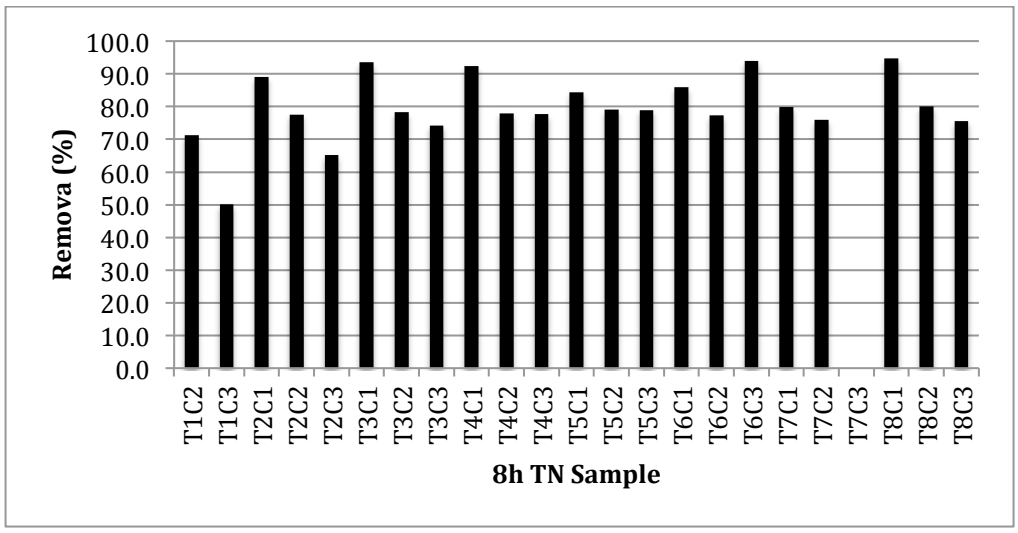

Figure D.17. Dissolved nitrogen (TN) removal percentages measured at each time point (T) for each column (C) for the wastewater effluent of the 8h dose interval.

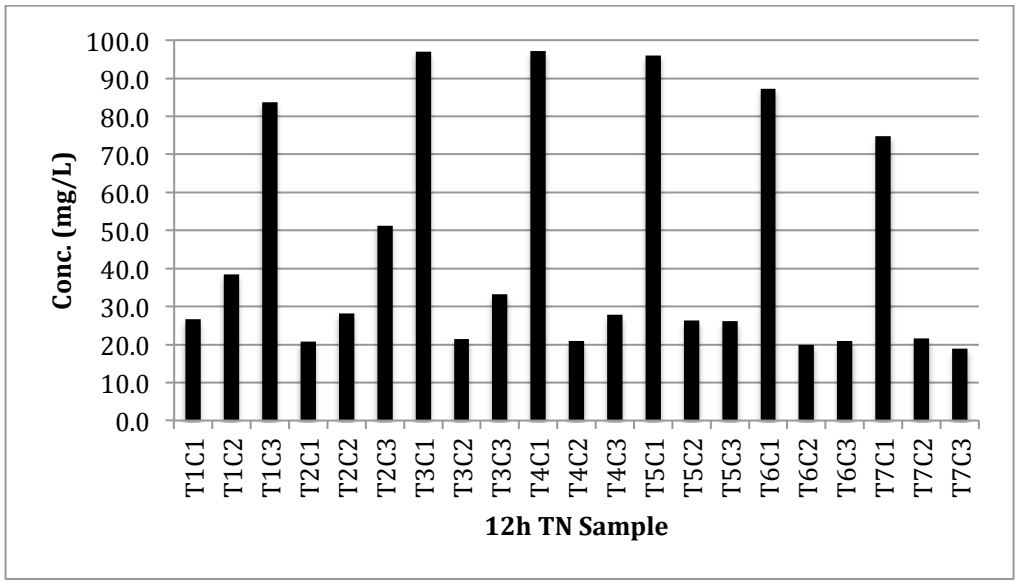

Figure D.18. Dissolved nitrogen (TN) removal percentages measured at each time point (T) for each column (C) for the wastewater effluent of the 12h dose interval.

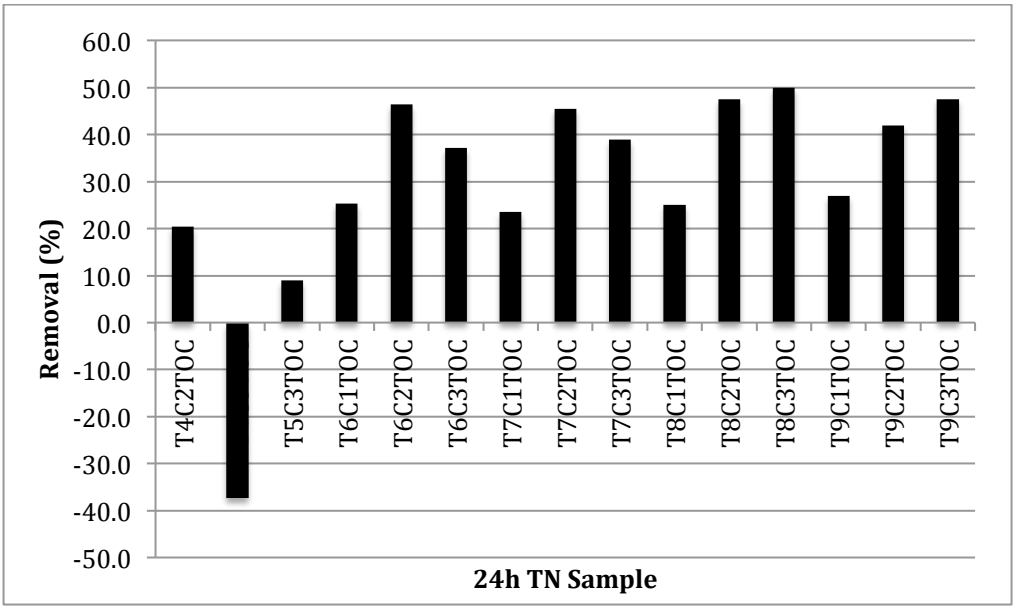

Figure D.19. Dissolved nitrogen (TN) removal percentages measured at each time point (T) for each column (C) for the wastewater effluent of the 24h dose interval.

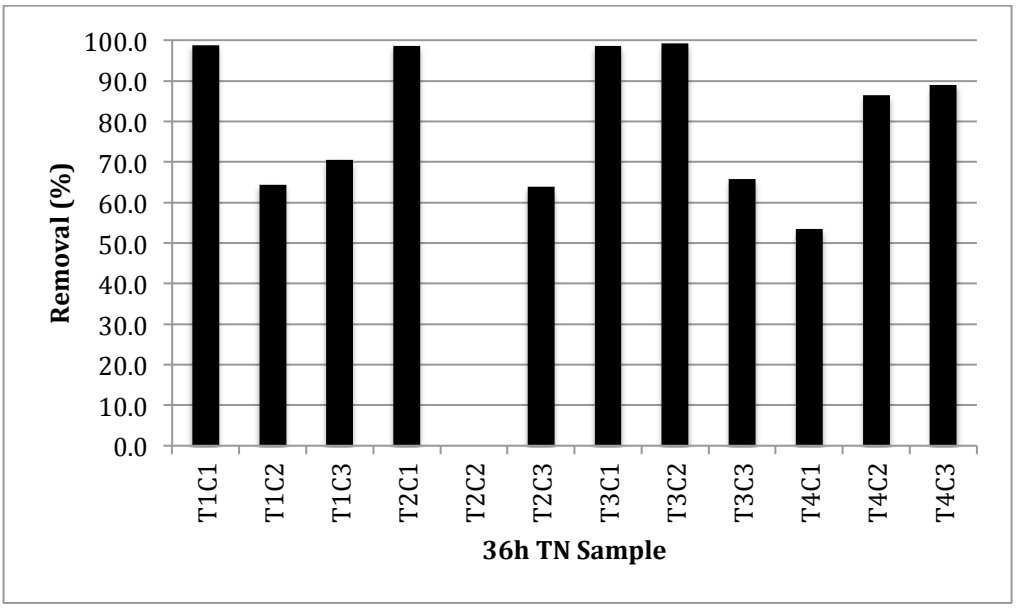

Figure D.20. Dissolved nitrogen (TN) removal percentages measured at each time point (T) for each column (C) for the wastewater effluent of the 36h saturated dose interval.

Table D.6. Batch study chemical concentrations.

		Stalite : Wastewater (g : mL)			
		120:600		600: 600	
Target Compound	Sample	1h Time Point Removal (µg/L)	30h Time Point Removal (µg/L)	1h Time Point Removal (µg/L)	24h Time Point Removal (µg/L)
ATZ	1	119	>90	NM	72
	2	102	>90	NM	75
	3	101	>90	78	68
	4	112	>90	65	66
CAF	1	98	84	NM	75
	2	78	86	NM	76
	3	85	87	79	71
	4	94	82	75	78
CBZ	1	109	109	NM	103
	2	101	96	NM	104
	3	104	105	101	94
	4	107	99	92	107
DEET	1	118	133	NM	39
	2	136	136	NM	45
	3	153	113	61	61
	4	104	134	55	43
E2	1	87	59	NM	83
	2	72	74	NM	65
	3	84	53	106	LOQ[a]
	4	72	60	23	LOQ[a]
4-NP	1	67	36	NM	72
	2	48	35	NM	63
	3	52	16	62	70
	4	46	41	62	72

[a]LOQ = limit of quantitation (10 µg/L)

REFERENCES

1. McDermott KJ. Monitoring the Removal of Estrogenic Activity from a Pilot-Scale Constructed Wetland Using the YES Assay. M.S. Thesis. University of North Carolina. 2017.

APPENDIX E: CHAPTER 4 DETAILED METHODS

APPENDIX E.1. Yeast Estrogen Screen (YES) Protocol

Note: The method and solution preparation were originally prepared by Paul Ebohon. The procedure is modified from the work of Routledge and Sumpter (1996) and Chen et al., (2007). The yeast strain (*Saccharomyces cerevisiae)* used for this assay was provided under agreement with Professor J.P Sumpter at Brunel University, UK. This protocol was written by Paul Ebohon at the University of North Carolina at Chapel Hill on January 4, 2011 and modified on July 9, 2011.

Part 1: Materials needed

96 well plate reader* (Molecular Devices, EMAX; Sunnyvale, California)

Centrifuge* (International Equipment Company; Needham Heights, Massachusetts)

Shaker table** (Barnstead International; Dubuque, Iowa)

Incubator** (Fisher Scientific; Dubuque Iowa)

Weighing scale (Sartorius; Goettingen, Germany)

Stirrer (Barnstead/Thermolyne; Dubuque, Iowa)

Disposable filter sterilization flasks (Corning Incorporated; Corning, New York)

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

Disposable 96 well flat bottom microplates (Greiner-Bio-One; Frickenhausen, Germany)

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

Falcon tube (Becton Dickinson Labware, Franklin Lakes, New Jersey)

Plate Sealing Film (Denville Scientific; Metuchen, New Jersey)

Disposable V-shaped wells for multichannel pipetting (USA Scientific; Ocala, Florida)

Vortex mixer (Barnstead/Thermolyne; Dubuque, Iowa)

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

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

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

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

20 -100 µL pipettor (Pipetman; Middleton, Wisconsin)

Weighing paper: 152 x 152 mm (Fisherbrand; Suwanee, Georgia)

1L reusable media/solution glass bottles (Corning Incorporated; Corning, New York)

250 mL screw cap Erlenmeyer glass flask (Kimble Chase Kontes; Vineland, New Jersey)

1, 5, 10 and 25 mL plastic disposable pipettes (Fisher Scientific; Raleigh, North Carolina)

*Located in room *2104 and **1213

Part 2: Chemicals Needed

Table E.1. Chemicals needed for YES assays.

Chemical	CAS #	Brand/Source used by Weinberg lab
Difco yeast nitrogen base	2014-11-30	BD; Sparks, MD
Ammonium sulfate	7783-20-2	Mallinckrodt; Paris, KY
Adenine sulfate	321-30-2	Acros Organics; New Jersey, NJ
Casamino acids	65072-00-6	Fisher Scientific; Fair Lawn, NJ
Dextrose anhydrous	50-99-7	Fisher Scientific; Fair Lawn, NJ
Bacto agar	2014-03-31	BD; Sparks, MD
Bacto peptone	2012-04-22	BD; Sparks, MD
Sucrose	57-50-1	Fisher Scientific; Fair Lawn, NJ
[a]$Na_2HPO4 * 7H_2O$	7782-85-6	Mallinckrodt; Paris, KY
[b]$NaH_2PO4 * H_2O$	10049-21-5	Mallinckrodt; Paris, KY
Potassium chloride	7447-40-7	Fisher Scientific; Fair Lawn, NJ
[c]$MgSO_4 * 7H_2O$	10034-99-8	Acros Organics; New Jersey, NJ
2-mercaptoethanol (βME)	60-24-2	Acros Organics; New Jersey, NJ
20% Sodium dodecyl sulfate	151-21-3	Fisher Scientific; Fair Lawn, NJ
[d]$CuSO_4 * 5H_2O$	7758-99-8	EM Science; Gibbstown, NJ
[e]o-NPG	369-07-3	Research Organics; Cleveland, OH
Sodium carbonate	497-19-8	Mallinckrodt; Paris, KY

[a]$Na_2HPO4 * 7H_2O$: Sodium hydrogen phosphate heptahydrate
[b]$NaH_2PO4 * H_2O$: Sodium dihydrogen phosphate monohydrate
[c]$MgSO_4 * 7H_2O$: Magnesium sulfate septahydrate
[d]$CuSO_4 * 5H_2O$: Cupric sulfate pentahydrate
[e]o-NPG: o-Nitrophenyl-β-D-galactopyranoside

Part 3: Preparation of solutions and buffers

Note: Solutions should be made in sterilized bottles or vials with laboratory grade water (LGW) and stored at room temperature unless otherwise stated in this SOP. Any solution should be discarded in the event of a visible color change or visible turbidity

Liquid URA-TRP media for yeast cells growth (500 mL):

- 3.35 g Yeast Nitrogen Base without amino acids and ammonium sulfate
- 2.5 g ammonium sulfate
- 2.5 mL adenine sulfate (4 mg/mL)
- 10 g anhydrous dextrose
- 2.5 g casamino acids
- Dissolve in 500 mL LGW
- Filter sterilize using a disposable filter sterilization flask and transfer into an autoclaved 1Lscrew top glass bottle before storing at room temperature.

URA-TRP Solid media for yeast cell propagation (500 mL):

- Add 10 g of bactoagar to 500 mL liquid Ura-Trp media
- 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 cooling until it can be handled without difficulty. Do not shake the content of the autoclaved bottle at this point because this would cause bubbles to be present in the media.
- Pour 15-20 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.

YPS Media used to make the diluted yeast solution (500 mL):

- 5 g Yeast Nitrogen Base without amino acids and ammonium sulfate

- 2.5 g peptone
- 50 g sucrose
- Filter sterilize using a disposable filter sterilization flask and transfer into an autoclaved 1 L screw top glass bottle before storing at room temperature.

Z-Buffer solution (1L):

- 16.1 g disodium hydrogen phosphate heptahydrate (60 mM final)
- 5.5 g sodium dihydrogen phosphate monohydrate (40 mM final)
- 0.75 g potassium chloride (10 mM final)
- 0.246 g magnesium sulfate heptahydrate (1 mM final)
- Adjust pH to 7.0 by using a 2 M potassium hydroxide solution
- Filter sterilize using a disposable filter sterilization flask and store at room temperature. Immediately before using, add 135 μL of β-mercaptoethanol (βME) per 50 mL of Z-buffer solution .βME cannot be added in advance because it becomes oxidized and loses its potency over time.

10% Sodium dodecyl sulfate (SDS) used to denature proteins prior to colorimetric measurement:

Note: Make in small batches because it will lose its potency after a month

- Transfer 5 mL LGW into a 250 mL screw top bottle
- Transfer 5mL 20% SDS into the bottle containing LGW and swirl contents
- Label bottle as 10% SDS and include intials , date and time of preparation

1M sodium carbontate solution (500 mL) that stops the reaction of β-galactosidase with o-NPG by shifting the reaction mixture to pH 11:

- Dissolve 59.5 g sodium carbonate in enough water to make 500 mL.
- Filter sterilize using a disposable filter sterilization flask and store at room temperature

Copper (II) sulfate solution (250 mL):

- Dissolve 0.122g $CuSO_4 * 5H_20$ in enough water to make 250 mL.

  Filter sterilize using a disposable filter sterilization flask and store at room temperature.

10% Ethanol (EtOH) used for serial dilution of the samples and the 4-nonylphenol standard:.

NOTE: In order to avoid possible volatilization of the EtOH from LGW, make this solution right before performing the assay.

Dissolve 1 mL ethanol in 9 mL of LGW.

17-β-estradiol (E2) stock 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 stock solution to a 10 mL volumetric flask with ethanol
- All stock solutions should be stored at -20°C

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

- Add 9 mL LGW to an amber vial
- Add 1 mL ethanol and 1 mL 1E-6M E2 stock solution to the vial
- Add an additional 9 mL LGW to the vial. Mix the contents well.
- Store at 4°C

NOTE: This E2 working standard solution can be used for at least two weeks; however, it is recommended to make it fresh before each assay.

Part 4: Yeast Cell Propagation and Assay Procedure:

1. Yeast cell propagation

I. Cell growth on solid Ura-Trp media: 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, 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 keep the yeast colonies going.

2. Assay Procedure

NOTE: Slightly different procedures must be followed depending on whether samples are prepared in 10 or 100% ethanol and other solvents. See details below based on what solvents your samples are prepared in.

DAY 1

II. NOTE: If your samples are prepared in 100% EtOH, you will need to prepare you r 96 well plates today in order to allow the solvent to evaporate overnight. *This assay procedure assumes duplicate analysis for each sample with nonylphenol being used as positive control, and 10% ethanol or suitable matirx as a negative control on every plate. A separate plate containing the samples, nonylphenol and negative control should also be assayed simultaneously with the assay media containing no yeast cells in order to determine if the samples reacts with o-NPG; thereby, generating false positive results. When analyzing Disinfection By-Products (DBPs), do not use this step because they do not evaporate completely after 24 hours*.

1) Add 200 µL of 5E-8M E2 standard (in 100% EtOH) to the first column of rows A and B (See Figure A.1).

2) Add 200 µL of sample (in 100% EtOH) in duplicate to row C-H. A total of 3 samples can be run on each plate (See Figure A.1).

3) Place the plates in the fume hood for 24 hours to completely evaporate solvent in the wells.

NOTE: Cover plates loosely with Kim wipes to prevent dust from falling in.

III. Grow yeast cells in liquid Ura-Trp media:

1) Aliquot 7 mL of liquid Ura-Trp media into a 50 mL sterile falcon tube.
2) Using a sterile wand, pick up one independent colony from a solid Ura-Trp plate and transfer it to the liquid media in the falcon tube.

3) Incubate the yeast cells at 30°C on a shaker table at ~200 rpm for 24 hours.

DAY 2

IV. Determination of yeast cell density and its dilution using YPS media

1) Vortex the falcon tube containing the 24 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. Also plate 100 µL (in triplicate) of the YPS media on the same plate. Read the plate at an absorbance of 600 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_{600YPS} from $Abs_{600Yeast}$

   b) Solve for x: $(Abs_{600Yeast} - Abs_{600YPS})(x\ \mu L) = (0.07)(100\ \mu L)$

   c) Solve for y: $(x\ \mu L)/(100 \mu L) = (y\ mL)/(33\ mL)$

   d) y mL of yeast + (33-y) mL of YPS = dilution of the yeast suspension need ed for the assay.

   e) Make the yeast dilution calculated above. In order to ensure that there is enough yeast solution; make 33 mL of diluted yeast suspension per YES plate.

   f) Check your yeast dilution to make sure that the Abs_{600} falls in the range of 0.06 and 0.08 (Once again, subtract Abs_{600YPS} to account for background).

   g) Add 100 µL of $CuSO_4$ solution per 10 mL to the diluted yeast solution and vortex to mix.

V. Prepare 96 deep well 1 mL plates: If your samples were prepared in 10% ethanol, ignore step 2 below.

NOTE:If your samples and nonylphenol working standard solution were prepared in 100% EtOH, you should have completed this step on Day 1 in order to allow evaporation overnight.

1) For samples prepared in 10 and 100% EtOH: Using a multichannel pipettor, add 100 µL of freshly prepared 10% ethanol to each well in columns 12-2 of a 96 deep well 1 mL plate.

2) For samples prepared only in 100% EtOH: Using a multichannel pipettor, add 200 µL of 10% EtOH to each of the wells in column 1. Aspirate each well thoroughly with the multichannel pipettor in order to resuspend the samples in the 10% EtOH.

3) For samples prepared in 10% EtOH: Add 200 µL of 2.50E-04M nonylphenol working standard solution to the first column of rows A and B (Wells A1 and B1). Add 200 µL of samples (prepared in 10% EtOH) in duplicate to row C-H. A total of 3 samples can be run on each plate (See Figure A.1).

4) 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 as pirating, then transfer 100 µL from column 2 to column 3. Continue the serial dilution across entire plate until you get to column 11. After mixing the contents of column 11, withdraw 100 u L that would be discarded as waste so that the column 12 wells containing the negative control as shown in the YES assay template (Figure A.1) has only 10% ethanol at this point.

<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="10" rowspan="2">4-nonylphenol standard in rows A and B</td><td rowspan="8">Negative control (10% ethanol)</td></tr>
<tr><td>B</td></tr>
<tr><td>C</td><td colspan="10" rowspan="2">Sample 1 in rows C and D</td></tr>
<tr><td>D</td></tr>
<tr><td>E</td><td colspan="10" rowspan="2">Sample 2 in rows E and F</td></tr>
<tr><td>F</td></tr>
<tr><td>G</td><td colspan="10" rowspan="2">Sample 3 in rows G and H</td></tr>
<tr><td>H</td></tr>
</table>

Figure E.1 YES Assay Template.

VI. Exposure of yeast cell to samples:

1) Add 300 µL of the diluted yeast solution to each well of the 96 deep well 1 mL plate containing the samples, nonylphenol and negative control. Ensure that you perform this addition by starting from column 12, which contains the negative

control. 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 for 3 days at 30°C while shaking at ~200 rpm. Ensure that you avoid opening and closing the closing the incubator until incubation period has elapsed.

DAY 5

VII. Measurement of optical densities (Endpoint):

1) Aliquot the amount of Z-buffer you will need into a 250 mL screw top bottle. You will need about 50 mL of Z-buffer per assay plate. Add 135 µL of βME per 50 mL of Z-buffer and mix thoroughly.

2) Prepare o-NPG assay buffer: Do not allow o-NPG to cool down to room temperature before use. Use as soon as you remove from freezer and return immediately. For each assay plate, dissolve 42 mg of o-NPG in 41 mL of the freshly-prepared Z-buffer + βME. o-NPG takes approximately 30 to 35 minutes to dissolve. Once dissolved, add 1 mL 10% SDS and an additional 525 µL of βME. Mix the contents of this solution thoroughly.

3) Using a multichannel pipettor, add 50 µL of Z-buffer (NOTE: Plain Z-buffer + βME and not the assay buffer) to each well of the 96 deep well 1 mL plate. Replace the plate sealing film on the plate if you observe any vapors or liquid on it. Mix the plate's contents at room temperature by shaking at ~300 rpm for 3-5 minutes on a shaker table.

4) Remove the plate sealing film and use a multichannel pipettor to add 400 μL of the o-NPG assay buffer to each well. NOTE: Ensure that no bubbles are present in the pipette tips and each has the same level of o-NPG assay buffer. Reseal the well with the same plate sealing film.

5) Incubate the plate at 30°C for 20 minutes while shaking at ~200 rpm.

6) Using a multichannel pipettor, add 200 μL of 1 M sodium carbonate to each well to stop the reaction.

7) Centrifuge the plate at room temperature for 10 minutes at 3000 rpm in order to allow the yeast cells settle to the bottom of the 96 deep well 1 mL plate.

8) Using an 8 channel multipipettor, withdraw 100 μL of clear supernatant from each well and place on a sterile 96 well flat bottom microplate. Ensure that you do not withdraw any yeast cell debris during this step. Avoid allowing bubbles in the 96 well flat bottom microplate because they will interfere with the plate reader's result.

9) Measure the absorbance of the contents of the plate above at 450 nm by using a plate reader. Export the 450 nm measurements into an excel spreadsheet and transfer the spreadsheet into a USB drive. Proceed to calculations below (See part V of this protocol). Once the data analysis is complete and you are sure that you do not need to re-measure the absorbance of the samples at 450 nm, proceed to step 11.

10) Using a multichannel pipette, thoroughly mix the contents of the 96 deep well1 mL plate so that the yeast cells are resuspended in the wells.

    a) Using a multichannel pipettor, mix the contents of column 12 by aspirating each well about 10 to 15 times.

    b) Transfer 100 μL of the contents of column 12 into a sterile 96-well flat bottom microplate.

    c) Push the top of multichannel pipettor's button so that the contents of the pipette tips are discarded on a Kim wipe. Continue this process until no bubbles are visible on the pipette tips. Repeat the process again for column 11 and work your way to column 1.

11) Measure the absorbance of the plate (in step 10) containing the resuspended yeast solution at 600 nm and place raw readings under template 4 that is set up exactly as template 2 under part V of this protocol. Compare the samples absorb ance at 600 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.

Clean up:

1) All plates and disposable containers that have been inoculated with yeast cells sho uld be autoclaved prior to disposal.

2) All glassware should be cleaned according to the glassware cleaning procedure, wrapped with aluminum foil and stored in their appropriate cabinet.

Pour unused assay media, and chemicals into properly labeled disposal container and store appropriately according to laboratory procedure.

VIII. Yeast Estrogen Screen Calculation Procedure to Determine EC_{50} for 4-nonylphenol

Calibration Curve

For the calibration curves, you should have two rows (A & B) containing 11 concentrations of nonylphenol, ranging from a pre-dilution concentration of 2.50E-03 M to 2.44E-07 M. Remember that you added 100 µL of the nonylphenol standard to the wells before diluting it with 300 µL of yeast suspension; therefore, the actual final concentrations/concentration factors are the pre-dilution concentrations divided by 4. In your calculations nonylphenol concentrations will range from 6.24E-05 to 6.09E-08 M.

Label an excel spreadsheet as shown below:

Template 1:

Column A: Labeled as compound with the corresponding samples listed as ran on the YES assay plate.

Column C: Labeled as absorbance at 450 nm

Column N: Labeled as negative control

Column P: Labeled as average of negative control

1) Transfer the raw absorbance readings into template 1 of the excel spreadsheet. The highest absorbance for nonylphenol should be at cells C2 and C3.

2) Average the absorbance 450 nm (Abs_{450}) of the negative controls in order to get a single negative control value.

3) Leave some spaces after template 1 and set up template 2 (in the same format as template 1 but exclude column P) on the same page of the excel spreadsheet. Template 2 will contain your corrected absorbances for E2, and samples.

4) Template 2: For each well on the plate, subtract the single Abs_{450} of the negative control from Abs_{450} of sample. This value (Abs_{450} of sample – Average Abs_{450} of negative control) will simply be referred to as "Abs 1" and "Abs 2".

5) Leave some spaces after template 2 and set up template 3 (as shown below) on the same page of the excel spreadsheet in order to plot the dose response curve for nonylphenol and samples.

Template 3: Columns may vary based on how you want your sheet set up**.**

Column A: Concentration.

Column B: Abs 1.

Column C: Abs 2.

You have 2 absorbances since each sample was run on 2 rows during the assay.
Column D: % Induction 1.

Column E: % Induction 2.

Column F: Top.

Column G: Bottom.

Column H: EC_{50}.

Column I: Lower 95th % confidence interval for EC_{50}.

Column J: Upper 95th % confidence interval for EC_{50}.

Column K: Hillslope.

Column L: EC_{10}.

6) Transfer the E2 concentrations into column A of template 3 with the highest concentration being on top.

7) Transfer the corrected absorbances at 450 nm into column B and C of template 3 and proceed to Appendix B.

APPENDIX E.2. MO Bio FastDNA Spin Kit Protocol

The DNA samples were extracted using the proprietary method by MO Bio (Germantown, MD) according to the instructions. All solutions and reagents were provided as part of the kit and prepared by MO Bio and acronyms refer to those provided by MO Bio.

1) Stalite samples were first mixed with 122 μl MT Buffer and 978 μl Sodium Phosphate Buffer and shaken for 1 h.
2) Samples were centrifuged for 5 min at 14000 g, 4 °C. The supernatant was removed.
3) PPS Buffer (1 ml) was added.
4) The sample was centrifuged for 5 min at 14000 g, 4 °C. The supernatant was recovered and Stalite was discarded.

5) DNA binding matrix (1 ml) was added to the supernatant and vortexed for 12 min at room temperature.
6) The sample was centrifuged for 2 min at 14000 g, 4 °C.
7) Guanidine thiocyanate 5.5 M (500 µl) was added and the sample was transferred in stages to a Spin Filter tube, sequentially spinning down the sample on a centrifuge for 2 min at 14000 g, 4 °C until all of the sample has been added.
8) SEWS-M (500 µl) is then added and the sample was centrifuged for 1 min at 14000 g, 4 °C then discarding supernatant, followed by 2 min at 14000 g, 4 °C, then again discarding supernatant.
9) The Spin Filter was then allowed to air dry for 5 min before adding 150 µl of DES solution then centrifuged for 1 min at 14000 g, 4 °C. The spin filter was discarded and the DNA stored at -20 °C.