A Survey of Endocrine Disrupting Chemicals (EDCs) in Marine Sediments, Influents/Effluents and Biosolids in Vancouver Wastewater Treatment Plants (WWTPs)

by

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Abstract

The objective of this study was to apply a panel of yeast bioassays in the quantification and identification of chemicals from 4 different classes of endocrine disrupting chemicals (EDCs) in marine sediments and wastewater samples from Vancouver wastewater treatment plants (WWTPs). In wastewater, estrogenic activity and AhR activity was detected in the ng/L range, while no glucocorticoid or androgenic activity was detected. There was also an observed general reduction in the estrogenic and AhR activity due to wastewater treatment. In marine sediments, estrogenic activity was detected in the ng/g range for 39% of samples, while AhR activity was detected in the µg/g range in 49% of samples. GC-MS analysis of select samples identified bisphenol A (BPA) in both wastewater and marine sediments, while dehydroabietic acid (DHAA) was found in only marine sediments. Overall, the yeast bioassay is a useful tool for use in biomonitoring of EDCs.

Keywords: Endocrine disrupting chemicals; Wastewater Treatment; Marine Sediments; Estrogens; Yeast Bioassay; AhR agonists

To all my friends and family who have supported me over the years

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List of Acronyms

%CV	Percent coefficient of variation
AhR	Aryl hydrocarbon receptor
AR	Androgen receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
CALUX	Chemically activated luciferase expression
DHT	Dihydrotestosterone
DOC	Deoxycorticosterone
E2	17β-estradiol
EC	Effective concentration
EDC	Endocrine disrupting chemical
EEQ	17β-estradiol equivalent
ER	Estrogen receptor
GC-MS	Gas chromatography mass spectrometry
GR	Glucocorticoid receptor
HAH	Halogenated aromatic hydrocarbon
HPLC	High performance liquid chromatography
IDZ	Initial dilution zone
LOD	Limit of detection
MLOD	Method limit of detection
NAP	β-naphthoflavone
NAPEQ	β-naphthoflavone equivalent
PAH	Polyaromatic hydrocarbon
PBDE	Polybrominated diphenyl ethers
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-p-dioxin
PCDF	Polychlorinated dibenzofuran
SEM	Standard error of the mean
T4	Thyroxine
WWTP	Wastewater treatment plant

Glossary

Effective concentration 50 (EC50)	The concentration of a drug, antibody, toxicant or other chemical which produces 50% of the maximal possible effect of that agonist after a specified exposure time
Endocrine disrupting chemical (EDC)	Chemicals that may interfere with the body's endocrine system and produce adverse development, reproductive, neurological and/or immune effects in both humans and wildlife
17β-estradiol equivalents (EEQs)	The collective estrogenic potency of the chemical contents within a sample expressed in terms of ng 17β-estradiol/g or L
Initial dilution zone (IDZ)	IDZ extends the lesser (in any direction) of one-quarter of the waterbody width or 100-m radius around the outfall. Receiving water quality objectives and guidelines do not apply within the IDZ
β-naphthoflavone equivalents (NAPEQs)	The collective AhR potency of the chemical contents within a sample expressed in terms of ng β-naphthoflavone/g or L

Chapter 1.

Introduction

The emergence of compounds capable of interfering with regular hormonal pathways in living organisms has provided a new challenge for conventional toxicity testing and the regulation of these compounds. Collectively classified as endocrine disrupting chemicals (EDCs), these compounds do not belong in any one group of toxic substances (*e.g.* pesticides, heavy metals, pharmaceuticals etc.), but rather contain chemicals within all of these groups. In addition, EDCs do not behave in the manner that traditional toxic substances do; where an incremental increase of the dose leads to an increase in effect. In fact, many EDCs may exhibit an effect at low doses, but may have no effect or a completely different effect at high doses (Vandenberg et al. 2012). This in turn makes it difficult and sometimes inappropriate to extrapolate results from high dose experiments to low dose effects. In particular, the extrapolation of high dose laboratory experiments to effects in the environment where concentrations are expected to be much lower.

1.1. Classes of EDCs

1.1.1. Estrogens

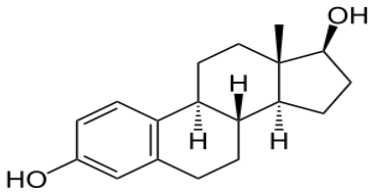
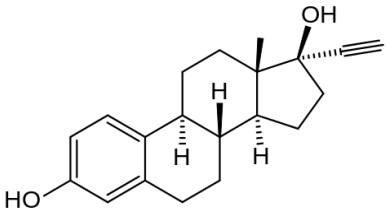
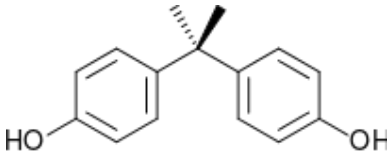
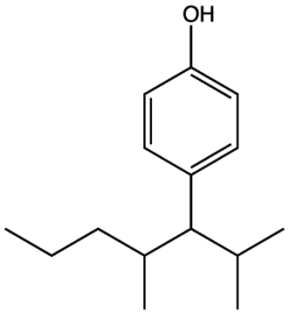
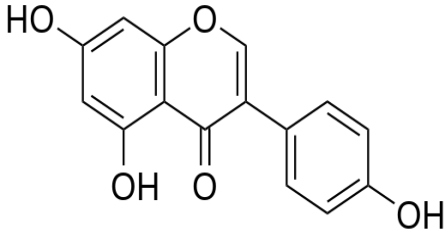
One of the main classes of EDCs is the estrogens and estrogen mimics. These are compounds both natural and synthetic that are capable of inducing an effect similar to that of the natural female hormone 17β-estradiol (E2). Other than E2, the most common estrogens detected in the environment include the synthetic estrogen 17α-ethynylestradiol (EE2), industrial surfactant nonylphenol (NP) and Bisphenol A (BPA) (a compound widely used in the manufacturing of plastic products see Table 1.1) (Ferguson et al. 2013, Lee et al. 2013). In addition to the synthetic estrogens mentioned,

there are also a large number of naturally occurring phytoestrogens that are present in the environment due to the consumption, processing and degradation of plant material. Compounds such as genistein, daidzein, biochanin A and formononetin are weak estrogens found in plants such as soy and other legumes (Rocha et al. 2013, Sassi-Messai et al. 2009).

Estrogens play an important role in the function and development of living organisms through the regulation of genes associated with the estrogen receptor. When estrogenic chemicals bind to the estrogen receptor, a receptor ligand complex is formed and acts as a transcription factor that binds to the estrogen response elements on DNA. This plays an important role in metabolism, behaviour as well as sexual development. Therefore, any upset in the balance of estrogens within an organism may affect the processes mentioned above.

One environmental effect often associated with estrogens found in the aquatic environment is the feminization of male fish. Rainbow trout exposed to EE2 have been reported to show increased feminization and vitellogenin production (Verslycke et al. 2002). A 7-year study on fathead minnows in the Experimental Lakes Area also showed that chronic exposure to low concentrations (~2 ng/L) of EE2 led to the development of intersex fish and adverse effects on gonadal development. It also showed that these effects did have an effect on the reproductive success of fish and ultimately lead to the collapse of the population in the lake (Kidd et al. 2007).

Table 1.1. Examples of Estrogenic Compounds

Estrogenic Compound	Chemical Structure	Use
17β-estradiol (E2)		**Natural female hormone**
17α-ethinyl estradiol (EE2)		**Synthetic estrogen found in oral contraceptives**
Bisphenol A (BPA)		**Used in the manufacturing of certain plastics**
Nonylphenol (NP)		**Industrial surfactants and detergents**
Genistein (GEN)		**Natural phytoestrogen found in legumes such as soybeans**

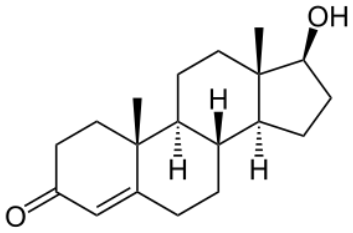
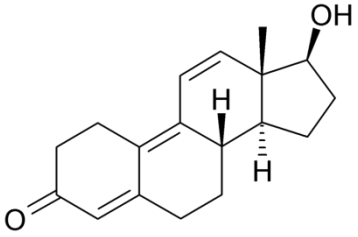
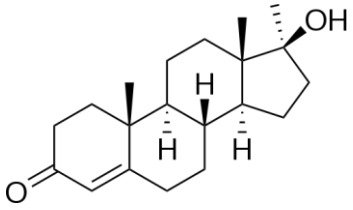
1.1.2. Androgens

Androgens are the primary group of male sex hormones. They are mainly responsible for the development of secondary male sexual characteristics such as muscle development and the inhibition of adipose tissue formation (Singh et al. 2006).

Therefore androgens are commonly found in farm animals as they may be used in the promotion of animal growth (Schiffer et al. 2004). Common androgens include trenbolone used in promotion of growth in livestock and the synthetic androgen methyltestosterone which is often used in the treatment of androgen deficient men (Table 1.2) (Morthorst et al. 2010 and Selzsam et al. 2005). Androgens exert their effect through a mechanism similar to that of estrogens. Upon binding of an androgen, the androgen receptor translocates to the nucleus of the cell and binds onto the section of DNA known as the androgen response element. This in turn regulates the expression of genes associated with androgens and the androgen receptor leading to effects mentioned above.

In fish, exposure to androgens may cause different adverse effects. For example, Zebrafish exposed to environmentally relevant concentrations of trenbolone exhibited irreversible masculinisation as well as a skewed sex ratio tending towards males (Morthorst et al. 2010). Fathead minnow exposed to feedlot effluents from concentrated animal feeding operations containing potent androgens resulted in the defeminisation of female fathead minnows (Orlando et al 2004). Androgens therefore, also represent an important class of EDC capable of inducing adverse effects in aquatic organisms.

Table 1.2. Examples of Androgenic Compounds

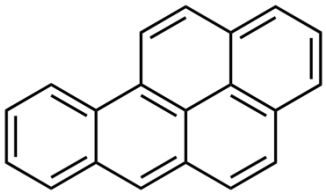
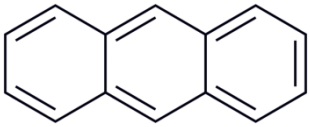
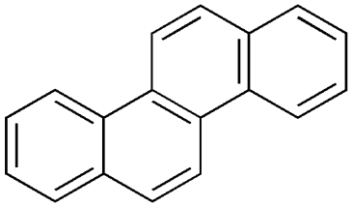
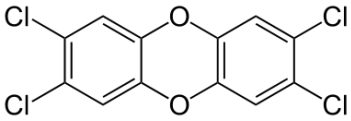
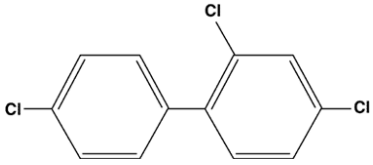
Androgenic Compound	Chemical Structure	Use
Testosterone		**Natural male hormone**
Trenbolone		**Synthetic androgen used mainly in livestock to increase muscle growth**
Methyltestosterone		**Synthetic androgen used to treat androgen deficiency**

1.1.3. Aryl Hydrocarbon Receptor (AhR) Agonists

Agonists of the AhR found in the environment are most often associated with industrial manufacturing or the combustion of oil or coal. Widely known AhR agonists include chemicals such as polycyclic aromatic hydrocarbons (PAHs), halogenated aromatic hydrocarbons (HAHs) such as polychlorinated biphenyls (PCBs) and polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzo-p-dioxins (PCDDs) (Denison et al. 2002). Examples of AhR agonists are listed in Table 1.3. Many of these compounds are known for their acute toxicity and potent carcinogenicity. This is due to the importance of the AhR in the regulation of xenobiotic metabolism. The binding of a ligand to the AhR forms a receptor ligand complex that forms a heterodimeric complex with the aryl hydrocarbon receptor nuclear translocator (ARNT). This complex regulates the expression of metabolic genes such as CYP1A1 and CYP1A2 which increases the breakdown of many AhR agonists. The metabolites of these agonists are quite often

toxic, leading to adverse effects within the cell (Ko et al. 1996, Martin and Klaasen 2010).

Table 1.3. Examples of AhR Agonists

AhR Agonist	Chemical Structure	Use
Benzo[a]pyrene		**Component of coal tar, PAH byproduct of combustion of organic materials**
Anthracene		**Component of coal tar, used in the production of dyes**
Chrysene		**Component of coal tar, found in the wood preservative creosote**
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)		**Industrial byproduct**
PCB 28		**Industrial coolants, plasticizer**

In addition to the acute toxic effects, agonists of the AhR can induce various endocrine disrupting effects. White whales that were found to have been exposed to PCBs had significantly reduced levels of thyroxine (T4), a hormone that contributes to the regulation of metabolism and protein synthesis (Villanger et al. 2011). Killifish exposed to benzo[a]pyrene, a potent carcinogen, had inhibited levels of the enzyme aromatase, the enzyme that normally converts androgens into estrogens. This leads to the interference of normal steroidogenesis within the fish and may lead to adverse effects in development (Patel et al. 2009). Other than through the normal AhR signalling

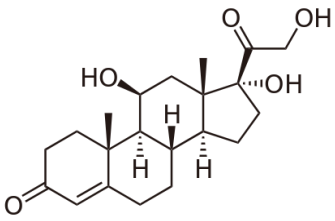
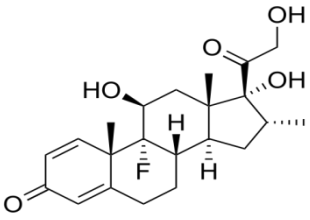
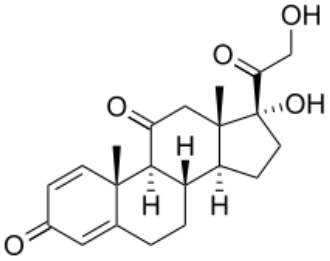
pathway, agonists of the AhR may also induce endocrine disrupting effects through the estrogen receptor pathway. Many PCBs have been shown to have an effect on estrogen signalling through an AhR-ER crosstalk mechanism which will activate the estrogen receptor (Calo et al. 2010). Some AhR agonists may therefore also be classified as estrogenic chemicals as they are capable of mimicking the effects of E2. This further highlights the difficulty of classifying EDCs as a whole, and predicting of biological effects due to EDC exposure.

1.1.4. Glucocorticoids

Glucocorticoids are a class of steroid hormones that function mainly to suppress the immune system, promote gluconeogenesis and regulate blood pressure (Kugathas and Sumpter, 2011). They exert their action by binding to the glucocorticoid receptor (GR) which up-regulates the expression of genes associated with the glucocorticoid response element (GRE). This is similar to the mechanism by which estrogens and androgens exert their effect within an organism. Due to the ability to suppress the immune system, many synthetic and natural glucocorticoids are prescribed as anti-inflammatory drugs, but this same immunosuppressant effect also has the potential to harm organisms exposed to glucocorticoids in the environment (Kitaichi et al. 2010).

Glucocorticoids in the aquatic environment may induce several adverse effects to fish. A study in fathead minnows exposed to the environmentally relevant concentrations of synthetic glucocorticoids prednisolone and beclomethasone dipropionate reported an increase in plasma glucose levels as well decreased levels of white blood cells. The suppression of the immune system may increase the susceptibility of fish to parasitic infections and other diseases which can have a large impact on the sustainability of fish populations (Kugathas and Sumpter, 2011). Examples of glucocorticoids are listed in Table 1.4.

Table 1.4. Examples of Glucocorticoids

Glucocorticoid	Chemical Structure	Use
Hydrocortisone		**Natural stress hormone, increases blood sugar, suppresses immune system**
Dexamethasone		**Anti-inflammatory drug**
Prednisone		**Anti-inflammatory drug**

1.2. EDCs and the Environment

There are 3 main anthropologenic sources by which EDCs may enter the environment and potentially pose a risk to wildlife. This includes municipal wastewater treatment plants (WWTPs), agricultural discharges and industrial effluents. First, WWTPs are a known point source for the release of EDCs into the environment. Many products being used today contain compounds that are known to be or suspected of being EDCs. Daily use of pharmaceuticals such as oral contraceptives and personal care products like lotions and creams in addition to the naturally excreted hormones within human waste contributes to the load of EDCs entering a WWTP (Boyd et al. 2004). Even though WWTPs are not designed to remove EDCs from the wastewater, microbial dependent treatments used in many WWTPs are capable of removing some types of EDCs, more specifically 30-70% of estrogenic activity from the wastewater

(Johnson et al. 2007). Still, EDCs contained within the waters exiting the drains of the average household may eventually reach the environment. It has been reported that the synthetic estrogen EE2, a common EDC and the main component of oral contraceptives, has been detected in aquatic environments at concentrations in the ng/L range (Wise et al. 2011). These concentrations, although low, have the potential to induce adverse behavioural effects in fish under laboratory conditions, including reduced aggression in male zebrafish and fathead minnows, and reduced interactions between male spined sticklebacks (Soffker and Tyler, 2012).

The second point source of EDC discharge is large agricultural and livestock operation. Livestock such as cattle, pigs and goats excrete large amounts of natural hormones in their wastes (Sarmah et al. 2006). In addition to the natural hormones, growth hormones and other synthetic steroids administered to increase the growth in these animals may also be excreted into the farm effluents (Schiffer et al. 2004). Effluents from agricultural facilities are generally untreated and are directly discharged into the environment. Agricultural operations may also use sewage sludge gathered from WWTPs as a source of fertilizer due to their abundance in phosphate (Deeks et al. 2013). But since many EDCs are hydrophobic and have a tendency to bind onto organic particles, EDCs may be concentrated in the sewage sludge during the treatment of wastewater (Lee et al. 2004). There is therefore potential for EDCs in sewage sludge to reach the aquatic environment through farm effluents, especially during rainy seasons.

The third source of EDCs in the environment are the effluents discharged from industrial facilities which may contain a wide range of EDCs such as PAHs, PCBs, PBDEs (polybrominated diphenyl ethers) and heavy metals (Hong et al. 2010). Despite regulatory legislation and discharge permits that limit the release of these chemicals, the persistent and bioaccumulative nature of these EDCs may lead to concentrations capable of inducing adverse effects to humans and wildlife.

EDCs discharged into the environment from the sources mentioned above typically enter surface waters posing a great risk to aquatic organisms. In general, industrial EDCs such as PAHs and PCBs have long half-lives in water and sediments ranging from months to years (Tansel et al. 2011). Natural and synthetic hormones generally have shorter half lives in water and sediment, ranging from hours to days. For

example, the natural female hormone 17β-estradiol (E2) has a typical half life of approximately 1 day in water, whereas the synthetic estrogen EE2, has a half life of 17 days (Jurgens et al. 2002). Even though hormones have much shorter half lives, the constant release of effluents from WWTPs and agricultural operations leads to a pseudo-persistence of these compounds in concentrations detectable in surface waters (Moschet, 2009). Studies from Germany, Japan, Netherlands and Italy found E2 concentrations in surface water up to 27 ng/L (Ying et al. 2002).

Although concentrations of EDCs in surface water remain relatively low (ng/L range) , many EDCs have high octanol water partition coefficients ($\log K_{ow}$) and tend to bind onto organic particles (Ying et al. 2002 and Nagpal, 1993). Many studies have found detectable concentrations of EDCs in marine and river sediments even in areas where the concentrations of EDCs in the surface water are not detectable (Peck et al. 2004, Hilscherova et al. 2002 and Levy et al. 2011). Sediments provide a sink where hydrophobic EDCs may accumulate as the degradation rates of many EDCs is substantially lower in sediments compared to surface water. In surface water, the reported half life of E2 is approximately 1 day, whereas the half life in anaerobic sediments increases to 70 days (Ying and Kookana, 2003). The accumulation of EDCs in river and marine sediments poses an increased risk to benthic species that spend the majority of the time in contact with the sediment. EDCs in sediments may also be released back into the surface water, or the sediment itself may be resuspended into the water column through processes such as bottom trawling, ultimately increasing the exposure to other aquatic organisms. Thus, when river sediments collected from an agriculturally intense watershed containing anti-estrogenic compounds were exposed to female fathead minnows, defeminisation of the fish was apparent (Jeffries et al. 2011). Sediments therefore represent a potential sink or source of EDCs in the environment.

1.3. Methods for EDC Detection

There are currently several commonly used methods in the detection of EDCs in environmental samples. These include chemical analysis such as high performance liquid chromatography (HPLC) or gas chromatography mass spectrometry (GC-MS), and cell based assays such as the yeast screening assays and chemically activated

luciferase expression (CALUX) assays (Nie et al. 2009, Leusch et al. 2010). Each of these methods have distinct advantages and disadvantages which makes the selection of a method dependent on the questions being asked.

Chemical analysis is generally more sensitive compared to cell based assays. In the detection of natural and synthetic estrogenics using high resolution GC-MS, the limits of detection (LODs) were reported as low as the pg/L range (Nie et al. 2009). In cell based assays, method limits of detection reported were between 0.1-5 ng/L depending on the type of cell used (Leusch et al. 2010). In addition to high sensitivity, chemical analysis can also identify the individual compounds within a mixture and quantify the concentrations of the target compounds.

Although chemical analysis has many desirable advantages, it is not without limitation. First and foremost, chemical analysis is expensive, meaning it is not an economical choice when considering the large number of samples that must be tested from the environment to reduce the effect of environmental variation on the quantification of EDCs. Quite often, internal standards are also used in chemical analysis to improve the accuracy of the results which further add to the cost when considering the potentially large number of EDCs that may be included in environmental mixtures.

In addition to being costly, the use of chemical analysis also requires the development of procedures to optimize the derivatization process for EDC detection (Nie et al. 2009). With the wide variety of EDCs that may be in an environmental mixture, different derivatization processes may be needed depending on the types of EDCs being targeted in the analysis. This may increase the time required for sample preparation, further increasing the cost. Even with optimized derivatization procedures, chemical analysis can only detect chemicals which are known to be EDCs. If the endocrine disrupting properties of a chemical present in a sample mixture is not known, it will not be included in the results as it was not a targeted compound.

Cell based assays provide an alternative method to chemical analysis for the detection of EDCs. General advantages of cell based assays include relatively simple procedures, inexpensive when compared to chemical analysis, and quick turnover rates which allows for the rapid screening of a large amount of samples at the same time

(Balsiger et al. 2010). But perhaps the biggest advantage of cell based assays is the direct biological response that is measured in the assay. Cell based assays gives a biological response based on the collective compounds within the mixture and therefore takes into account potential chemical-chemical interactions. A measured biological response also gives direct information on the potential effect of exposure to an organism (Young, 2004). This is compared to chemical analysis which identifies and quantifies individual compounds but gives no indication of the potential effects to an organism.

With all the advantages of cell based assays, there remain a few issues to overcome. One of the main issues is the potential for toxicity of the sample to the cells used in the assay. As environmental samples contain a mixture of chemicals, the potential for highly polluted samples to contain chemicals that are toxic to the cells is substantial (Leusch et al. 2010). This would require further cleanup procedures to remove the toxic substances but may also reduce the levels of the target compounds leading to an underestimation in the final result. The biological variability of cell based assays have also lead to questions regarding the uncertainty when interpreting the results, although adequate replication of experiments may help to overcome some of the variation (Andersen et al. 1999).

The two main types of cell based assays are the yeast cell assays and mammalian cell assays (Hilscherova et al. 2002, Bistan et al. 2011, Houtman et al. 2007, Leusch et al. 2010 and Balsiger et al. 2010). Mammalian cells are generally more sensitive to EDCs and therefore provide a lower limit of detection. They are also more relevant in terms of equating the response seen in the assay to effects expected to be seen in organisms that are exposed. This is due to the natural endogenous hormones contained within the mammalian cell which may interact with the EDCs within the sample, whereas yeast cells transformed to express the hormone receptor do not contain a hormone system (Leusch et al. 2010). The drawbacks in using mammalian cell based assays are the requirements of sample sterilization, longer incubation periods and general higher costs compared to yeast based assays. Mammalian cells also naturally express many different types of hormone receptors which may affect or interfere with the expression of the reporter gene. Therefore, the response seen in the results of these assays may not be based solely on the activity of the class of EDCs being targeted with the assay (Balsiger et al. 2010). In comparison, yeast cells provide the benefit of shorter

incubation periods, lower costs and higher resistance to contamination with the trade-off of being less sensitive. Yeast cells also provide the added advantage of fewer false positives as endogenous hormones which may cause a false positive are not present. Therefore, the response seen will be entirely dependent on the concentration of EDCs in the sample activating the hormone receptor. Yeast bioassays have also been developed to assay environmental samples without the need for extraction, concentration and sterilization (Balsiger et al. 2010). The main disadvantage of yeast based assays however, is that yeast cells genetically modified with the a human hormone receptor, may not react the same way as a normal human cell when exposed to agonists that should activate said receptor. For example, hydrocortisone is a potent agonist of the human glucocorticoid receptor. This compound when exposed to yeast cells modified to express the human glucocorticoid receptor induced virtually no response (Garabedian and Yamamoto, 1992). This may be due to degradation of the compound by the yeast cells or a decrease in the affinity of the receptor for these ligands. Either one of these mechanisms may lead to underestimation of the concentrations of EDCs within the sample. But it should be noted that the major EDCs that are expected to be found in the environment are detectable using yeast based assays (Bistan et al. 2011, Balsiger et al 2010 and Hilscherova et al. 2002).

The disadvantages of chemical analysis and cell based assays make the use of just one method insufficient when assessing the endocrine disrupting properties of an environmental sample. Interestingly, the advantages of each method appear to compensate for the disadvantage of the other. Cell based bioassays may be used to screen a large number of samples to identify and prioritize highly polluted sites. While chemical analysis can be done on the prioritized sites to identify the main chemicals of potential concern. Therefore the use of both methods in the assessment of environmental samples provides information that will lead to a more accurate assessment of EDC concentrations in the sample as well as the chemicals most likely to induce adverse effects to living organisms.

1.4. Research Objective and Study Area

There are many reports which link EDC exposure to adverse reproductive effects and/or decline fish population (Verslycke et al. 2002 and Kidd et al. 2007). It is therefore important to determine EDC concentrations in environmental samples, especially those collected near WWTPs. To this end, our laboratory has compared the use of the yeast estrogenic screening (YES) assay and the E-screen assay in the screening of influents and effluents from WWTPs operated by Metro Vancouver for estrogenic chemicals (Nelson et al. 2007). We have also used the YES assay to survey the concentrations of estrogenic chemicals in WWTPs across Canada (Shieh et al. 2011) and to monitor estrogenic chemicals in fish and shellfish samples from Pakistan (Hunter et al. 2012).

The main objectives of the current study were: (a) to apply a panel of yeast bioassays in detecting and quantifying four different classes of EDCs (AhR agonists, glucocorticoids, estrogenic and androgenic chemicals) in WWTP and environmental samples, (b) to examine the effects of wastewater treatment on EDC concentrations in the wastewater, de-watered biosolids, marine sediments, and initial dilution zone (IDZ) boundary waters, (c) to identify compounds chemically in these samples, which may contribute to the EDC activity of the bioassay, and (d) to conduct a preliminary risk assessment of EDCs for marine sediments near the WWTPS based on the yeast bioassay results. Hitherto, available sediment and wastewater guidelines are based on the concentrations of single chemicals. Because the yeast bioassays are able to provide a response for multitude of chemicals having a similar mode of action, they may be used to develop guidelines for EDC mixtures.

Chapter 2. Materials and Methods

2.1. Sample Collection

Wastewater samples were collected from 5 different WWTPs coded A-E (Table 2.1) operated by Metro Vancouver using ISCO Avalanche refrigerated autosampler in 2012 and 2013. Twenty-four hour time-weighted composite wastewater samples were taken over a period of at least 3 days and transferred to 1 L amber glass bottles. At the time of collection of WWTP E samples in 2013, a disinfection trial was in progress which resulted samples that have undergone a different treatment compared to the WWTP E samples of 2012.

Table 2.1. Characteristics of WWTPs Sampled

WWTP	Annual Volume Treated (Billion L)	Population Served	Sewage Treatment Process
A	32	180 000	Anaerobic Digestion
B	207	600 000	Anaerobic Digestion
C	175	1 000 000	Trickling Filters/Anaerobic Digestion
D	26	172 000	Trickling Filters/ Anaerobic Digestion
E	4.5	27 000	Trickling Filters/ Anaerobic Digestion

Dewatered bio-solid samples were also collected in from the storage tanks and kept in 250 ml amber glass jars. However, biosolid samples were only collected from WWTP A, WWTP C and WWTP D as WWTP B and WWTP E did not process solids at

their location. All samples were kept on ice and/or refrigerated at 4 ^{o}C before being delivered to Simon Fraser University for extraction and analysis. Samples were extracted within 48-72 h upon receipt, and not more than 96 hours from the time of collection to minimize possible chemical degradation.

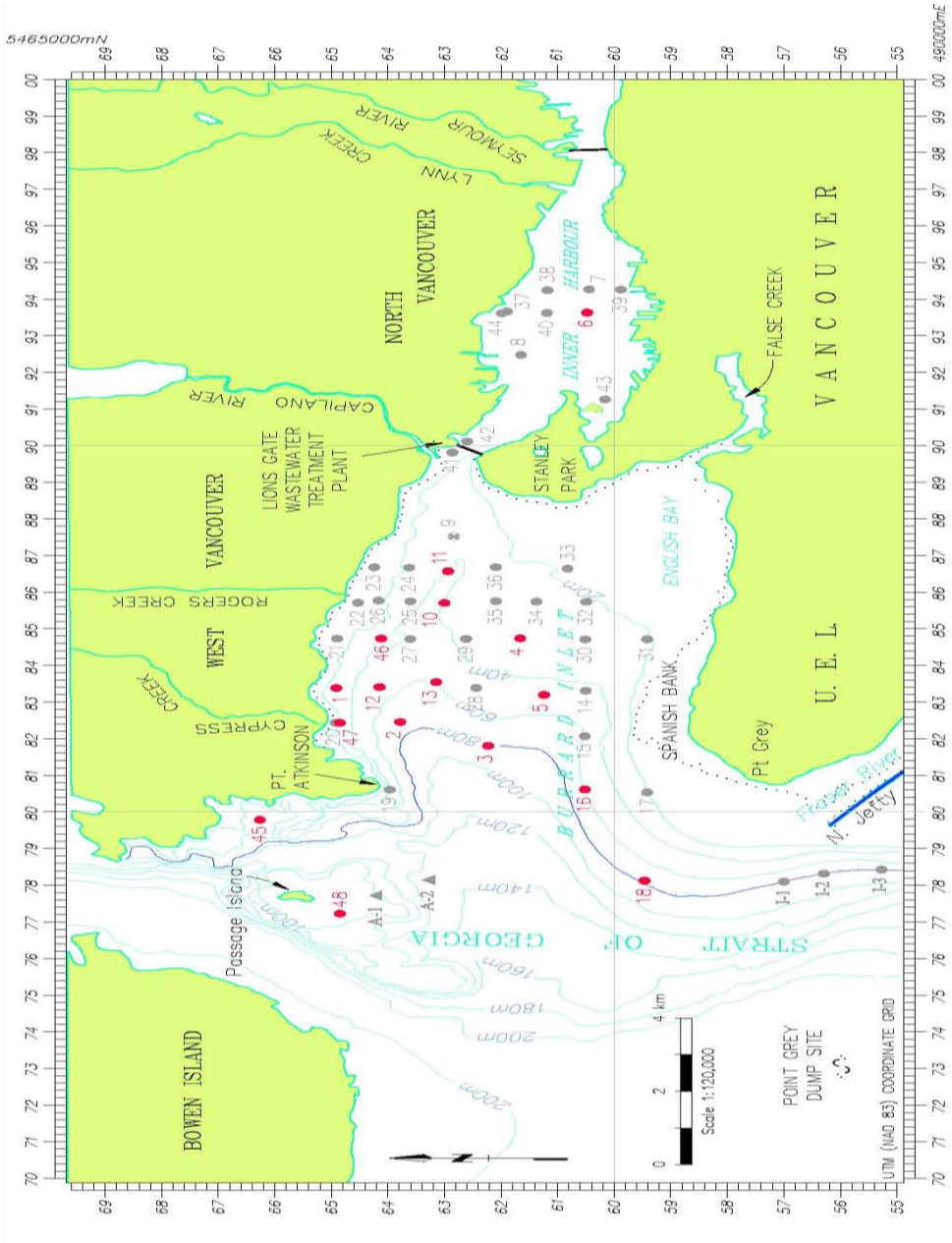


Figure 2.1. Map of WWTP A Stations
Image provided by Metro Vancouver

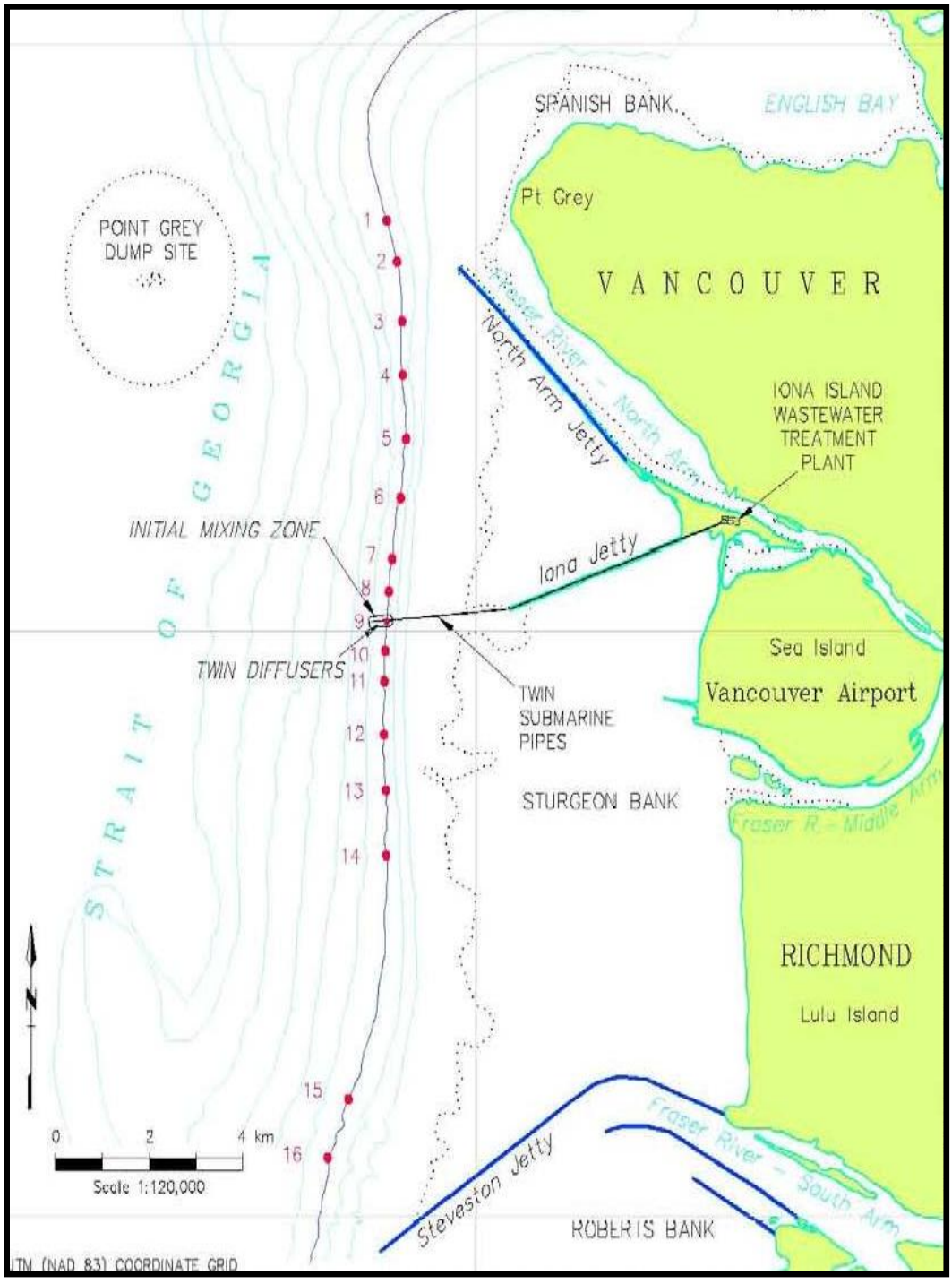


Figure 2.2. Map of WWTP B Stations
Image provided by Metro Vancouver

Marine sediment samples were collected in 2012 and 2013 by Metro Vancouver's consulting teams using a 0.1 m^2 stainless steel Van Veen sampler

(McPherson et al. 2013a,b). At each station three grabs samples were collected. The top 2 cm of each grab was transferred to a stainless steel bowl where it was mixed until the color and consistency was homogenous. Pre-cleaned 250 ml amber glass jars were filled with sediment leaving no headspace. Samples were collected from the receiving environment around the WWTP A and WWTP B outfalls at 16 stations each. In addition, two stations were sampled in duplicate (fresh casts / not a split sample) for a total of 18 samples for each of the two programs. Station locations are mapped on Figures 2.1 and 2.2. All samples were kept on ice and/or stored at 4 ^{o}C before delivery to Simon Fraser University within 72 h of sample collection. Samples were extracted within 72 h upon receipt and assayed within 48 h after extraction to minimize possible chemical degradation.

IDZ water samples were collected in 2012 by ENKON Environmental Limited for Metro Vancouver using a 10-L or 12-L Teflon-lined “Go-Flow” sampler at the WWTP A IDZ boundary at a target sampling depth of 15 m and WWTP C IDZ boundary at a target sampling depth of 3-5 m. Pre-cleaned, 1 L amber glass bottles were filled leaving no headspace. Five samples were collected from each IDZ boundary. One reference sample was also collected for each program for a total of 12 samples. One IDZ boundary sample was sampled in duplicate (ie. split sample). Final effluent samples were also collected during the IDZ boundary sample collection at both WWTPs to provide a comparison to the IDZ boundary samples. All samples collected were kept on ice before delivery and stored at 4^{o}C before extraction. Samples were extracted within 96 h of collection and assayed within 48 h after extraction to minimize potential for chemical degradation.

2.2. Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma Aldrich (Oakville, ON, Canada). The chemicals standards for the bioassays were: 17β-estradiol (E2) for the estrogen receptor (ER) assay, β-naphthoflavone (NAP) for the aromatic hydrocarbon receptor (AhR) assay, deoxycorticosterone (DOC) for the glucocorticoid receptor (GR) assay and dihydrotestosterone (DHT) for the androgen receptor (AR) assay.

L-Histidine (98% purity), L-leucine (98% purity), L-trytophan (98% purity), uracil (99% purity), Difco yeast nitrogenous base w/o amino acids and ammonium sulphate (BD Bioscience, Mississauga, ON, Canada), anhydrous dextrose (Merck Canada, Kirkville, QC, Canada), synthetic complete amino acid dropout mix minus histidine, leucine, uracil and tryptophan were purchased from MP Biomedicals, Solon, OH, USA.

2.3. Chemical Standards and Media Solutions

Stock solutions for the chemical standards were prepared in methanol at a concentration of 2.72E+6 ng/ml and stored at -4 ^{0}C until use. New stock solutions were prepared each month and tested in the assay once per week to confirm potency. Dilution series for the standard stock solution ranging from 2.72E+2 ng/ml to 2.72E-4 ng/ml were prepared for the bioassays. Agar and media were prepared according to Balsiger et al. (2010) and differed in the yeast strain and the amino acids added to the culture medium. For example, DSY-219 SC-UW referred to the DSY-219 yeast strain and the amino acids not used in the culture medium, SC-UW therefore, refers to the media prepared without uracil and tryptophan. The yeast strains and the culture media used in the present study were: DSY-219 in SC-UW media for the ER assay, DSY-1345 in SC-UWH media for the GR assay, DSY-1555 in SC-LUW media for the AR assay and MCY-038 in SC-W media for the AhR assay. All yeast strains were kindly provided by Dr. Marc B. Cox (University of Texas at El Paso).

2.4. Sample Extraction and Dilution

Wastewater samples were extracted according to Huang and Sedlak (2001). Fifty ml of the wastewater sample were poured into clean 200 ml glass beakers. The C18 extraction discs (Empore 3M, London, ON, Canada) were preconditioned with 10 ml methanol for 1min, followed by 10 mL of distilled water for 1min. The 50 ml wastewater sample was filtered through the preconditioned extraction disc and eluted with 10 ml of methanol after soaking for 2 min. The eluent was collected in a clean test tube and evaporated to dryness under a gentle stream of nitrogen.

Biosolid and marine sediment samples were extracted using the methods described by Temes et al. (2002). Five grams of the biosolid/marine sediment sample was measured into a clean glass extraction tube. Ten ml of ethyl acetate was added into the sample and shaken by hand for 2 min. The extraction tubes were then set on a shaker at low for 10min before being centrifuged at 2000 rpm for another 10 min. The ethyl acetate portion was collected in a clean test tube and the process was repeated two more times for a total collection of 30 mL of ethyl acetate. This portion was evaporated to dryness under a gentle stream of nitrogen. The evaporated residues were reconstituted in 500 µl of methanol and stored at -4 ^{o}C until use.

As the extracts of the biosolid samples may contain sulphur and cause toxicity to the yeast cells, a simple and rapid sulphur removal procedure described by Jensen et al. (1977) was used to reduce the potential of this toxicity.

Dilutions series were prepared for each sample extract at 1.0, 0.8, 0.2, 0.02, 0.002 and 0.0002 of the original extract concentration before analysis.

2.5. Yeast Bioassay

The procedures for the yeast bioassay were modified from Balsiger et al. (2010). Each of the yeast strains used in the assays contained a receptor for a particular class of endocrine disrupting chemical (Estrogens, Androgens, Glucorticoids and Aromatic Hydrocarbons). The receptors upon binding to an agonist in the test extract induced an upregulation in the production of the *β*-galactosidase enzyme within the yeast cells. The amount of β-galactosidase produced within the yeast cells was directly proportional to the potency of the total estrogens, androgens, aromatic hydrocarbons or glucocorticoids in the test extract. Upon addition of a buffer and substrate mixture, the yeast cells were lysed resulting in the release of *β*-galactosidase which catalyzed a reaction with the added substrate. The reaction produced UV light as a measurable signal. The UV light intensity was proportional to the amount of β-galactosidase produced.

Table 2.2. Summary of the 4 Assays

Assay Type	Standard Compound	Yeast Strain	Culture Media	Media used for Dilution
Estrogen	17β-estradiol	DSY-219	SC-UW	SC-UW
Androgen	Dihydrotestosterone	DSY-1555	SC-LUW	SC-LUW
Glucocorticoid	Deoxycorticosterone	DSY-1345	SC-UWH	SC-UWH
AhR Agonists	B-naphthoflavone	MCY-038	SC-W	SC-W w/ Galactose

H, L, U, and W represents histidine, leucine, uracil and tryptophan respectively. All media were made with dextrose as the sugar with the exception of the dilution media for the AhR assay where galactose is used.

The experimental procedures for the four assays were very similar but with different yeast strains and culture medium used in each assay (Table 2.2). On the first day, one colony of a yeast strain was inoculated into 10 ml of the culture medium. The culture was incubated overnight at 30 ^{o}C with shaking. On the morning of day two, the culture was diluted to an optical density of 0.08 at a wavelength of 600 nm. This diluted culture was again incubated at 30 ^{o}C until an optical density of 0.1 at 600 nm was reached. The incubation typically took 2-2.5 h. Upon the completion of the incubation, 1 µl of each concentration of the sample and standard dilution series were aliquotted onto an opaque bottom 96 well cell culture plate followed by 100 µl of the yeast culture. The contents of each well were mixed by pipetting gently up and down before incubation at 30 ^{o}C for 2 h. Several min before the completion of the incubation, Tropix Gal-screen buffer was prepared by diluting the substrate with Buffer B at a ratio of 1:24 and kept on ice until use. The mixture was added into each well of the 96 well plate in 100 µl aliquots and mixed gently with a pipet before a final 2 h incubation at room temperature. Following the last incubation, the plate was read on a Multilabel Plate Reader (Perkin Elemer, Woodbridge, ON, Canada) to determine the UV activity induced by the sample in each well. All samples were tested in triplicates.

2.6. Calculation of Data

The UV light intensities obtained from the plate reader were plotted as dose response curves for both the standard and sample dilution series on Graphpad (La Jolla, CA, USA) Prism 5. As the sample concentrations were unknown, the concentrations

were instead plotted as the dilution factors mentioned above (Section 2.4). From the resulting dose-response curves, EC50s and the slopes of each curve were obtained. For some samples, theundiluted extracts showed absorbance readings below the background value indicating the presence of unknown chemicals which caused toxicity to the yeast cells. These samples were not included in the calculation. Thus the EC from samples not affected by toxicity were used to calculate the endocrine disrupting potency of the samples with the equation from Lorenzen et al. (2004) The results were expressed as endocrine disruptor equivalents where "endocrine disruptor" is one of the standard compounds (β-naphthoflavone, 17β-estradiol, deoxycorticosterone or dihydrotestosterone):

Below is an illustrative example to calculate β-naphthoflavone equivalents (NAPEQ) in the AhR assay (Lorenzen et al. 2004):

NAPEQ (ng/ml) = [β-naphthoflavone EC_{50}(ng/ml) / extract EC_{50} (unitless)] * [volume of assay medium (ml) / (volume of extract tested (µl)] * [volume of stock extract (µl) / volume of unknown water sample (ml)]. **(1)**

The EC50 obtained from the dose-response curves also were used to calculate the EC20s and EC30s of both the sample and standard curves using the following equation:

ECX = [X/(100-X)^(1/H)] * EC50 Where X is either 20 or 30 and H is the hill slope of the curve. **(2)**

Using these values, the estimates from equation 1 using the EC20s, EC30s and EC50s were averaged to take into account the linear portion of the sample and standard curves providing a more accurate estimate of the sample potency.

2.7. Statistical Analysis

The final results were reported as the mean concentration equivalents of a standard compound ± standard error of the mean (SEM). Data <MLOD were graphed as half of the MLOD value. To make further statistical comparisons, a Student's *t*-test was

used to determine significance at $p < 0.05$ for wastewater influents compared to effluents, and year to year differences between marine sediment and biosolid samples. The Pearson's R Test was used to determine if there were relationships between the results of each bioassay and also the measured EDC concentrations in marine sediments and the distance from the WWTP outfall.

The LOD of each assay was taken as the EC20 of the standard curve because the EC20 is the lowest concentration at which the assays can reliably differentiate from background activity (Lorenzen et al. 2004). Using the EC20s as the LOD and the amount of standard added to the sample, the method limit of detection (MLOD) for water was determined in each yeast bioassay. Samples/replicates that tested <MLOD were included in the calculation of mean EEQs as one half of the MLOD. An examination of the limits of detection of our assays showed that they were generally within the ranges reported in the literature (Table 2.3), with perhaps the exception of the glucocorticoid assay where no comparable values could be found.

Table 2.3. MLODs of Bioassays for Aqueous Samples

Assay	Current Study (ng/L)	Other Studies (ng/L)	Reference
Estrogen	1.28±0.26	0.1-5	Di Dea Bergamasco et al. (2011), Leusch et al. (2010)
Aromatic Hydrocarbon	50.5±10.1	49.9-59.1	Miller et al. (1997), Olivares et al. (2011)
Androgen	17.4±3.5	17.3-78.7	Bovee et al. (2007), Eldridge et al. (2007)
Glucocorticoid	94±19.0	N/A	N/A

2.8. GC-MS Analysis

Sediment and WWTP final effluent samples with high EDC contents in the yeast bioassays were also extracted and analyzed with GC-MS (Nie et al. 2009) which consisted of a Model 7890A gas chromatograph and a model 5975C VL MSD mass spectrometer (Agilent Technologies, USA). The carrier gas used was 99.99% helium at a flow rate of 1.5 ml/min. The GC oven temperature was programmed to start at 100^{o}C

for 1 minute before an increase to 200 ^{o}C at 10 ^{o}C/min, followed by an increase to 260 ^{o}C at 15 ^{o}C/min, and a final increase to 300 ^{o}C at 3 ^{o}C/min. The MS was operated in full scan mode to determine the unknown compounds within the sample.

Chapter 3. Results

3.1. Glucocorticoid and Androgen Assays

All samples collected in 2012 were tested for glucocorticoids and androgenic chemicals in addition to estrogenic chemicals and aromatic hydrocarbon receptor agonists. All samples tested for glucocorticoid and androgen activity were <MLOD in both assays.

In 2013, selected samples (WWTP A: 1, 3, 45, 49, 51, 52; WWTP B: 3, 7, 9, 16, 17, 18) containing the highest and lowest EEQs or NAPEQs from 2012 were assayed for glucocorticoids and androgens to confirm the 2012 results. Again, samples tested in 2013 were <MLOD for both assays.

3.2. E2 and AhR Assay Verification

Distilled water samples spiked with three different concentrations of E2 and *β*-naphthoflavone were extracted and assayed to examine the accuracy of the E2 and AhR assays as well as the recovery rate of the extraction method. Results of the study showed that assay-determined 17β-estradiol equivalents (ng/mL) and NAPEQs (ng/mL) were very close to the actual E2 and *β*-naphthoflavone concentrations (Figure 3.1 and 3.2). In addition, the calculated percent coefficients of variation (% CV) for both assays were used to assess the precision of the assays and the variability of the assays. For the E2 assay, the %CV was 51.2, 47.6 and 56.6 for each of the three spiked concentrations. For the AhR assays, %CVs was 15.4, 11.0 and 20.5 for each of the concentrations tested. The %CVs calculated were similar across the 3 test concentrations for both assays, suggesting that there was minimal plate-to-plate variation for each assay. However, the mean %CV of 51.8% for the E2 assay indicated that there was a considerable amount of variation in the estimated EEQs.

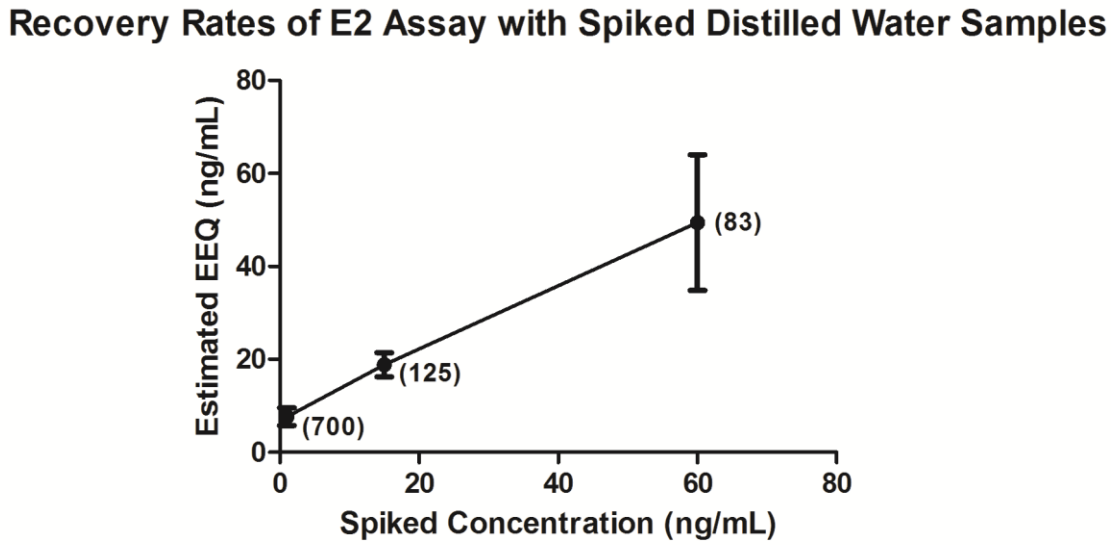


Figure 3.1. E2 Assay Results of Spiked Distilled Water with 3 Different E2 Concentrations.

Each point represents the mean ± SEM of three separate assays. Percent recovery in brackets.

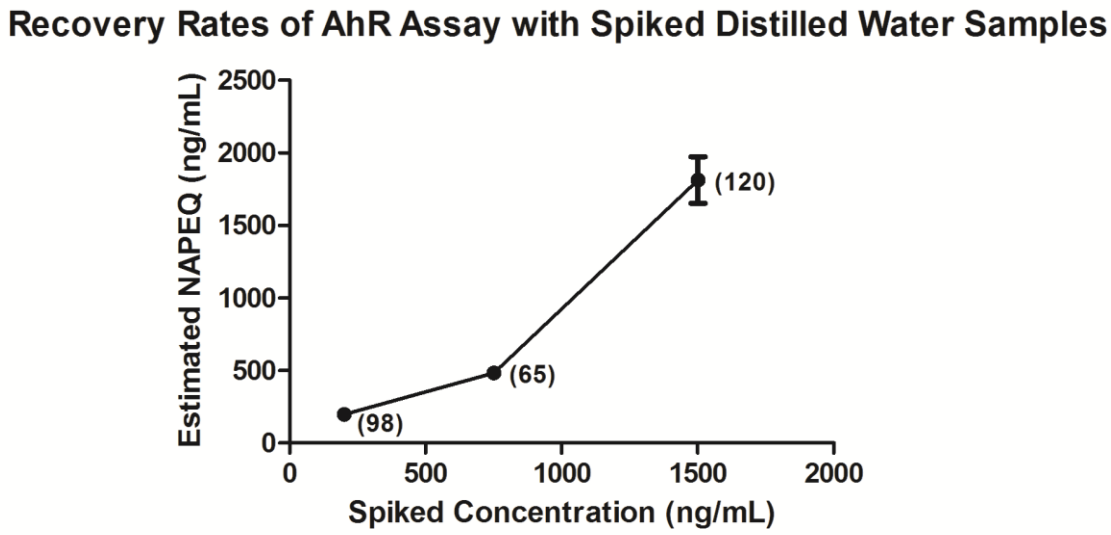


Figure 3.2. AhR Assay Results of Spiked Distilled Water with 3 Different NAP Concentrations.

Each point represents the mean ± SEM of three separate assays. Percent recovery in brackets

3.3. Effects of Storage

As there is a period of time between sample collection and the running of the assay, there is potential for degradation of target compounds which may affect the results of the assay and/or the GC-MS analysis. Select marine sediment samples where EDC activity was detected from 2012 for both WWTP A and WWTP B were assayed at times approximately 1 month and 2 months after collection to determine the effects of storage on the concentration of estrogenic and AhR activity. For the E2 assays (WWTP A 3, 6, 10, 12, 13, 18, 45, 49, WWTP B 2, 9, 10, 11, 14, 15) estrogenic activity was still detectable after 1 month of storage at 4^{o}C. The WWTP A samples did not change significantly ($p < 0.05$) in the estimated EEQ while the samples at WWTP B had a statistically significant ($p < 0.05$) increase of 60% in EEQ after 1 month of storage. Estrogenic activity was no longer detectable when assayed 2 months after the sample collection date (Figures 3.3 and 3.4).

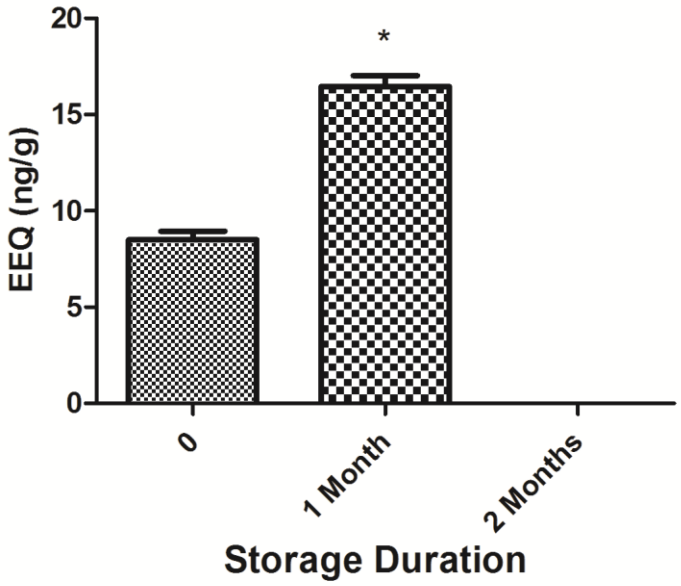


Figure 3.3. Effects of Storage on EEQs of WWTP B Marine Sediments.
Bars represent the mean ± SEM of the 6 samples tested. An * indicates a significant effect of storage on the marine sediment samples ($p < 0.05$)

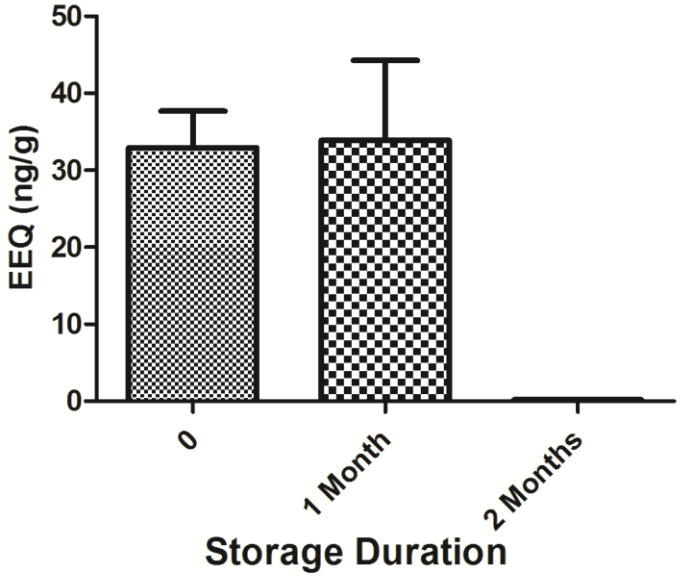


Figure 3.4. Effects of Storage on EEQs of WWTP A Marine Sediments.
Bars represent the mean ± SEM of the 8 samples tested. An * indicates a significant effect of storage on the marine sediment samples ($p < 0.05$)

For the AhR assays (WWTP B 2, 14, 15, 16, WWTP A 3, 6, 10, 12, 13, 18, 45, 49), samples at both WWTP A and WWTP B showed no significant changes ($p < 0.05$) in the AhR activity even after 2 months of storage (Figures 3.5 and 3.6).

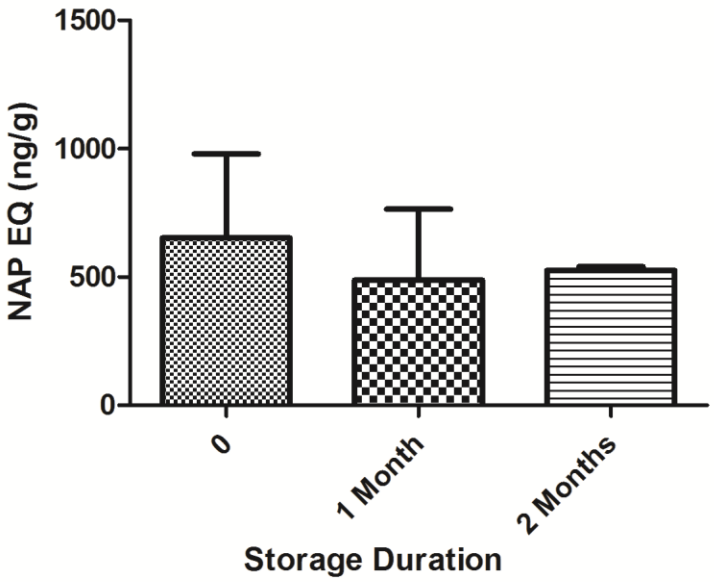


Figure 3.5. Effects of Storage on NAPEQs of WWTP B Marine Sediments.
Bars represent the mean ± SEM of the 4 samples tested. An * indicates a significant effect of storage on the marine sediment samples ($p < 0.05$)

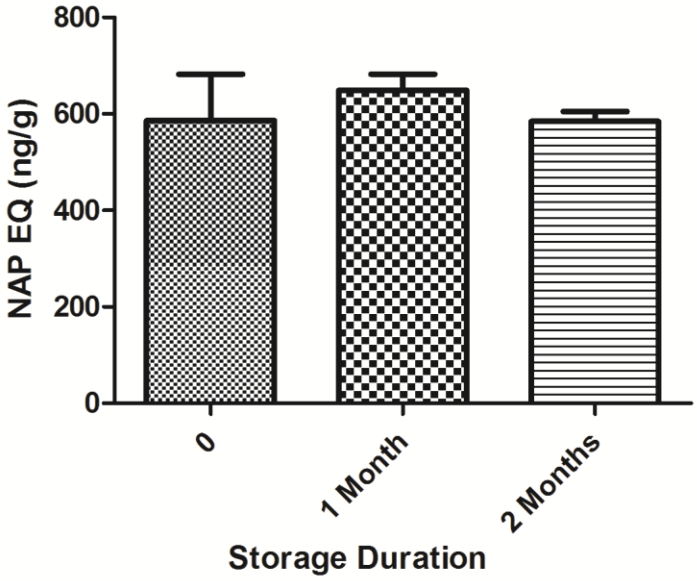


Figure 3.6. Effects of Storage on NAPEQs of WWTP A Marine Sediments.
Bars represent the mean ± SEM of the 8 samples tested. An * indicates a significant effect of storage on the marine sediment samples ($p < 0.05$)

3.4. Wastewater Analysis for EDCs

3.4.1. Estrogenic Chemicals

The YES bioassay showed that there were detectable concentrations of estrogenic chemicals in many raw influent and final effluent samples (Figure 3.7). Of the 5 WWTPs from which wastewater samples were collected, 3 WWTPs (WWTP A, WWTP B, WWTP C) showed a statistically significant ($p < 0.05$) decrease of EEQs in the final effluents when compared to the raw influents. In contrast the final effluents of WWTP D had detectable estrogenic chemicals although the raw influents did not show any estrogenic chemicals. Also, WWTP C was the only WWTP where raw influents and final effluents had detected estrogenic activity in both sampling years with an average decrease of 85% in the estrogenic activity of the final effluent compared to the raw influent. At WWTP E, toxicity of the raw influent to the yeast cells was observed in both sampling years (2012 and 2013), while toxicity due to the final effluent was only observed in the first year (2012) of sampling. The reduction of toxicity in the second year (2013) of sampling coincided with the implementation of a chlorination process which occurred between the two sampling seasons. However, variation in samples, weather and industrial activity may have also lead to the observed result. Regardless, estrogenic chemicals were detected in these effluents, but whether the concentrations were above or below that of the raw influents was unknown. Toxicity was also observed to a lesser degree in the raw influents collected at WWTP A. This was seen in all raw influents in the 2012 but only in samples collected on 2 of the 4 days sampled in 2013. This suggests that there is variation in the wastewater quality input to and processed by the WWTPs daily.

A year-to-year comparison of the results showed no obvious differences between the estimated EEQs of the two sampling years other than the aforementioned changes at WWTP E (Table 3.1 and 3.2). Although the 2013 WWTP B raw influents had detectable estrogenic activity, the detection was only seen in 1 of the 3 sampling days. The remaining WWTP B raw influents and final effluents showed EEQs <MLOD for both sampling years. Collectively, the highest EEQs estimated in the raw influents were from WWTP C, with an estimate of 4.8±0.21 ng/L in 2012 and 9.8±3.7 ng/L in 2013, whereas the lowest estimates were <MLOD for both sampling years at WWTP D. Although

WWTP D raw influents had EEQs below the <MLOD, the final effluents had the highest estimated EEQs out of all effluents tested for both sampling years with estimates of 1±0.005 ng/L in 2012 and 2±0.45 ng/L in 2013. The lowest EEQs seen in all of the final effluents came from the WWTP B which were below <MLOD for both sampling years.

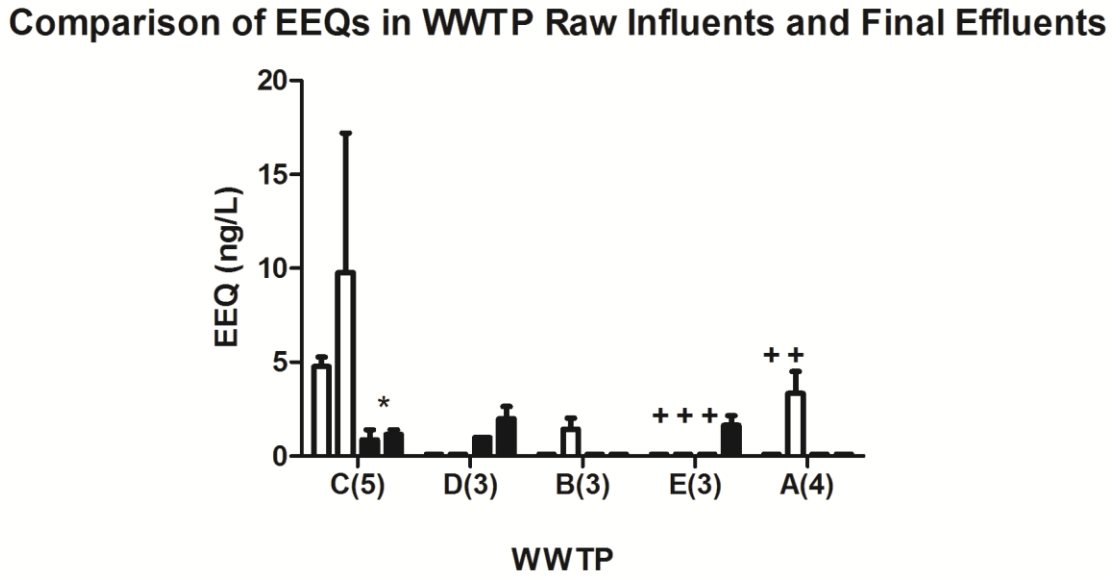


Figure 3.7. E2 Assay Results of WWTP Raw Influents and Final Effluents.
Bars represent the mean ± s.e.m. of the samples collected over (n) sampling days. Each WWTP contains 4 bars where the first and third represent results from 2012, and second and fourth bars from 2013. An * indicates a significant difference between influent and effluent results ($p < 0.05$). A + indicates toxicity observed in at least one sample. Raw Influents ☐ , Final Effluents ■

Table 3.1. Ranges of EEQs (ng/L) in WWTP Raw Influents

	Raw Influent EEQs (ng/L) 2012			Raw Influent EEQs (ng/L) 2013		
	Low	**High**	**Mean**	**Low**	**High**	**Mean**
WWTP C	1.9	6.2	4.7 (5)	2.2	19	9.7 (5)
WWTP D	<MLOD	<MLOD	<MLOD (3)	<MLOD	<MLOD	<MLOD (4)
WWTP B	<MLOD	<MLOD	<MLOD (3)	<MLOD	5.7	1.4 (4)
WWTP E	T	T	T (4)	T	T	T (5)
WWTP A	T	T	T (5)	T	4.1	3.3 (5)

T= toxicity observed, (n) = number of samples

Table 3.2. Range of EEQs (ng/L) in WWTP Final Effluents

	Final Effluent EEQs (ng/L) 2012			Final Effluent EEQs (ng/L) 2013		
	Low	**High**	**Mean**	**Low**	**High**	**Mean**
WWTP C	<MLOD	4.4	0.8 (5)	0.6	1.5	1.1 (5)
WWTP D	0.3	1.3	0.9 (3)	<MLOD	2.4	2.0 (4)
WWTP B	<MLOD	<MLOD	<MLOD (3)	<MLOD	<MLOD	<MLOD (4)
WWTP E	T	T	T (4)	1.0	2.4	1.6 (5)
WWTP A	<MLOD	<MLOD	<MLOD (5)	<MLOD	<MLOD	<MLOD (5)

T=toxicity observed, (n) = number of samples

3.4.2. AhR Agonists

The aromatic hydrocarbon receptor (AhR) assays detected AhR agonists present in the raw influents and final effluents of the majority of samples tested (Figure 3.8). WWTP C and WWTP D showed statistically significant ($p < 0.05$) decreases in the estimated β-naphthoflavone equivalents (NAPEQs) in the final effluents when compared

to the raw influents, whereas WWTP B and WWTP A raw influents and final effluents had estimated NAPEQs that were not significantly different. At WWTP C, the average decrease in AhR activity in the final effluent was approximately 75%, whereas WWTP D had a similar average decrease of 74%. The results of the AhR assay for WWTP E samples were affected by toxicity much like the results of the E2 assay. Toxicity was observed in all samples from WWTP E except for the effluents of 2013 which all tested <MLOD. In 2012, the range of NAPEQs estimated ranged from <MLOD (WWTP B) to 234±1.8 ng/L (WWTP D) in the raw influents and <MLOD (WWTP B) to 197±17 ng/L (WWTP A) in the final effluents. In 2013, estimates ranged from 81±52 ng/L (WWTP B) to 434±217 ng/L (WWTP C) in the raw influents and <MLOD (WWTP E) to 88±3.4 ng/L (WWTP D) in the final effluents.

A comparison of results between the 2012 and 2013 showed no significant temporal differences ($p < 0.05$) in the NAPEQs estimated in samples from WWTP A, WWTP C and WWTP D (Table 3.3 and 3.4). At WWTP B, differences were observed between the 2012 and 2013 samples, as the raw influents and final effluents collected in 2012 all tested <MLOD, but AhR activity was detected in both in 2013.

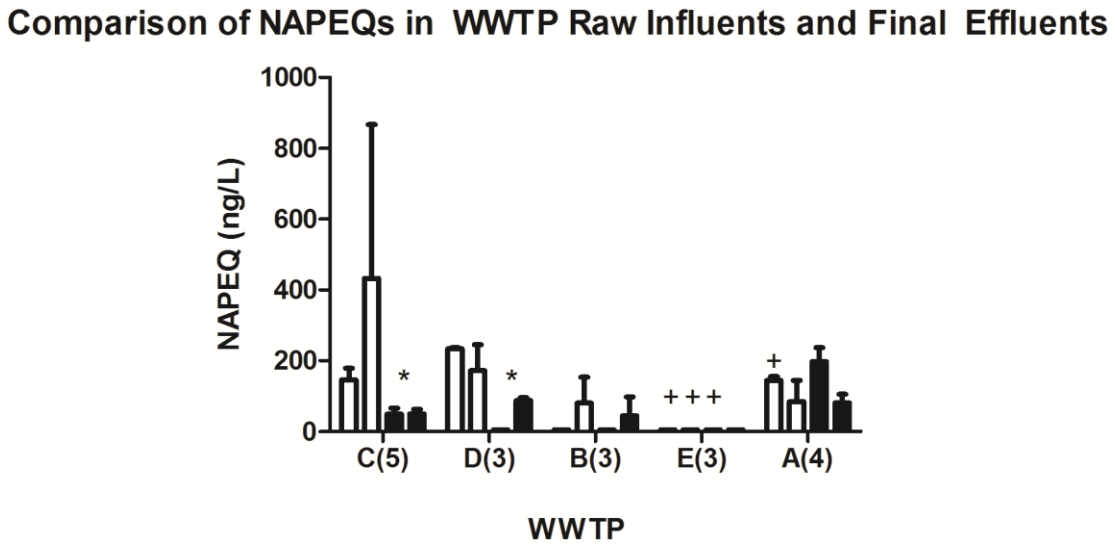


Figure 3.8. Ahr Assay Results of WWTP Raw Influents and Final Effluents.
Bars represent the mean ± s.e.m. of samples collected over (n) sampling days. Each WWTP contains 4 bars where the first and third represent results from year 2012, and second and fourth bars from 2013. An * indicates a significant difference between influent and effluent results ($p < 0.05$). A+ indicates toxicity observed in at least one sample. Raw Influents ☐, Final Effluents ■

Table 3.3. Range of NAPEQs (ng/L) in WWTP Raw Influents

	Raw Influent NAPEQs (ng/L) Year 1			Raw Influent NAPEQs (ng/L) Year 2		
	Low	**High**	**Mean**	**Low**	**High**	**Mean**
WWTP C	58	232	146 (5)	65	971	432 (5)
WWTP D	220	244	234 (3)	105	274	172 (4)
WWTP B	<MLOD	<MLOD	<MLOD (3)	<MLOD	132	81 (4)
WWTP E	T	T	T (4)	T	T	T (5)
WWTP A	T	266	144 (5)	<MLOD	168	84 (5)

T=toxicity observed, (n) = number of samples

Table 3.4. Ranges of NAPEQs (ng/L) in WWTP Final Effluents

	Final Effluent NAPEQs (ng/L) Year 1			Final Effluent NAPEQs (ng/L) Year 2		
	Low	**High**	**Mean**	**Low**	**High**	**Mean**
WWTP C	<MLOD	131	49 (5)	<MLOD	77	50 (5)
WWTP D	<MLOD	<MLOD	<MLOD (3)	82.37	96	88 (4)
WWTP B	<MLOD	<MLOD	<MLOD (3)	<MLOD	121	44 (4)
WWTP E	T	T	T (4)	<MLOD	<MLOD	<MLOD (5)
WWTP A	<MLOD	303	198 (5)	<MLOD	101	80 (5)

T=toxicity observed, (n) = number of samples

3.5. Biosolid Analysis for EDCs

3.5.1. Estrogenic Chemicals

The biosolids from WWTP A, WWTP C and WWTP D all showed estrogenic activity based on the E2 yeast assay (Figure 3.9). Of the three WWTPs, biosolid

samples from WWTP D appeared to contain the highest average estrogenic activity with EEQs of 45±6.3 ng/g and 48±8.6 ng/g in 2012 and 2013 respectively. These were comparable to WWTP C with EEQs of 11±6.5 ng/g in year 2012 and 28.2±4.9 ng/g in 2013. At WWTP A, the toxicity that was observed in some of the wastewater samples was also seen in the biosolid samples. Of the 4 samples collected at WWTP A in each of the sampling years, only 1 of the 4 samples in 2012 had detectable estrogenic activity where the other 3 exhibited toxicity. All samples in 2013 showed toxicity to the yeast cells. However, the detection had an estimated EEQ of 123.7 ng/g, which was the highest estimate among all the biosolid samples assayed. Overall, there is no significant year to year ($p < 0.05$) variation in EEQs estimated in the samples collected at each WWTP.

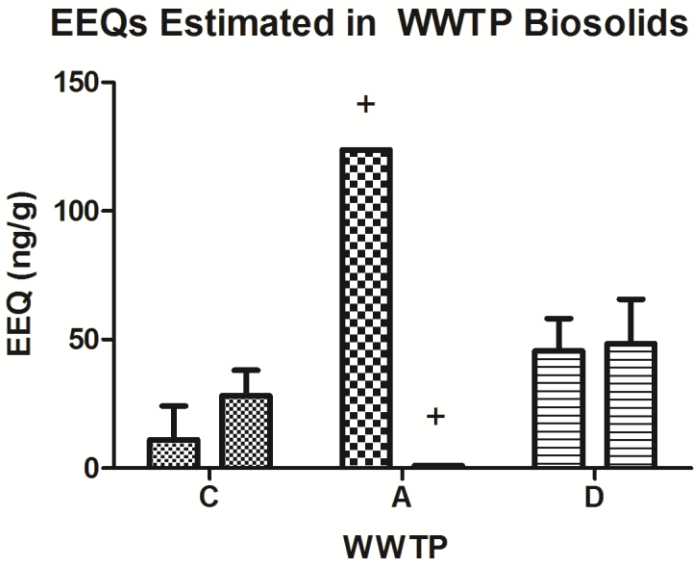


Figure 3.9. E2 Assay Results of WWTP Biosolids.
Bars represent the mean of 3-4 samples ± s.e.m. of samples collected over 3-4 sampling days. Each WWTP contains 2 bars where the first represents 2012 and the second represents 2013. An * indicates a significant difference between year 1 and year 2 estimates ($p < 0.05$). + indicates toxicity observed in at least one sample

3.5.2. AhR Agonists

The results of the AhR assay for the biosolids differed to the E2 assay results markedly (Figure 3.10). Although toxicity was also seen in some of the WWTP A samples, 3 of the 4 samples in 2012 had detectable AhR activity, while only 1 showed

toxicity. In 2013, 1 sample had detectable AhR activity while the other 3 showed toxicity. The NAPEQs estimated in the WWTP A biosolid samples ranged from 6716 ng/g to 9174 ng/g, which were higher than the NAPEQs seen in the other two WWTPs. WWTP C biosolids contained the second highest estimates of NAPEQs at 3373±1342 ng/g in 2012 and 3081±728 ng/g in 2013. WWTP D biosolid samples contained the lowest estimated NAPEQs of the three WWTPs with 672±142 ng/g in 2012 and 1450±271 ng/g in 2013.

When the results were compared year to year, there was no significant variation ($p < 0.05$) between 2012 and 2013 samples collected from WWTP C and WWTP D. In terms of NAPEQs estimated, there was also no significant variation seen at WWTP A ($p < 0.05$), but there appeared to be an increase in toxicity to the yeast cells in the biosolid samples collected during 2013. This difference was seen for both the E2 and AhR assays for the biosolid samples.

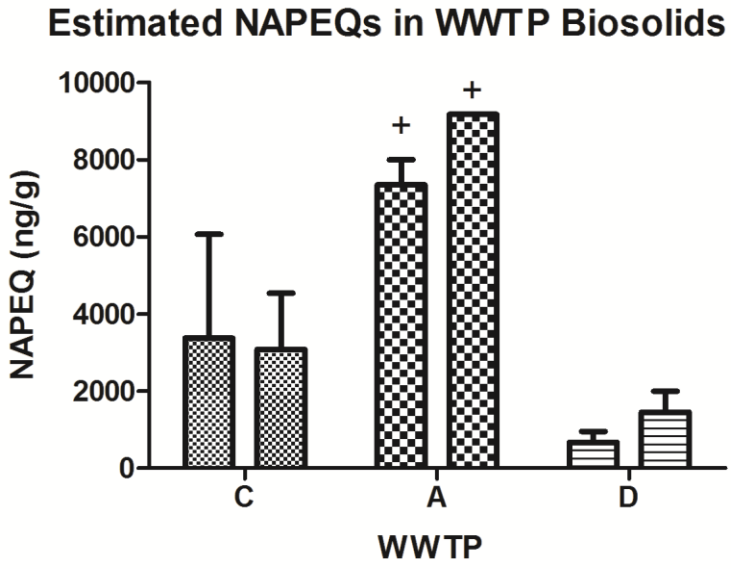


Figure 3.10. AhR Assay Results of WWTP Biosolids.
Bars represent the mean of 3-4 samples ± s.e.m. of samples collected over 3-4 sampling days. Each WWTP contains 2 bars where the first represents year 2012 and the second represents 2013. * indicates a significant difference between year 1 and year 2 estimates ($p < 0.05$). + indicates toxicity observed in at least one sample

3.6. IDZ Analysis for EDCs

Water samples collected at the IDZ boundary in the receiving environment of WWTP A and WWTP C were assayed for both AhR agonists and estrogenic chemicals. At both the WWTP A and WWTP C IDZs, no AhR activity was detected by the AhR assay for the 5 stations sampled (data not shown). For the E2 assay, 2 stations in the WWTP C IDZ had detectable estrogenic activity with 3.38±1.2 and 1.83±0.3 ng EEQs/L (Figure 3.11) whereas no estrogenic activity was detected in WWTP A IDZ samples (Data not shown). The EEQs estimated from WWTP C IDZ samples were higher than the average EEQ of 0.6±0.08 ng/L seen in the final effluents of WWTP C indicating that there are likely other sources contributing to the estrogenic activity seen at the WWTP C IDZ.

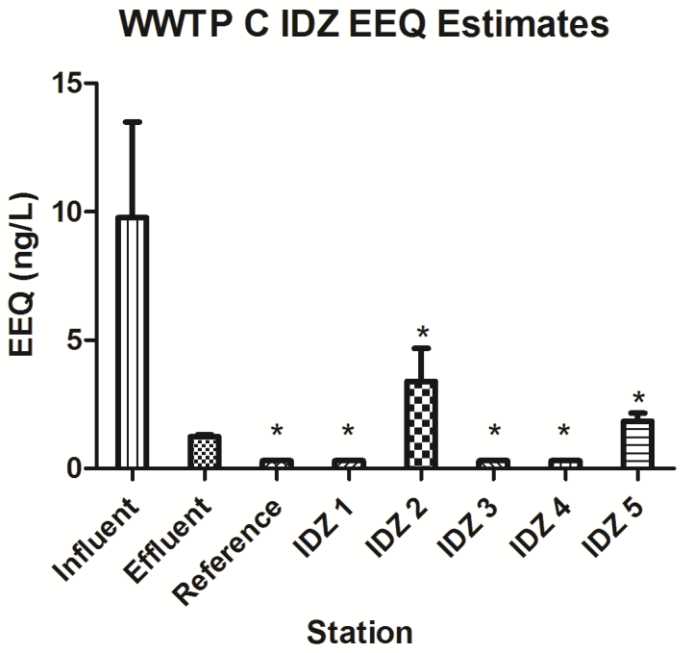


Figure 3.11. E2 Assay Results of WWTP C IDZ samples and Effluents from 2012.
Bars represent the mean ± s.e.m. of three lab assay replicates. * indicates a significant difference between effluent and IDZ results ($p < 0.05$)

3.7. Marine Sediment Analysis for EDCs

3.7.1. Estrogenic Activity

In 2012, 8 of the 16 stations or 50% of the stations from WWTP B stations had detectable estrogenic activity, while only 6 of 16 stations or 38% of the stations of WWTP A showed estrogenic activity. Although there were more detects in the WWTP B stations, the EEQs at WWTP A stations were much higher than those of WWTP B stations. The EEQs at WWTP B stations ranged from 3.8±0.9 - 16.5±4.8 ng/g with a median value of 9.3 ng/g (Figure 3.12) while the EEQs at WWTP A stations ranged from 1.6±0.3 – 51.9±11 ng/g with a median value of 28.5 ng/g (Figure 3.13).

In 2013, 7 of the 16 stations or 44% of the stations from WWTP B showed estrogenic activity, while 6 of the 16 stations or 38% of the stations showed estrogenic activity at WWTP A. Much like the first sampling year, the marine sediments from WWTP A had higher EEQs compared to those from WWTP B. The EEQs in WWTP B marine sediments ranged from 7.2±2.1 - 12.4±5.5 ng/g with a median value of 9.5 ng/g, while the EEQs of WWTP A samples ranged from 12.0±2.7 - 49.4±17.4 ng/g, with a median value of 27.0 ng/g.

When the results of the two sampling years were compared, there was some yearly variation seen in both study areas. At WWTP B, 4 stations had detections in both sampling years with estimated EEQs that were not significantly ($p < 0.05$) different between the two years. In addition, 4 stations had a decrease in EEQ to <MLOD between 2012 and 2013, whereas 3 stations that tested <MLOD in 2012 showed detectable estrogenic activity in 2013. Similar to WWTP B, WWTP A also had 4 stations that had detectable estrogenic activity in both sampling years with no significant ($p < 0.05$) difference in the EEQs between the two sampling years. There were also 2 stations that had an increase in the EEQ from <MLOD and 2 station that had a decrease in EEQ to <MLOD. Overall, there was no significant correlation between the EEQs at each station and the distance of the station from the WWTP outfalls of either WWTP (R^2 = 0.006 for WWTP B and 0.018 for WWTP A) .

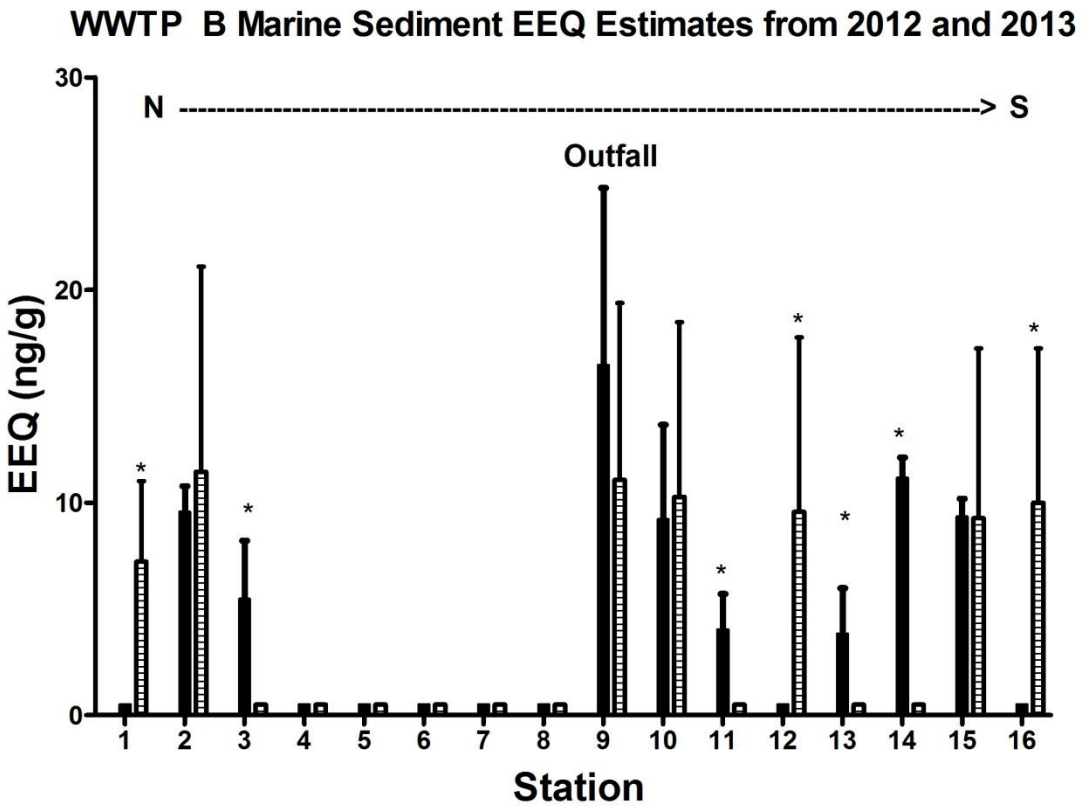


Figure 3.12. E2 Assay Results of WWTP B Marine Sediments.
Bars represent the mean ± s.e.m. of three assay replicates. * indicates a significant difference between 2012 and 2013 results ($p < 0.05$). Year 1 ■, Year 2 ▤

Table 3.5. Summary of E2 Assay Results for Marine Sediments

	WWTP B		WWTP A	
	Year 1	**Year 2**	**Year 1**	**Year 2**
% of Samples Positive	50%	44%	38%	38%
Highest EEQ (ng/g)	16	12	52	49
Lowest EEQ (ng/g)	3.8	7.2	1.6	12
Median EEQ (ng/g)	9.3	9.5	29	27
# of Sites Positive for both Years	4/16 (25%)		4/16 (25%)	

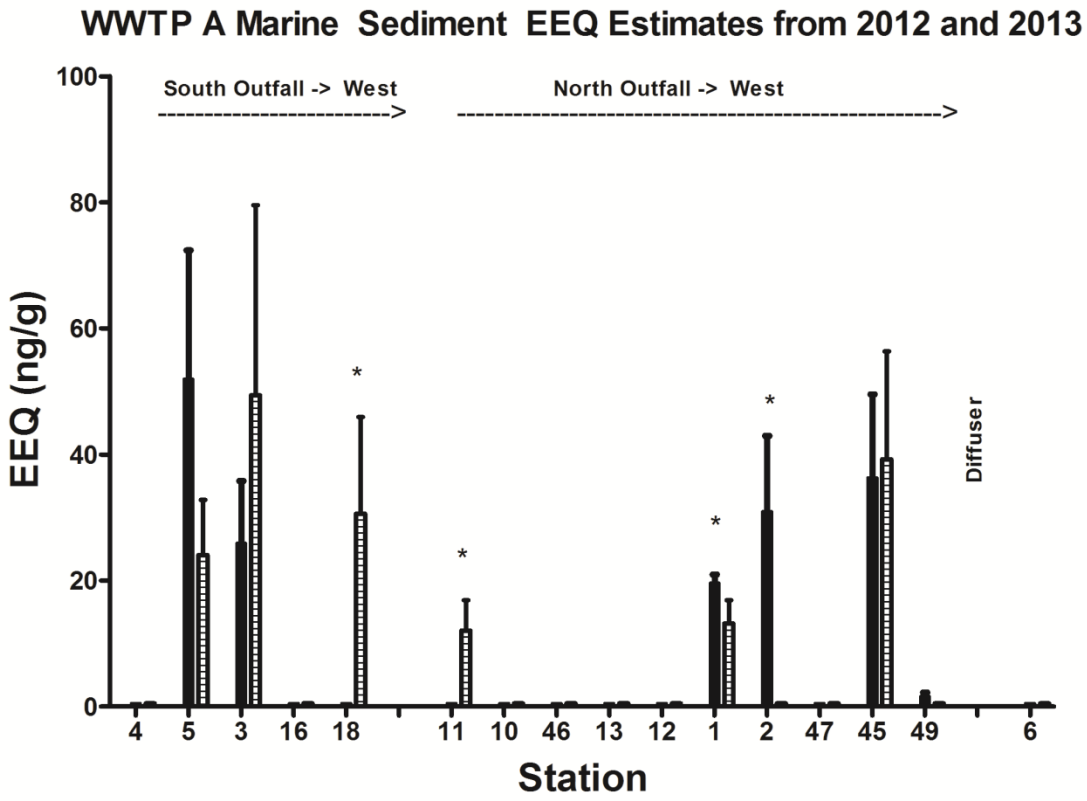


Figure 3.13. E2 Assay Results of WWTP A Marine Sediments.
Bars represent the mean ± s.e.m. of three assay replicates. * indicates a significant difference between 2012 and 2013 results ($p < 0.05$). Year 1 , Year 2

3.7.2. AhR Activity

Although the E2 assay results showed a similar number of stations that had detectable estrogenic activity at both study areas, the AhR assay results showed considerable differences between WWTP B and WWTP A. In 2012, only 2 of the 16 (13%) WWTP B stations showed detectable AhR activity with NAPEQs of 394±49 ng/g and 533±18 ng/g (Figure 3.14). This was comparable to the 11 out of the 16 (69%) WWTP A stations that showed NAPEQs ranging from 264±137 - 1575±229 ng/g with a median value of 506 ng/g (Figure 3.15).

In 2013, there was a similar trend: 7 out of 16 (44%) WWTP B stations had detectable AhR activity while WWTP A had 12 of 16 (75%) stations that showed AhR activity. At WWTP B the NAPEQs ranged from 104±28 - 519±82 ng/g with a median of 445 ng/g, while at WWTP A, NAPEQs ranged from 288±76 - 1396±653 ng/g and a

median of 558 ng/g. Overall, the range of NAPEQs for each study area remained similar from year 1 to year 2 but the number of stations with AhR activity increased in the second year. Of the 16 stations sampled at WWTP B, 6 stations had an increase in NAPEQs from <MLOD in 2012, while only 1 stations showed a decrease to <MLOD. There was also 1 station where AhR activity was detected in both sampling years with estimated NAPEQs that were not significantly different ($p < 0.05$).

Altogether, 9 stations at WWTP A had AhR activity detected in both sampling years. Of these 9 stations, 1 had a significant ($p < 0.05$) decrease, while the remaining 8 do not have NAPEQs that changed significantly ($p < 0.05$) from year to year. There were 2 stations where AhR activity was detected in 2012 and 2 other stations where AhR activity was detected in 2013. Overall, there is no significant correlation between the NAPEQ estimated and the distance of the station from the WWTP A outfall ($R^2 = 0.230$). Although the stations located on the north side of the outer Burrard Inlet tended to have higher AhR activity detection rates for both sampling years, but this was not related to the WWTP effluent as the NAPEQs estimated did not change significantly ($p < 0.05$) with increasing distance from the outfall. At WWTP B, there was no significant relationship between the NAPEQs estimated and the distance from the WWTP B outfall at station 9 ($R^2 = .07$). Only WWTP B station 16, which was considered a background reference station had detectable AhR activity in both sampling years. Although WWTP B stations 4, 5, 6 and 8 also had detectable AhR activity in 2013, they were all non-detects in 2012. It was therefore unclear if the NAPEQs estimated in these stations were influenced by the WWTP B outfall.

When the results of the E2 and AhR assays for the marine sediments (Figures 3.12 and 3.13) were compared, there was no significant correlation between the detected EEQs and NAPEQs at either WWTP B ($R^2 = 0.019$) or WWTP A ($R^2 = 0.152$) samples. Of the 16 WWTP B stations, only 4 stations had detectable estrogenic and AhR activity in the same sampling year. At WWTP A, 6 of 16 stations had detectable estrogenic and AhR activity in the same sampling year.

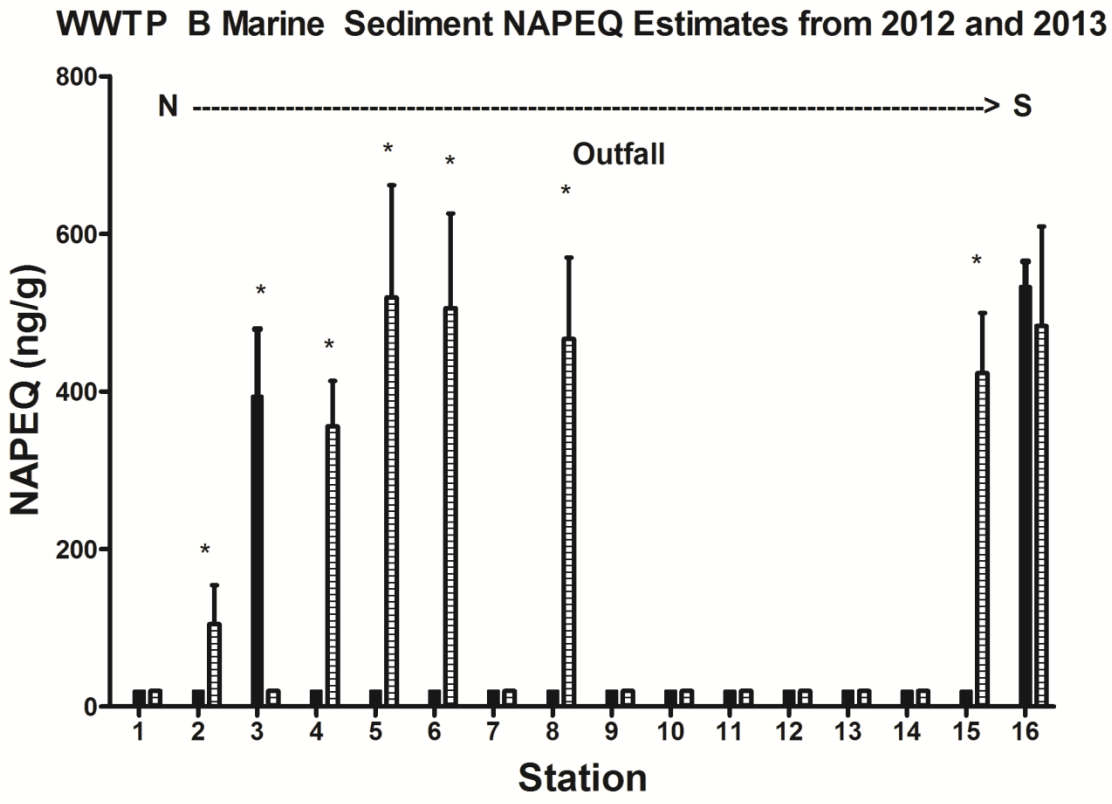


Figure 3.14. AhR Assay Results for WWTP B Marine Sediments.
Bars represent the mean ± s.e.m. of three assay replicates. * indicates a significant difference between 2012 and 2013 results ($p < 0.05$). Year 1 ■ , Year 2 ▤

Table 3.6. Summary of AhR Assay Results for Marine Sediments

	WWTP B		WWTP A	
	Year 1	**Year 2**	**Year 1**	**Year 2**
% of Samples Positive	13%	44%	69%	75%
Highest NAPEQ (ng/g)	533	606	1575	1396
Lowest NAPEQ (ng/g)	394	355	264	288
Median NAPEQ (ng/g)	464	466	506	558
# of Sites Positive for both Years	1/16 (6%)		9/16 (56%)	

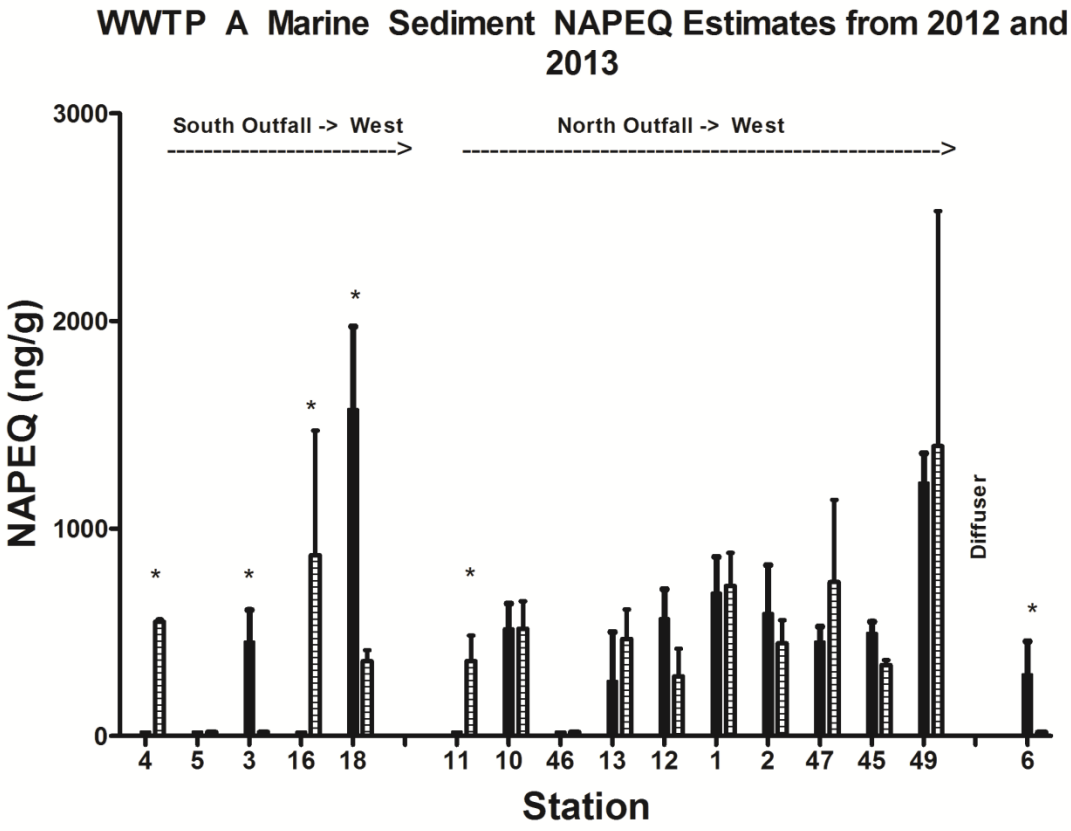


Figure 3.15. AhR Assay Results for WWTP A Marine Sediments.
Bars represent the mean ± s.e.m. of three assay replicates. * indicates a significant difference between 2012 and 2013 results ($p < 0.05$). Year 1 ■, Year 2 ▤

3.8. GC-MS Analysis

Selected samples from 2013 were analyzed for estrogenic and AhR agonists with GC-MS based on the detection of such chemicals in the yeast bioassay. Several samples were combined to form a composite sample to potentially increase the probability of identifying EDCs in the extract. The samples tested were wastewater effluents from WWTP C (composite of 4 effluents collected), wastewater effluents from WWTP A (composite of 4 effluents collected), marine sediments from WWTP B (composite of stations 9, 10 16) and marine sediments from WWTP A (composite of stations 5, 45, 49). GC-MS analysis of composite final effluents from WWTP C and WWTP A effluent samples and marine sediments from WWTP B and WWTP A regions identified BPA in all four composite samples.

Dehydroabietic acid was identified in both the marine sediment samples from WWTP B and WWTP A but not detected in the WWTP C and WWTP A composite final effluents.

Chapter 4. Discussion

4.1. E2 and AhR Assay Verification and Effects of Storage

The distilled water samples spiked with a chemical standard showed that the extraction method had a 65% recovery (Figure 3.1 and 3.2). However, the samples showed a large variation in the estimated EEQs, especially when the spiked concentration was very low. As such the 700% E2 recovery in the 1 ng/mL sample (Figure 3.1) most likely is due to variation in the assay replicates with a 52% mean %CV. Therefore, sufficient replication is needed by the assay to minimize the effect of variation and allow for the removal of assay outliers. In comparison, the AhR assay has been shown to be both precise and accurate with an average recovery rate of 95% (Figure 3.2) across the three spiked concentrations with %CVs ranging from 10-20%.

Previous work in our laboratory shows that sample extracts can be stored at 4^{o}C without significant ($p < 0.05$) changes in the estimated EEQs for a period of approximately 2 weeks, while wastewater samples under the same storage conditions maintain similar EEQs for up to 2 months (Shieh, 2011). As the samples in this study have been extracted and assayed well within this timeframe, the effects of storage on the E2 assay results are expected to be small.

Based on the results of the stored marine sediments (Figs. 3.3-3.6), storage effect on the assay results are minimal as estimated EEQs and NAPEQs do not change significantly ($p < 0.05$) for at least 1 month with the exception of the estrogenic activity of WWTP B marine sediments. Since all assays have been run within one week after receiving the samples, the overall effect of storage on the assay results is expected to be small. An increase in estrogenic activity in WWTP B samples after storage may be due to the conversion of less active estrogenic chemicals to more active estrogenic compounds such as the de-conjugation of glucoronic acid and/or sulfate conjugates of estrogens by bacteria and other micro-organism which (Lee et a. 2004). There was also

an apparent change in the top layer of the sediment, which suggests that oxidation of compounds in the sediment may also contribute to the increased estrogenicity.

For the AhR assay (Figures 3.5 and 3.6), there is no significant ($p < 0.05$) effect of storage on the NAPEQs estimated in marine sediments even after 2 months of storage which is not surprising as many AhR agonists are persistent in the environment due to their structural stability and resistance to degradation.

4.2. EDCs in Wastewater Samples

4.2.1. Estrogenic Chemicals

WWTP treatment may increase or decrease the estrogenicity of the final effluent discharged into the environment. In this study, three WWTPs (WWTP A, WWTP B and WWTP C) show an apparent decrease in the estrogenicity of the final effluent in comparison to the raw influent, while WWTP D is the only WWTP with a statistically significant increase in estrogenicity in the final effluent (Figure 3.7). The 85% decrease in estrogenicity seen at WWTP C may be due to contributions from residential areas making up most of the load. As such, the majority of the estrogenic compounds in the wastewater would be natural hormones such as E2 of which up to 98% can be removed by conventional wastewater treatment methods (Servos et al. 2005). At WWTP D, the EEQs in the final effluent were the highest of the five WWTPs tested despite estimated EEQs <MLOD in the raw influents of both sampling years (Tables 3.1 and 3.2). Several factors may contribute to the non-detects of EEQs in the raw influents. One factor is the reduced bioavailability of estrogenic compounds to the yeast cells due to the conjugation of estrogenic compounds or sorption to organic particles (Atkinson et al. 2012). As the yeast assay is based on the binding of free estrogens onto the receptor to induce a response, any change in the chemical form or bioavailability of the estrogens may prevent the yeast cells from detecting the estrogenic substance. However, subsequent treatment of the wastewater may lead to de-conjugation or release of the estrogenic compounds from organic particles through reactions catalyzed by bacteria in the wastewater (D'Ascenzo et al. 2003). This will result in an increase in the estrogenicity detected in the final effluents. A second factor may be the presence of anti-estrogenic

compounds which may bind to the estrogen receptor but do not induce a response, therefore preventing the estrogenic chemicals from exerting their effect. Anti-estrogenic compounds have been reported in wastewater samples and may include pharmaceutical compounds such as the anti-breast cancer drug tamoxifen (Fang et al. 2012). If wastewater treatment is able to remove the anti-estrogenic compounds from the raw influents, then the yeast cells would be able to detect estrogenic compounds in the effluent samples.

At WWTP B, only 1 raw influent sample shows estrogenic activity while all other raw influents and final effluents show <MLOD activity (Tables 3.1 and 3.2). This suggests that there is some daily variation in the EEQ contents of the influents. Therefore, a carefully designed sampling program, such as the one used in this study (sampling spans multiple days, 24 h composite samples, duplicate samples etc.), should be included to account for these variations. The results at WWTP B also show that population size may not be the best indicator of the estrogenicity of a given WWTP. WWTP C which serves approximately 1 million residents shows the highest EEQs in raw influents among all samples with detectable estrogenic activity. WWTP B, which serves over 600000 people, has only 1 sample with detectable estrogenic activity over the two sampling years. In addition, WWTP A and WWTP D which are similar to each other in the population size served and volume of wastewater processed, have very different estrogenic profiles. Whereas WWTP A has raw influents that show detectable estrogenic activity and final effluents with no detectable estrogenic activity, the opposite is observed at WWTP D. The variation seen in the EEQs of the raw influent across all 5 WWTPs therefore suggests that the composition of the wastewater received by each WWTP is very different (Figure 3.7). This means the type of estrogenic compounds in the raw influent is more likely to have the greatest effect on the estrogenicity of the final effluent because different estrogenic compounds are reduced to different extents (Ye et al. 2012). The use of the yeast bioassay alone to assess and compare the effectiveness of different wastewater treatment methods does not seem appropriate if the composition of estrogenic chemicals in raw influents differ among the WWTPs. In other words, two WWTPs employing the same treatment methods would show different effectiveness in the reduction of estrogenic activity in the wastewater if one WWTP has raw influent containing mainly natural estrogens which are quite readily removed (Servos et al. 2005)

while the other WWTP has raw influent containing compounds such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) which are not readily removed by conventional wastewater treatment methods (Rattanaoudom et al. 2012).

At WWTP E, the toxicity affects the results of both sampling years (Tables 3.1 and 3.2). As a result, I am only able to obtain results for the final effluent of the second sampling year. Although WWTP E services only 27000 people, it has an EEQ in the effluent similar to those of WWTP C and WWTP D. This may be due to contributions of estrogenic chemicals from industrial and/or agricultural sources. Again, this suggests the population size alone is not a good indicator of estrogenicity. The source and type of estrogenic compounds may be more important factors to consider.

Overall, the range of assay EEQs in the final effluents of the five WWTPs servicing the Greater Vancouver Area is within the range of EEQs reported in other studies (Table 4.1) and appears to be at the lower end of the range. It may simply be that estrogenicity is relatively low in the effluents of Vancouver WWTPs, but difference in sampling time and methodology among the many studies may have an effect on the estimated estrogenicity. Twenty-four hour composites, 8 hour composites and grab samples collected at different times of the day are likely to have different estrogenic compositions because of population movement during the day and industrial/other contributions to the raw influents of the WWTPs (Johnson et al. 2000). Although the EEQs estimated in the raw influents are very different among the 5 WWTPs, the average EEQs in the final effluents of the WWTPs where estrogenic activity is detected are surprisingly similar. The detected effluent EEQ concentration range (0.32±0.002 - 4.41±0.8 ng/L) is within the reported range of EEQs which have resulted in adverse effects such as feminization of fish (Kidd et al. 2007), and reduction of gonadal growth and development of secondary sexual characteristics (Pawlowski et al. 2004). However, the adverse effects listed here are likely to occur only if the sample consists mainly of EE2. Furthermore, the actual concentrations in the surface water are likely to be much lower due to the dilution of the effluent in the receiving environment.

Previous YES studies involving the same 5 WWTPs from our laboratory have found that EEQs in the final effluent may range from 30-1400 ng/L (Nelson et al. 2007). These EEQs differ to the current results by several orders of magnitude. A possible

explanation for the difference in the results may be that the composition of the wastewater differs due to dilution effect of precipitation, changes in the sources or treatment methods of wastewater and/or variation in the sensitivity of the assays used in the studies. In addition, a change in the extraction and assay method may also contribute to the difference in the detected EEQs of these studies. Most notably, Nelson et al. (2007) extracted total sample volumes of 1 L using liquid-liquid extraction compared to just 50 mL using solid-phase extraction in the current study. The EEQs estimated by Nelson et al. (2007) include results from both the YES assay and the E-SCREEN assay which would lead to higher estimated EEQs because the E-SCREEN assay which uses a human breast cancer cell line, generally produces EEQs that vary from the EEQ of the YES assay by up to 4 fold higher. Although the present assay EEQs are not consistent with results of the previous study, they are very similar to the range of chemical concentrations determined by chemical analyses (0.5-4.0 ng/L) (Nelson et al. 2007). A second study from our laboratory looking at wastewater effluents of 13 WWTPs across Canada reported a range of EEQs of 1.55-54.1 ng/L and a mean EEQ of 16.8 ng/L (Shieh, 2011), which is similar to the range of EEQs reported here.

Although the toxicity seen in samples from WWTP A and WWTP E affected the results, the samples affected are mostly raw influent samples. This suggests that wastewater treatment has lead to a reduction in compound(s) that contribute to the observed toxicity. As a result, there is likely to be little or no effect of toxicity on the EEQs detected in the final effluents.

Table 4.1. EEQs in WWTP Final Effluents from Other Studies

Country	Range of EEQs (ng/L) in Final Effluent	Reference
Canada	0.32-4.41	Current Study
Canada	1.55-54.1	Shieh, 2011
Canada	0.2-14.7	Servos et al. 2005
China	1.28-3.26	Fang et al. 2012
Portugal	0.95-24.2	Sousa et al. 2010
United Kingdom	0.28-13.8	Baynes et al. 2012

4.2.2. AhR Agonists

The AhR assay of wastewater samples indicates that AhR activity was reduced in the five WWTPs (Figure 3.8). Apparent reduction averages about 75% at WWTP C and 74% at WWTP D which is comparable to the 73-96% reduction seen in WWTPs in France (Dagnino et al 2010). The NAPEQs in the final effluents are within the range of those reported in other studies (Table 4.2). Although the AhR activity is reduced significantly ($p < 0.05$), the tendency for AhR agonists to bind onto organic compounds may affect estimation of the actual NAPEQ concentrations in the final effluents. In addition to the dissolved phase, WWTP final effluents also contain suspended solids which have not been removed from wastewater during the treatment process. Therefore, AhR agonists may be bound to the suspended solid particles and discharged into the environment along with the final effluent. Ultimately, this leads to an underestimation of the actual concentrations discharged into the environment (Allinson et al. 2011).

At WWTP E, toxicity affects the results of the AhR assay much like the E2 assay, but at WWTP A there is less toxicity seen as only 1 raw influent sample shows signs of toxicity (Tables 3.3 and 3.4). As the same sample extract is used in the AhR and E2 assays, it is likely that the yeast used in the AhR assay is much more resistant to toxicity compared to the yeast used in the E2 assay. This may be a product of the difference

between the ages of the yeast culture plates used, or differences in sensitivity to certain chemicals between the two yeast strains.

The fact that some unknown compounds in a WWTP sample have the potential of inducing toxicity to yeast cells means that they may cause adverse effects to wildlife if discharged into the environment. However, the absence of toxicity seen in the 2013 WWTP E effluent samples suggests that wastewater treatment may have removed most of the toxic compounds or that there are daily/seasonal variations in the input of the toxic compound into the wastewater influent. Further research is needed to identify the chemicals that caused toxicity in the bioassays.

There are very few studies on the removal efficiency of AhR activity in WWTP samples. The NAPEQs estimated in the wastewater samples are of less importance compare to the types of AhR agonists present. In particular, dioxin and dioxin like chemicals such as PCBs and PCDDs are known to be potent toxins that can induce a range of adverse effects from reproductive to developmental disorders (Janosek et al. 2006). Regardless, the range of NAPEQs seen in the final effluents of this study is comparable to those found in Germany and Australia (Table 4.2). Whether these concentrations will lead to adverse effects in wildlife will depend on factors such as the type of AhR agonist in the effluent and whether these compounds are persistent or bioaccumulative.

Table 4.2. NAPEQs in WWTP Final Effluents from Other Studies

Country	Range of NAPEQs (ng/L) in Final Effluent	Reference
Canada	45-302	Current Study
Australia	16-279	Allinson et al. 2011
Germany	387-741	Stalter et al. 2011

4.3. EDCs in Biosolids

Bacteria and other micro-organisms are used in WWTPs to remove nutrients from wastewater and to degrade organic compounds which may include some EDCs (Johnson et al. 2007). Degradation of EDCs by micro-organisms likely contributes to the apparent reduction of estrogenic and AhR activity in wastewater, but it is unlikely to account for all of the 70-90% reduction seen in the current and other studies (Stalter et al. 2011 and Dagnino et al. 2010). Primary treatment of wastewater involves the removal of solids and other organic particles which are later processed into biosolids. As many EDCs are likely to be lipophilic and adsorbed onto the particulates and/or suspended particles during wastewater treatment, the removal of these will also take EDCs out of the wastewater. This means that the apparent reduction of AhR and estrogenic activity seen in the wastewater is not all due to complete removal, but may be in the biosolids produced at the WWTPs (Lorenzen et al. 2004). Continued treatment of biosolids at the WWTP can lead to a further reduction in the concentrations of EDCs through processes such as microbial degradation, but little work with the yeast bioassay has been conducted in this area due to the potential for toxicity to the yeast cells (Citulski and Farahbakhsh. 2010).

The biosolid samples from the 3 WWTPs all show detectable concentrations of estrogenic chemicals and AhR agonists (Figures 3.9 and 3.10). At WWTP C, the apparent reduction of both estrogenic and AhR activity in the wastewater and the detection of both in biosolids support the idea that not all of the apparent reduction seen in the wastewater is due to complete removal and/or microbial degradation. At WWTP D, the raw influents do not have detectable estrogenic activity in the E2 assay (Figure 3.7), but the biosolids do contain detectable concentrations of estrogenic chemicals (Figure 3.9). A possible explanation for this is that the estrogenic chemicals are already bound to organic particles in the wastewater before entering the WWTP. The removal of these particles during primary treatment would collect the estrogenic chemicals into the biosolids (Dagnino et al. 2010), while reactions catalyzed by bacteria may cause the release of estrogenic chemicals from the bound particle or deconjugation of estrogenic chemicals leading the detections seen in the final effluents.

The biosolids at WWTP A show signs of toxicity much like the wastewater influents. The toxic compounds are likely removed from the wastewater and collected in the biosolids leading to the observed toxicity. In the 2013 biosolid samples, there is an increase in toxicity in the biosolid samples correlating to decrease in toxicity seen in the wastewater samples. There may have been an increase in the removal of the toxic compounds from the wastewater into the 2013 biosolid samples leading to an increase in toxicity in the biosolid samples and/or a lower toxic compound concentration in the wastewater. In either case, biosolids are a sink for EDCs and other compounds which need to be considered in any assessment as they are frequently applied as fertilizer in agricultural operations (Deeks et al. 2013).

Overall, the biosolid samples at WWTP A contain the highest concentrations of both AhR and estrogenic activity among the 3 WWTPs (Figures 3.9 and 3.10). The EEQs (R^2 = 0.44) and NAPEQs (R^2 = 0.04) estimated do not have a significant correlation with the population size served (Table 2.1). Rather the main factors most likely to affect the concentrations of EDCs in the biosolids are retention time, treatment temperature, oxygen availability and other factors affecting micro-organism growth and metabolism (Wilson et al. 2011).

4.4. EDCs in IDZ Samples

At the IDZ boundary of WWTP A, neither AhR nor estrogenic activity are detected in the water samples (data not shown), suggesting that the EDCs detected in the final effluent are diluted to <MLOD in the receiving environment. Although the yeast bioassay does not detect any estrogenic or AhR activity in the IDZ water samples, a chemical analysis study conducted by Metro Vancouver shows low concentrations of estrogenic compounds and PAHs (Enkon, 2013a). However, common estrogenic compounds such as NP, E2 and EE2 are either not detected, or if detected, do not meet criteria for quantification (NDR). Of the 19 PAHs included in the 2012 WWTP A IDZ study (Enkon, 2013a), all are below available CCME water quality guidelines. The concentrations of individual PAHs ranged from < 0.01- < 0.05 µg/L. These results are consistent with the non-detects seen in the present E2 and AhR assays. The corresponding E2 and AhR effluent activity is <MLOD (except in 2012 for AHR), and

would be further reduced using an average dilution factor of 250:1 at WWTP A IDZ (Enkon, 2013a).

At the WWTP C IDZ, 2 of the 5 stations have detectable estrogenic activity (Figure 3.11) while no stations have detectable concentrations of AhR agonists (data not shown). The assumption that concentrations of EDCs in wastewater effluents would be diluted to concentrations below detection limits is not always appropriate. Areas where water flow may be reduced or where the water is relatively enclosed can lead to the accumulation of EDCs in the water and a reduction in the dilution effect (Allinson et al. 2011). The average predicted dilution at the WWTP C IDZ boundary is 40:1 under low river flows and 60:1 under high river flows, compared to the ~250:1 predicted average dilution at the WWTP A IDZ boundary. The estimated dilution at the WWTP C IDZ boundary during the sampling events on March 6 and March 14 ranged from 25:1 to 110:1 and 13:1 to 59:1, respectively (Enkon 2013b). However, based on E2 and AhR activities in these effluent grab samples and the 24-h composite samples (Figures 3.7, 3.8 and 3.11), the concentration at the WWTP C IDZ boundary would be predicted to be <MLOD for these dilutions and the worst case minimum dilution of 7:1

Other studies also have reported estrogenic activity in the receiving waters of WWTPs. Tang et al. (2012) have shown EEQs ranging from 1.05-17.60 ng/L in China, Ferguson et al. (2013) have reported a range of EEQs from 2.95-18.9 ng/L in Australia, while Jones-Lepp et al. (2012) have reported a range of EEQs from 0.04-2.4 ng/L in the United States. Our estimates of EEQs in the WWTP C IDZ samples ranged from 1.83±0.3 - 3.38±1.2 ng/L for the 2 of 5 samples with detectable estrogenic activity. Interestingly, these EEQs are higher than the average EEQ seen in the corresponding WWTP final effluent of 1.16±0.1 ng/L. This is suggestive of sources upstream such as agricultural operations that may have contributed to the estrogenic activity observed in the samples from the IDZ. The 2013 WWTP C IDZ study identifies several estrogenic compounds within the IDZ water sample, including NP (100 - 110 ng/L), E2 (1.88 - 2.59 ng/L), EE2 (< 0.405 – NDR < 0.839 ng/L) and E1 (8.93 - 11.9 ng/L). Although the concentrations of these estrogenic compounds in the WWTP C IDZ are comparatively higher than those at the WWTP A IDZ, they do not exceed the CCME water quality guidelines as the freshwater guideline for nonylphenol is 1000 ng/L.

4.5. EDCs in Marine Sediments

4.5.1. Estrogenic Chemicals

The mouth of the Fraser River is an area where large volumes of sediments are deposited from upstream sources. It is therefore expected that EDCs which bind to sediment particles will be deposited here as well. At both the WWTP A and WWTP B, ~40% of the marine sediment samples collected had detectable concentrations of estrogenic activity (Figures 3.12 and 3.13), with EEQs ranging from 1.6±0.3 - 51.9±11 ng/g (Table 3.5). Our range of estimated EEQs are comparable to those from studies in China which has reported EEQs ranging from 5.44-36.72 ng/g (Chen et al. 2012) and 9.8-101 ng/g (Zhao et al. 2011) as well as a study in Germany which has reported values of 15-23 ng/g (Schmitt et al. 2012) in marine sediment. But our results differ by up to several orders of magnitude when compared to studies that examined riverine sediments which reported values of 0.001-1.2 ng/g in the Czech Republic (Hilscherova et al. 2002) and 0.022-0.029 ng/g in the United Kingdom (Peck et al. 2004). The difference in EEQs between marine and riverine sediments may be attributed to the flow of the river carrying suspended organic particles and sediments downstream where it is deposited in the estuary. As EDCs are likely bound onto these particles, they are carried downstream and deposited in the estuary as well (Ferguson et al. 2013).

A comparison of the EEQs in wastewater effluent demonstrates that both WWTP A and WWTP B have no detectable estrogenic activity in the final effluents for both sampling years (Figure 3.3). This suggests that the estrogenic activity seen in the marine sediments may not have come from the effluents of the WWTPs. However, Peck et al. (2004) have reported that even in areas where surface water and final effluents show low to non-detectable concentrations of estrogenic activity, the sediments in the receiving environment may still show detectable estrogenic activity. It is likely that the overall estrogenic activity detected at both WWTP A and WWTP B are due in part to the effluents discharged from WWTPs in addition to other sources from upstream such as agricultural and industrial operations.

Results of the marine sediments show that E2 activity is detected more often in WWTP B samples, than WWTP A samples, while the activity tends to be lower at

WWTP B (Figures 3.12 and 3.13). The difference in results between these two sites is explainable by the EDC sources upstream and the geographic location. Both the Burrard Inlet and the Fraser River receive discharges from different industries. In addition, the Fraser River may also receive estrogenic chemicals from cattle farms and agricultural operations. These and other factors such as the persistence of the compounds, tendency of the compounds to bind onto organic particles and/or the concentrations of discharged estrogenic compounds may have affected the concentrations of estrogenic activity detected in the marine sediments downstream. The different geographic location of the two sites may have also affected the estrogenic activity seen in the marine sediments. WWTP B discharges into the Strait of Georgia leading to a larger dispersal of the effluent. In addition, the Fraser River flows into three main arms before meeting the Strait of Georgia. This may lead to the dispersal of upstream estrogenic compounds from the Fraser River over a much wider area in the WWTP B region, resulting in a larger number of detections but lower EEQs in the marine sediments. In contrast, at WWTP A, the final effluent is discharged into the Burrard Inlet which is a partially enclosed waterway, and also has other potential sources of EDCs. Together with the inflow of water from the Strait of Georgia and the binding of EDCs to sediment particles, EDCs may have accumulated within a smaller area around the entry of the Burrard Inlet. This may explain why relatively higher concentrations of estrogenic activity are observed in the few detections in marine sediment samples of WWTP A compared to WWTP B.

Overall, results of the E2 assay on marine sediments for both WWTP B and WWTP A (Figures 3.12 and 3.13) confirm other studies that marine sediments around WWTPs may contain EDC contaminants (Peck et al. 2004 and Ferguson et al. 2013). As EDCs accumulate in the marine sediments, they pose not only risks to benthic organisms that live in contact with the marine sediments, but also result in a potential source of EDCs as the EDCs may be re-introduced back into the water column if they are re-suspended by tidal movements or shipping activity (Gomes et al. 2011 and Ferguson et al. 2013). It is therefore important to monitor estrogenic activity in marine sediments and not focus solely on industrial/WWTP/agricultural effluents and/or surface waters.

A comparison of the EEQs measured by the yeast bioassay and the chemical EEQs calculated using the relative potencies (Table 4.3) for E1, E2, E3, EE2, NP,

equilin, equilenin, mestranol, 17α-estradiol and 17α-dihydroequilin which are detected in the 2012 sediments effects survey (McPherson et al. 2013a,b) shows that chemical EEQs are lower compare to the YES bioassay EEQs. Whereas the bioassay EEQs may range from 1.6±0.3 - 51.9±11 ng/g (Table 3.5) and only show detectable estrogenic activity in ~40% of the marine sediments using the yeast assay (Figures 3.12 and 3.13), chemical EEQs may range from 0.574-3.964 ng/g and estrogenic chemicals are detected in all of the marine sediments from WWTP A and WWTP B. In addition, WWTP A marine sediments have lower chemical EEQs which range from 0.574-2.844 ng/g with a median of 0.947 ng/g when compare to WWTP B where chemical EEQs range from 2.412-3.964 ng/g with a median of 3.471 ng/g. This result differs to the yeast assay, where the bioassay EEQs at WWTP A are generally high than those at WWTP B. The difference between the bioassay EEQs and chemical EEQs may be due to other estrogenic compounds within the sediments that are not measured by the chemical analysis. As the bioassay EEQ is a collective response of the yeast to all of the estrogens in the sample including possible interactions, it is expected to be higher when compared to chemical EEQs which are calculated with only the known detected estrogenic chemicals and does not take into account possible chemical interactions. Although the chemical EEQs do not completely agree with the bioassay EEQs, the narrow range of chemical EEQs at both WWTP B and WWTP A also show no significant relationship between the chemical EEQs measured and the distance of the station from the outfall (WWTP B R^2 = 0.163, WWTP A R^2 = 0.057) which is consistent with the bioassay results.

Table 4.3. Relative Potency of Estrogenic Chemicals

Chemical	Relative Potency	References
17β-estradiol	1	Blair et al. 2000, Kuhl 1998.
Estrone	0.073	
Estriol	0.097	
17α-estradiol	0.030	
Equilin	0.400	
17α-dihydroequilin	0.180	
Equilenin	0.070	
Mestranol	0.022	
17α-ethynylestradiol	2	
Nonylphenol	0.0003	

4.5.2. AhR Agonists

The AhR assay results of the marine sediments differ to the E2 assay results; the number of samples where AhR activity is detected, is higher in WWTP A samples (~73%) than WWTP B samples (~25%) (Figures 3.14 and 3.15). The WWTP A samples also have higher average NAPEQ which are not surprising considering the final effluents of WWTP A have the highest AhR activity among the 5 WWTPs when detected (*i.e*., only 3 samples are > MLOD) (Table 3.4). Although AhR agonists in the WWTP final effluent may have contributed to the AhR activity seen in the marine sediment samples, there are other possible sources. For example, the Burrard Inlet is home to one of the busiest ports in North America with a large volume of ship traffic. Combustion emissions as well as transport of coals and petroleum products may have introduced PAHs into the water that eventually settled in the marine sediments (Soclo et al. 2000). It is likely that a

large portion of the AhR activity seen in the WWTP A marine sediment is related to the ship traffic in Burrard Inlet.

In addition to the higher AhR activity in the samples at WWTP A, a large volume of ship traffic may also increase the disturbance to the marine sediments. Re-suspension of the sediment and changes in water flow may cause movements of contaminant bound sediments leading to some of the year to year variation in results seen at WWTP A. At WWTP B, there is less ship traffic so it is unlikely that the year to year variation seen here is due to anthropogenic disturbances. The open waters at WWTP B are more likely susceptible to tidal movements which may have caused the re-suspension and movement of sediments from one site to another (Gomes et al. 2011).

Overall, the range of NAPEQs (264±137 - 1575±229 ng/g) estimated by the yeast bioassay (Table 3.6) are within the range of total PAHs concentrations calculated from the chemical analytical results reported in the 2012 Metro Vancouver WWTP outfall sediments effects survey (McPherson et al. 2013 a,b). At WWTP A, total PAHs range from 341 to 1772 ng/g with a median of 988 ng/g while at WWTP B, total PAHs reported ranged from 144 to 781 ng/g with a median of 202 ng/g (McPherson et al. 2013a,b). A screening level risk assessment based on the yeast assay NAPEQs is conducted after converting them to benzo[a]pyrene equivalents (BAPEQs) and comparing the results with the Canadian Sediment Quality Guidelines (CSQGs) for benzo[a]pyrene. Based on the EC50s, EC30s and EC20s of the standard curves for benzo[a]pyrene and β-naphthoflavone in the AhR assay, it is determined that benzo[a]pyrene is approximately 3.5 times more potent than β-naphthoflavone, yielding a relative potency factor of 0.285 for NAP. Of the combined fourteen samples where AhR activity was detected (2 WWTP B, 12 WWTP A) in 2012, BAPEQs range from 85-479 ng/g with a median of 144 ng/g. Moreover, 13 of the 14 estimated BAPEQs are above the interim CSQG value for benzo[a]pyrene in marine sediment (88.8 ng/g), although all of them were well below the probable effects level (concentration above which adverse effects are expected to occur frequently) of 763 ng/g (CCME, 1999). In 2013, 21 marine sediment samples showed detectable AhR activity in the yeast bioassay with BAPEQs ranging from 82-398 ng/g with a median of 133 ng/g. Of the 21 positives results, 18 have an estimated BAPEQs above the interim CSQG value, although all of them are well below the probably effects level. Based on the results of the screening level risk assessment study, it is concluded

that there may be potential risks for adverse effects for aquatic organisms exposed to WWTP A marine sediments. To compare the assay BAPEQs to chemical BAPEQs, measured concentrations of individual PAHs from the 2012 Metro Vancouver WWTP Outfall sediments effects survey (McPherson et al. 2013 a,b) are converted to BAPEQs using PAH toxic equivalency factors (Table 4.4). The chemical BAPEQs of the 16 WWTP B stations range from 17-109 ng/g with a median of 28 ng/g, and only one station is above the CSQG. The 16 WWTP A stations however, have chemical BAPEQs ranging from 27-265 ng/g with a median of 155 ng/g, and 13 stations are above the CSQG. Although the chemical BAPEQs are lower than the bioassay BAPEQs, the general trend for both datasets show benzo(a)pyrene equivalent concentrations above the CSQG value in WWTP A marine sediments. The conclusion from both datasets is that there is a potential for adverse effects occurring in aquatic organisms exposed to AhR agonists such as PAHs in WWTP A marine sediments, while there is minimal risk for adverse effects occurring in aquatic organisms exposed in WWTP B marine sediments.

Table 4.4. PAH Toxic Equivalency Factors (TEF)

PAH	TEF	References
Naphthalene	0	ATSDR, 2009
Acenaphthylene	0.001	
Acenaphthene	0.001	
Fluorene	0.001	
Phenanthrene	0.001	
Fluoranthene	0.001	
Anthracene	0.01	
Pyrene	0.001	
Benz[a]anthracene	0.1	
Chrysene	0.001	
Benzo[b/j/k]fluoranthene	0.01	
Benzo[e]pyrene	1.001	
Benzo[a]pyrene	1	
Perylene	0	
Dibenz[a,h]anthracene	1	
Indeno[1,2,3-cd]pyrene	0.1	
Benzo[ghi]perylene	0.01	
1-Methylnaphthalene	0	
2-Methylnaphthalene	0	
2,6-Dimethylphenanthrene	0.001	

Very few studies have examined the AhR activity in marine sediments using yeast based assays, making it difficult to compare our results with other studies. However, several studies have looked at the total PAH content in marine sediments. Tolosa et al. (1996) have reported total PAH ranges of 420-760 ng/g and 1200-2400 ng/g in two French coastal regions, Grimalt et al. (1984) have shown total PAH concentrations of 1300-2300 ng/g in Spain. Wakeham et al. (1996) report an average of total PAH concentration of 1500 ng/g in the marine sediments of Romania and Bates et al. (1984) show there is an average of 1100 ng/g total PAHs in marine sediments of the USA. Total sediment PAH concentrations are typically high in urban coastal regions and low in more remote waters (Fernandez et al. 1999). Nevertheless, the range of NAPEQs in the present study is comparable to the total PAHs seen in the other studies.

In many marine and river sediments PAH studies, samples are collected from areas known to be contaminated by high industrial actvity. Total PAH ranges of 84.4-14938 ng/g have been reported in China (Guo et al. 2007), 1132-39951 ng/g in the Czech Republic (Hilscherova et al. 2002), 13000-18000 ng/g in Poland (Fernandez et al. 1999) and 206-9570 ng/g in Finland (Leskinen et al. 2008). On average, total PAHs values are higher in areas where industrial operations discharge into enclosed water bodies such as lakes (Fernandez et al. 1999). Therefore the range of AhR activity reported in the present study is at the low range of contamination compared to other studies, but the types of PAHs and other AhR agonists contained in sediments still needs to be considered when assessing the potential risk and effects of exposure of the sediment to living organisms as different AhR agonists exert different effects (Giesy et al. 2002).

4.6. Non-Detection of Glucocorticoids and Androgens

The non-detection of androgens and glucocorticoids in the water and sediment samples (data not shown) is interesting as numerous studies have reported the presence of both androgens and glucocorticoids in WWTP samples (Leusch et al. 2006, Van der Linden et al. 2008 and Chang et al. 2011). It is estimated that androgens also

make up the majority of steroidal hormones found in WWTP effluents (Chang et al. 2011) However, there have also been reports of the absence of androgenic activity in WWTP samples and the presence of anti-androgenic compounds in both WWTPs and marine sediments (Mnif et al. 2012, Fang et al. 2012 and Zhao et al. 2011). There are a number of compounds that are potentially anti-androgenic. In addition to anti-androgenic drugs like flutamide, some PAHs are also known to be anti-androgenic. Sediments containing PAHs have been reported to mask the androgenic effect in the yeast androgen assay. It has been shown that when sample extracts were fractionated and the PAH portion was removed, the extract showed detection of androgenic activity. The presence of PAHs in the samples may have masked the androgenic activity of the samples (Weiss et al. 2009). Future studies using the yeast bioassay should consider fractionation of samples to assist in determining the presence of androgen activity.

The non-detects with regards to glucocorticoids may be due to low concentrations in wastewater and sediments. It is possible that further concentration of the sample may lead to detection of glucocorticoid activity (Mnif et al. 2012). This may be due to different physicochemical properties of the glucocorticoids . For example, compounds such as hydrocortisone are expected to bind onto organic particles based on log K_{oc} values, whereas other compounds such as dexamethasone are expected to stay in the aqueous phase (Pubchem, 2013). Separation of glucocorticoid mixtures into the aqueous and organic phases may lower the glucocorticoid activity leading to the observed non-detects. Future studies should focus on the development of an extraction method which is specifically designed for glucocorticoids.

4.7. GC-MS Analysis for EDCs

GC-MS analysis was unable to detect natural estrogenic compounds such as E2 in the wastewater effluents (WWTP A and WWTP C) and sediment (WWTP A and WWTP B) samples analyzed. However, a synthetic estrogenic chemical, BPA, is found in WWTP A and WWTP C effluents as well as marine sediments from the WWTP A and WWTP B region (Appendix H). BPA was not detected in a pilot study in 2011 that reported BPA between <489 to < 746 ng/L in the effluent and <471 to <474 ng/L at the IDZ of WWTP A. However, BPA concentrations in the reference sample, travel blank

and field blank also range from <478 to <500 ng/L (Enkon 2012). In addition, the marine sediments had concentrations of BPA ranging from <394 to <439 ng/g at WWTP A and <470 to <501 ng/g at WWTP B (Golder 2012 a,b). Therefore, BPA is not likely to contribute significantly to the overall detected EEQs even if the BPA concentration were equal to the detection limit assuming a relative potency of 0.00009 (Li et al. 2004).

Although public awareness of BPA as a potentially toxic substance to humans has increased in recent years, and production of products containing BPA has decreased, BPA is still used in the manufacturing of many plastic products. Other studies have also reported the presence of BPA in WWTP effluents and marine sediments due to the incomplete removal of BPA from wastewater treatment (Lee et al. 2013 and Ye et al. 2012). BPA removal rates in conventional WWTPs ranged from 65-79% compared to the 85-99% removal of natural estrogenic hormones such as estrone (E1), E2 and estriol (E3) (Ye et al. 2012). Moreover, a previous study from our laboratory using gas chromatograph-high resolution mass spectrometer (GC-HRMS) has reported the detection of a large number of estrogenic compounds including the natural estrogens E1 (1.3-27.2 ng/L), E2 (0.1-11.2 ng/L) and E3 (≤ 4.9-8.9 ng/L) as well as industrial estrogenic compounds like nonylphenol (207.5-1287.3 ng/L) and BPA (2.9-61.1 ng/L) (Nelson et al. 2007). The discrepancy in the results between our current and previous studies is likely due to the higher sensitivity of the GC-HRMS used in the earlier study and the generally low concentrations of different estrogenic compounds in the wastewater effluents. Although the current study is unable to detect estrogenic compounds other than BPA using GC-MSD, our previous study detected the presence of numerous estrogenic compounds using GC-HRMS in wastewater effluents.

The presence of BPA in the environment is of concern as numerous studies have reported adverse effects associated with BPA exposure. These include interference with sexual development and behaviour in rats (Kubo et al. 2003), reduction in the number of offspring in Daphnia (Jeong et al. 2013) and interference with reproduction and development in the fathead minnow (Staples et al. 2011) among others. Staples et al. (2011) have reported a chronic 444 day no observed effect concentration (NOEC) of 16 µg BPA/L on F2 hatching success which is equivalent to an EEQ of 1.44 ng/L relative potency (Li et al. 2004). This NOEC is below the EEQs measured in the IDZ samples of 1.83 and 3.38 ng/L, but given that the reported BPA concentrations in the effluents and

IDZ samples are in the ng/L range and well below the NOEC of 16 µg BPA/L, it is unlikely for BPA to pose a significant risk to aquatic organisms. However, it is possible that other estrogenic chemicals may accumulate in the marine sediment in concentrations that may induce adverse effects in benthic organisms.

All marine sediments and wastewater effluents used in the GC-MSD analysis had detections of AhR activity in the yeast AhR bioassay, but GC-MSD analysis failed to identify any PAHs or other potential AhR agonists. This may be due to the use of the full scan mode in our GC-MS analysis as opposed to the selective ion monitoring mode (SIM). As full scan sets the instrument to detect a wider range of unknowns, it is less sensitive compared to the SIM mode which programs the instrument to look for specific unknowns and therefore has a lower detection limit. However, previous studies conducted by Metro Vancouver from 2000-2009 at WWTP B and 2006-2009 at WWTP A have identified PAHs at all stations where marine sediments are collected, including anthracene, retene and napthalene. The total PAHs measured at WWTP B in 2009 range from 180-500 ng/g while at WWTP A in 2009, total PAHs range from 250-2600 ng/g. At WWTP B, there were 5 individual PAHs that were found to be above Canadian Council of Ministers of the Environment (CCME) sediment quality guidelines (SQGs) at one or more stations, while at WWTP A, all 17 PAHs for which CCME SQGs were available, were above the guideline at one or more stations. (McPherson et al. 2010 a,b) Even though we are unable to identify any PAHs using GC-MSD analysis, the fact that the range of NAPEQs we estimated from the AhR assay are comparable to the total PAHs in historical data at the same site suggests the total PAH concentrations in the sediments at both sites have remained relatively unchanged. In other words, the NAPEQs estimates in the present study indicate they may pose health risks to the aquatic organisms living in the WWTP B and WWTP A sampling area.

Although no specific PAHs have been detected in the present study, one chemical that has been identified in the marine sediments at both WWTP A and WWTP B is dehydroabietic acid (DHAA) (Appendix H), a resin acid that is naturally found in many coniferous trees (Martin et al. 1999). DHAA is commonly found in pulp and paper mill effluents and is readily reduced by microorganisms under anaerobic conditions into retene, a PAH (Ramanen et al. 2010, Leppanen and Oikari, 2001 and Martin et al. 1999). The presence of DHAA in the sediment suggests that the AhR activity seen in the

yeast AhR bioassay is due in part to the formation of retene which had also been identified in a previous study conducted by Metro Vancouver. In 2012, retene concentrations reported in marine sediment samples from WWTP A ranged from 17.9 to 105 ng/g, whereas concentrations at WWTP B ranged from 23.2 to 65.8 ng/g (Golder, 2013). The absence of DHAA from WWTP effluents indicates that sources other than WWTPs are contributing to the AhR activity found in the marine sediments. This may include pulp and paper mills, ship activity and other upstream industrial activities.

The endocrine disrupting properties of DHAA have been studied in organisms such as the Rainbow trout (*Oncorhynchus mykiss*). Exposure of rainbow trout to DHAA resulted in a reduction in vitellogenin levels indicating a potential anti-estrogenic effect of DHAA (Orrego et al. 2010). A yeast two-hybrid assay testing resin acids including DHAA confirms the anti-estrogenic potential of these chemicals. Although it was reported that the mechanism of anti-estrogenicity of DHAA is not receptor-mediated as DHAA does not show any affinity to the estrogen receptor, the YES bioassay may still be inhibited (Terasaki et al. 2009). It is a possibility that the presence of DHAA in the marine sediment had an effect on the EEQs estimated in the yeast estrogenic bioassay, leading to an underestimation of the total estrogenic activity.

The identification of DHAA in marine sediments suggests that pulp and paper mill effluents likely contributed to the load of EDCs measured through the yeast bioassay. This may explain some of the estrogenic activity seen in the results as natural phytoestrogens such as genistein have been detected in wood pulp at concentrations in the µg/kg range and both treated and untreated mill effluents in the µg/L range (Kiparissis et al. 2001). The presence of phytoestrogens in pulp mill effluents has been thought to be a contributing factor in the reduction of reproductive capacity in fish and other aquatic organisms. It has been shown that Japanese medaka exposed to genistein show increased instances of gonadal intersex as well as alterations to secondary characteristics (Kiparassis et al. 2003). In addition, genistein has also been reported to induce apoptosis in zebrafish embryos through an ER independent pathway, but can also activate 3 different zebrafish ERs leading to changes in the expression of aromatase, an enzyme important in estrogen biosynthesis (Sassi-Messai et al. 2009). It is therefore important to not only monitor concentrations of synthetic estrogens, but also

phytoestrogens as they are also capable of inducing adverse effects in aquatic organisms.

4.8. Application of the Yeast Bioassay

The results of this study show that the yeast bioassay can be successfully applied to different environmental samples for EDC detection. In addition, the assay is suitable to screen for a large number of samples due to its relatively short run time of 6 hours. Other yeast or mammalian cell based assays used in the detection of EDCs have run times generally ranging from 2-4 days (Bistan et al. 2011, Dagnino et al. 2010, Sonneveld et al. 2005). The high throughput of samples allow for quick identification of potentially contaminated sites where samples can be collected for further chemical analysis.

Although the yeast bioassay is applicable in the detection of EDCs as well as providing an estimation of potency, it should not be used as a standalone tool for assessing EDC concentrations in environmental samples. Due to the variability of the yeast assay, the estimated EDC concentrations should not be taken at face value even if sufficient replication reduces the variation. The estimations are meant to identify sites with the detectable concentrations of EDCs for further studies through other techniques such as chemical analysis. Another issue encountered in this study is the presence of compounds toxic to the yeast cells. Even though there are cleanup procedures such as the sulphur removal procedure applied to the biosolids in this study, it is difficult to know which cleanup procedure should be used when the structure of the toxic compounds in the sample are not known. A second complication with the use of cleanup procedures is the potential to remove target compounds from the sample extract. As no extraction process is 100% efficient, any additional procedures applied to the sample extract may cause loss of the target compounds, leading to an underestimation of the concentration in the sample. Nonetheless, the yeast bioassay remains effective as a screening tool for EDCs in both WWTP and environmental samples, though less effective when sample activities are generally low.

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Appendix A.

E2 and AhR Assay Results for Wastewater

2012 E2 Assay Results

WWTP	EEQ (ng/L) Influent	Standard Deviation	EEQ (ng/L) Effluent	Standard Deviation
WWTP C	1.99	1.95	< MLOD	-
	6.46		< MLOD	
	6.21		4.41	
	5.72		< MLOD	
	3.49		< MLOD	
WWTP D	< MLOD	-	0.32	0.57
	< MLOD		1.32	
	< MLOD		1.32	
WWTP A	T	-	< MLOD	-
	T		< MLOD	
	T		< MLOD	
	T		< MLOD	
	T		< MLOD	
WWTP B	< MLOD	-	< MLOD	-
	< MLOD		< MLOD	
	< MLOD		< MLOD	
WWTP E	T		T	
	T	-	T	-
	T		T	
	T		T	

2013 E2 Assay Results

WWTP	EEQ (ng/L) Influent	Standard Deviation	EEQ (ng/L) Effluent	Standard Deviation
WWTP C	9.37	7.42	1.08	0.30
	2.28		0.69	
	19.96		1.17	
	< MLOD		1.36	
	7.48		1.50	
WWTP D	< MLOD	-	1.52	0.62
	< MLOD		< MLOD	
	< MLOD		< MLOD	
	< MLOD		2.44	
WWTP A	T	1.28	< MLOD	-
	T		< MLOD	
	T		< MLOD	
	2.51		< MLOD	
	4.16		< MLOD	
WWTP B	< MLOD	-	< MLOD	-
	< MLOD		< MLOD	
	5.79		< MLOD	
	< MLOD		< MLOD	
WWTP E	T	-	1.30	0.52
	T		2.40	
	T		1.77	
	T		1.69	
	T		1.07	

2012 AhR Assay Results

WWTP	NAPEQ (ng/L) Influent	Standard Deviation	NAPEQ (ng/L) Effluent	Standard Deviation
WWTP C	97.84	68.47	45.58	44.02
	232.43		< MLOD	
	170.35		131.16	
	58.51		< MLOD	
	172.82		70.46	
WWTP D	237.61	12.78	< MLOD	-
	244.83		< MLOD	
	219.97		< MLOD	
WWTP A	< MLOD	105.75	138.51	91.15
	< MLOD		151.63	
	266.27		302.55	
	79.80		< MLOD	
	85.58		< MLOD	
WWTP B	< MLOD	-	< MLOD	-
	< MLOD		< MLOD	
	< MLOD		< MLOD	
WWTP E	T		T	
	T	-	T	-
	T		T	
	T		T	

2013 AhR Assay Results

WWTP	NAPEQ (ng/L) Influent	Standard Deviation	NAPEQ (ng/L) Effluent	Standard Deviation
WWTP C	65.30	434.13	< MLOD	12.78
	971.48		< MLOD	
	96.81		< MLOD	
	< MLOD		< MLOD	
	598.01		76.98	
WWTP D	139.54	72.94	92.20	6.83
	274.09		82.37	
	105.01		82.54	
	171.10		95.83	
WWTP A	T	-	< MLOD	-
	T		< MLOD	
	T		< MLOD	
	T		< MLOD	
	T		< MLOD	
WWTP B	< MLOD	33.24	< MLOD	38.17
	< MLOD		< MLOD	
	132.29		121.13	
	29.43		< MLOD	
WWTP E	T	-	< MLOD	-
	T		< MLOD	
	T		< MLOD	
	T		< MLOD	

Appendix B.

E2 and AhR Assay Results for Biosolids

E2 Assay Results

WWTP	EEQ (ng/g) 2012	Standard Deviation	EEQ (ng/g) 2013	Standard Deviation
WWTP C	25.15	4.19	20.62	9.89
	< MLOD		22.24	
	< MLOD		42.36	
	19.78		27.62	
WWTP D	26.65	12.72	62.83	17.23
	54.55		55.47	
	50.40		51.81	
	50.45		23.48	
WWTP A	123.78	-	T	-
	T		T	
	T		T	
	T		T	

AhR Assay Results

WWTP	NAPEQ (ng/g) 2012	Standard Deviation	NAPEQ (ng/g) 2013	Standard Deviation
WWTP C	7274.07	2685.74	4590.16	1457.21
	1900.26		4031.13	
	2968.91		2141.26	
	1350.84		1562.13	
WWTP D	1043.41	284.89	784.82	542.37
	418.25		1503.06	
	746.12		1405.77	
	483.14		2109.76	
WWTP A	6716.91	646.92	9174.84	-
	8009.75		T	
	7318.85		T	
	T		T	

Appendix C.

E2 and AhR Assay Results for IDZ

WWTP C IDZ E2 and AhR Assay Results

Station	EEQ (ng/L)	Standard Deviation	NAPEQ (ng/L)	Standard Deviation
R1	< MLOD	-	< MLOD	-
R2	< MLOD	-	< MLOD	-
1	< MLOD	-	< MLOD	-
2	3.38	2.23	< MLOD	-
3	< MLOD	-	< MLOD	-
4	< MLOD	-	< MLOD	-
5A	1.67	0.73	< MLOD	-
5B	2.09	0.82	< MLOD	-
Effluent	1.66	1.11	138.38	98.32

WWTP A IDZ E2 and AhR Assay Results

Station	EEQ (ng/L)	Standard Deviation	NAPEQ (ng/L)	Standard Deviation
R1	< MLOD	-	< MLOD	-
R2	< MLOD	-	< MLOD	-
1	< MLOD	-	< MLOD	-
2	< MLOD	-	< MLOD	-
3	< MLOD	-	< MLOD	-
4	< MLOD	-	< MLOD	-
5A	< MLOD	-	< MLOD	-
5B	< MLOD	-	< MLOD	-
Effluent	< MLOD	-	< MLOD	-

Appendix D.

E2 Assay Results for WWTP B Marine Sediments

Station	EEQ (ng/g) 2012	Standard Deviation	EEQ (ng/g) 2013	Standard Deviation
1	< MLOD	-	7.2	3.78
2	9.55	1.23	12.45	12.43
3	5.46	5.70	< MLOD	-
4	< MLOD	-	< MLOD	-
5	< MLOD	-	< MLOD	-
6	< MLOD	-	< MLOD	-
7	< MLOD	-	<MLOD	-
			<MLOD	-
8	< MLOD	-	< MLOD	-
	<MLOD	-		
9	16.46	15.64	11.07	9.49
10	9.21	4.45	10.26	10.28
11	4.03	1.67	< MLOD	-
12	< MLOD	-	9.56	11.50
13	3.83	2.15	< MLOD	-
14	11.16	0.96	< MLOD	-
15	9.33	0.85	9.27	10.57
	13.09	4.67		
16	< MLOD	-	9.99	10.99
			10.99	2.50

Appendix E.

E2 Assay Results for WWTP A Marine Sediments

Station	EEQ (ng/g) 2012	Standard Deviation	EEQ (ng/g) 2013	Standard Deviation
4	< MLOD	-	< MLOD	-
5	51.94	30.56	24.02	8.75
	30.94	12.54		
3	25.94	18.99	49.42	41.19
16	< MLOD	-	< MLOD	-
18	< MLOD	-	30.63	28.077
11	< MLOD	-	12.055	4.82
10	< MLOD	-	< MLOD	-
46	< MLOD	-	< MLOD	-
13	< MLOD	-	< MLOD	-
12	< MLOD	-	< MLOD	-
1	19.58	1.38	13.19	3.68
	<MLOD	-	13.20	2.911
2	32.94	12.01	< MLOD	-
47	< MLOD	-	< MLOD	-
45	36.31	26.63	39.24	33.71
			<MLOD	-
49	1.63	0.62	< MLOD	-
6	< MLOD	-	< MLOD	-

Appendix F.

AhR Assay Results for WWTP B Marine Sediments

Station	NAPEQ (ng/g) 2012	Standard Deviation	NAPEQ (ng/g) 2013	Standard Deviation
1	< MLOD	-	< MLOD	-
2	< MLOD	-	104.68	49.31
3	394.00	85.00	< LOD	-
4	< MLOD	-	255.75	57.66
5	< MLOD	-	519.40	143.26
6	< MLOD	-	505.83	119.89
7	< MLOD	-	< MLOD	-
			< MLOD	-
8	< MLOD	-	466.74	103.08
	< MLOD	-		
9	< MLOD	-	< MLOD	-
10	< MLOD	-	< MLOD	-
11	< MLOD	-	< MLOD	-
12	< MLOD	-	< MLOD	-
13	< MLOD	-	< MLOD	-
14	< MLOD	-	< MLOD	-
15	< MLOD	-	423.05	76.55
	< MLOD	-		
16	533.00	32.00	483.13	126.33
			360.82	82.56

Appendix G.

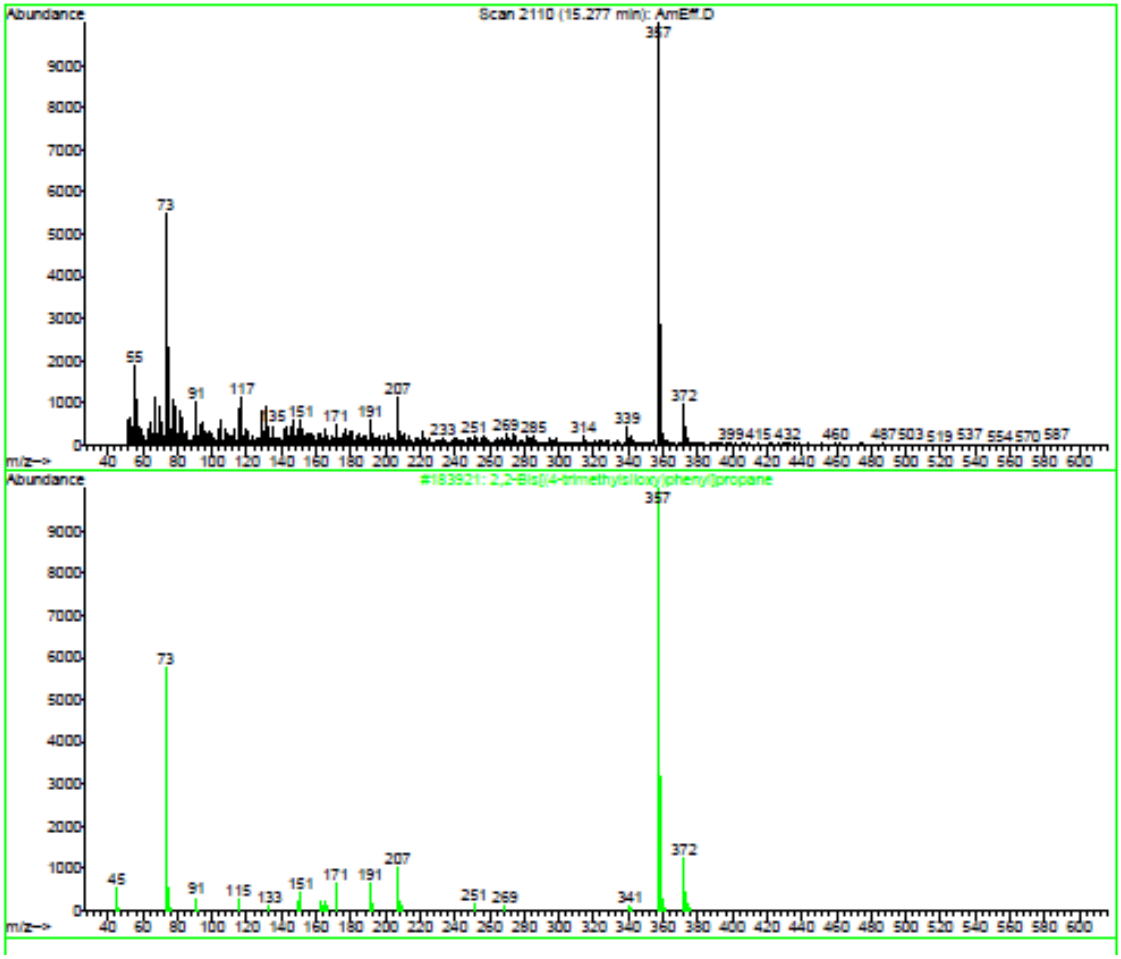
AhR Assay Results for WWTP A Marine Sediments

Station	NAPEQ (ng/g) 2012	Standard Deviation	NAPEQ (ng/g) 2013	Standard Deviation
4	< LOD	-	551.15	11.14
5	< MLOD	-	< MLOD	-
	< MLOD	-		
3	453.00	155.00	< MLOD	-
16	< MLOD	-	871.35	601.13
18	1575.00	398.00	360.76	53.43
11	< MLOD	-	361.13	123.52
10	517.00	121.00	518.05	131.50
46	< MLOD	-	< MLOD	-
13	264.00	238.00	467.97	143.26
12	565.00	142.00	288.07	132.62
1	689.00	174.01	723.23	159.57
	398.00	88.32	909.88	565.08
2	591.00	233.00	447.13	111.26
47	455.00	72.00	741.88	396.64
45	496.00	55.00	342.32	23.67
			564.36	256.03
49	1219.00	144.00	1396.43	1132.59
6	297.00	159.00	< MLOD	-

Appendix H.

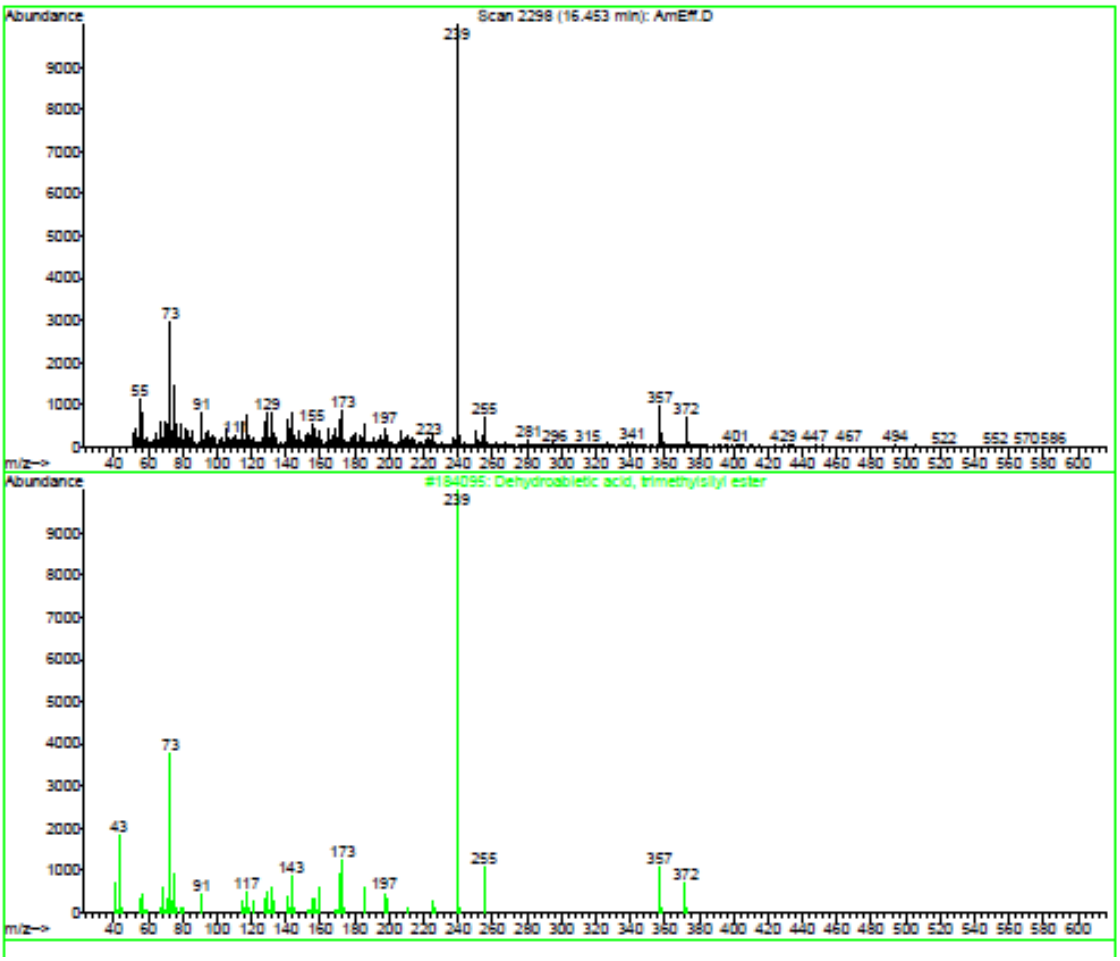
GC-MS Library Searches for BPA and DHAA

Library Searched : C:\Database\NIST08.L
Quality : 98
ID : 2,2-Bis[(4-trimethylsiloxy)phenyl]propane



Bisphenol A

Library Searched : C:\Database\NIST08.L
Quality : 92
ID : Dehydroabietic acid, trimethylsilyl ester



Dehydroabietic acid