

**SCREENING OF ENDOCRINE DISRUPTING  
COMPOUNDS USING ESTROGEN RECEPTOR  
TRANSCRIPTIONAL ACTIVATION *IN VITRO*  
BIOASSAYS**

by  
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B.Sc. Simon Fraser University, 2002

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## ABSTRACT

Many endocrine disrupting compounds (EDCs) are released from wastewater treatment plant (WWTP) effluents into surface waters worldwide. The objectives of this research were: (1) to explore the use of *in vitro* estrogen-receptor transcriptional activation (ERTA) assays to screen for estrogenic EDCs in aquatic samples, (2) to investigate WWTPs as a source of EDCs into the aquatic environment, and (3) to examine whether these bioassays can be applied in monitoring estrogenic EDCs in fish. ERTA assay analysis of reference estrogens and WWTP samples, using both mammalian and yeast cell lines had less than 5-fold inter-assay variation compared to 17 $\beta$ -estradiol equivalent (EEQ) values. WWTP samples had EEQ values consistently higher than 1 ng/L, a concentration that could potentially cause endocrine disruption in fish. ERTA assay analysis of fortified tissue samples correlated with doses; however, the recovery was low (1-3%), and future studies may focus on improving the extraction procedure.

## **DEDICATION**

For my family, who offered me unconditional love and support throughout the course of this thesis. For Kristina, my soul-mate, my inspiration, my best friend.

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## LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
BCME	British Columbia Ministry of Environment
BPA	Bisphenol A
C <sub>i</sub>	Initial Concentration
DHEP	Di(2-ethyl) phthalate
DFO	Department of Fisheries and Oceans
E1	Estrone
E2	17 $\beta$ -estradiol
E3	Estriol
EC <sub>50</sub>	Effective Concentration which has 50% of the maximal activity
EDC	Endocrine Disrupting Compound
EDL	Estimated Detection Limit
EE2	Ethinylestradiol
EEF	17 $\beta$ -Estradiol Equivalence Factor
EEQ	17 $\beta$ -Estradiol Equivalence Quotient
EPA	US Environmental Protection Agency
E-Screen	Estradiol Screen (Soto et al., 1995)
GC/MS	Gas chromatography mass spectrometry
GVRD	Greater Vancouver Regional District
HR-GCMS	High-resolution gas chromatography mass spectrometry

IDZ	Initial Dilution Zone
LOD	Limit of Detection
LOEL	Lowest Observable Effect Concentration
Langley	Northwest Langley (WWTP)
Lion's	Lion's Gate (WWTP)
Lulu	Lulu Island (WWTP)
MAX	Maximal Activity
NET	Norethindrone
NOEL	No Observable Effect Concentration
NP	Nonylphenol
ng	Nanogram
nM	Nanomolar
NWL	Northwest Langley (WWTP)
pg	Picogram
pM	Picomolar
R <sup>2</sup>	Correlation coefficient
R1881	Methyltrienolone
WWTP	Waste Water Treatment Plant
YES	Yeast Estrogen Screen

# CHAPTER 1 INTRODUCTION

## 1.1 Endocrine Disruption

The phenomena of endocrine disruption has been observed around the world, in many forms of life, including humans (Colborn and Clement, 1992), other mammals (Kirk et al., 2002) and aquatic organisms (Jobling, 2003). Environmental exposure to low and often analytically undetectable (ng/L) concentrations of endocrine disrupting compounds (EDCs) has caused physiological malfunction, such as sex reversal in fishes (Jobling, 1998). The number of anthropogenic and natural EDCs, released into the environment, is unknown since few chemicals have been tested for hormonal activity (Colborn and Clement, 1992). Many of the identified EDCs of potential concern have been detected in surface waters around the globe (Colborn and Clement, 1992).

The endocrine system is a highly sensitive information signalling system which uses chemicals messengers, or hormones, within an organism to regulate many vital functions. These chemical messengers are secreted by glandular tissue into the circulatory system, typically in very small concentrations (ng/mL or pg/mL). Hormones bind to receptors on cell surfaces or within the cell and exert important growth, homeostatic and regulatory effects (EPA, 1997). EDCs may elicit their effects through a variety of pathways including interference with the production, secretion, transport, action or elimination of hormones (Witorsch, 2000).

The majority of known environmental EDCs are estrogen mimics; therefore, the focus of most EDC research involves estrogen receptor interactions (Soto et al. 1995; NIEHS, 2002). Estrogen mimics elicit effects *via* estrogen receptor (ER) binding, which regulates transcriptional activity (Soto et al. 1995). Transcription is a process in which DNA is copied into complementary RNA, which then leads to the translation of the genetic code and synthesis of end-products, such as enzymes. When an estrogen mimic binds to the ER, the ER undergoes a conformational change and interacts with soluble cell factors to form a complex. This complex directly activates the estrogen-response element (ERE), a sequence of DNA located within the promoter region of a given gene, initiating the expression of that gene (EPA, 1997; NIEHS, 2002).

Among the many natural and synthetic estrogens identified in the environment, 17 $\beta$ -estradiol (E2) has been selected the standard EDC for the development of the *in vitro* bioassays because it is the most potent natural estrogenic chemical. As with the steroid hormones, 17 $\beta$ -estradiol is derived from cholesterol, thus many similarities in structure exist (Figure 1.1). E2 is primarily produced in the ovaries, and to a lesser extent in the testes; however, E2 is also produced in fat cells and brain cells (EPA, 1997; NIEHS, 2002). In testes, E2 is produced through steroidogenesis, which includes aromatization of testosterone into E2 (Figures 1.2).

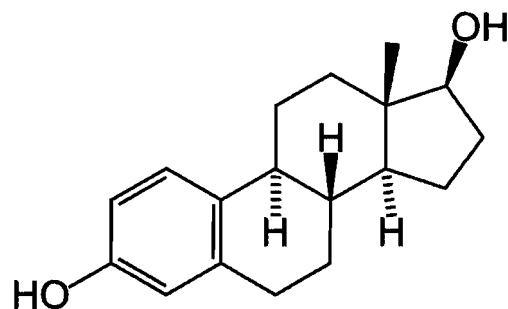


Figure 1.1. Structure of 17β-Estradiol

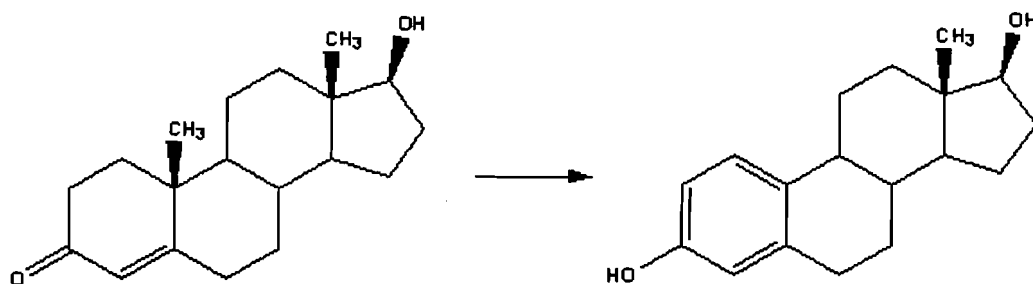


Figure 1.2. Aromatization of Testosterone into 17β-Estradiol

17β-estradiol is low in water solubility. It has a log  $K_{ow}$  of 4.01. Therefore, it does not exist in free form in water but is associated with organic materials (Table 1.1). It is not a persistent molecule, thus exists only temporarily in aqueous media, soils, sediments and biota (Ascenzo et al., 2003).



**Table 1.1. Physical and chemical properties of 17- $\beta$ -estradiol**

Property	Value
Molecular formula	C <sub>18</sub> H <sub>24</sub> O <sub>2</sub>
Molecular weight	272.3864
Melting point	178.5°C
Vapour pressure	1.3 x10 <sup>-8</sup> mm Hg
Log K <sub>ow</sub>	4.01
Water solubility	3.6 mg/L
Sigma # (CAS)	E4285

EDCs distribution in the environment is governed by their physical and chemical properties; many EDCs are persistent organic pollutants (Jobling, 2003). The chemical composition of EDCs varies widely in the environment, although some similarities exist in municipal wastewater (Servos et al., 2004). In general, the fate of EDCs lies in the aquatic environment where they may associate with organic matter or exist freely in the water column (Ascenzo et al., 2003). Although not long lived in its free form, estradiol is long lived in the conjugated form (Ascenzo et al., 2003).

In the aquatic environment, exposure of organisms to EDCs has been linked to endocrine effects in male fish such as vitellogenin induction and feminized reproductive organs (Aherne and Briggs, 1989; Purdom et al., 1994; Routledge et al., 1998; Tyler et al., 1998). It is suggested that industrial and municipal effluents as well as urban and agricultural runoff are the major sources of EDCs discharged into the aquatic environment (Desbrow et al., 1998; Snyder et al., 1999; Boyd et al., 2003). For example, when rainbow trout (*Oncorhynchus mykiss*) were kept in cages close to the discharges of WWTP effluents, vitellogenin synthesis was induced in the male fish (Harries et al.,

1997). Elevated levels of vitellogenin and decreased serum testosterone were also reported in male carp (*Cyprinus carpio*) caught near WWTP discharges (Folmar et al., 1996). Vitellogenin elevation and gonadal intersex also were observed in roach (*Rutilus rutilus*) and flounder (*Platichthys flesus*) caught near WWTP discharge sites (Jobling et al., 1998; Allen et al., 1999). In a study by Hansen et al. (1998), 70% of the fish sampled in watersheds receiving WWTP discharges, were female. These observations are consistent with the hypothesis of chemically induced feminization of fish at sites near WWTP discharges.

In response to the potential hazard of EDCs in the aquatic environment, several screening programs have been implemented using a variety of chemical analyses, *in vitro* and *in vivo* bioassays. Analytical methodologies based on gas chromatography-mass spectrometry or gas chromatography-tandem mass spectrometry have been developed and used for the ultra-trace determination of target EDCs in the aquatic environment (Desbrow et al. 1998; Johnson et al., 2000). Analytical techniques based on liquid chromatography-tandem mass spectrometry have also been used successfully for the determination of estrogens in different matrices (Draisci et al. 1998). Although chemical analysis can reveal the presence of EDCs in the aquatic environment, most chemical analysis is focused towards the determination of target substances in the matrices of interest. Considering the large number of EDC substances that can be present in complex environmental matrices, target chemical analyses could be limited in providing a complete account of all EDCs present in a specific environmental matrix. Moreover, mixture interaction is not taken into consideration and the biological effects of the chemical mixture cannot be determined. In contrast, *in vitro* bioassays which are based

on the interaction between the EDCs and the estrogenic receptors can determine the total estrogenic activity of EDCs in a mixture (Legler et al., 1999; Routledge and Sumpter 1996).

## **1.2 Estrogen-Receptor Transcriptional-Activation Assays**

A variety of *in vitro* ER-based assays have been developed to aid in the screening of estrogenic mimics. Various cell lines are utilized; some cells contain endogenous ER, whereas others must be transfected with foreign DNA, typically of human origin. Yeast assays consist of many different yeast strains, transfected with ER from humans, mice and even rainbow trout. Mammalian assays include different human cell lines, including a variety of carcinoma strains, and non-human mammalian cell lines such as hamsters, mice, monkeys, rats. In general, ERTA assays consist of a test substance interacting with the ER, resulting in transcriptional activation and a measurable endpoint. Typical endpoints include cell proliferation (cell-counts) and the production of  $\beta$ -galactosidase enzymes.

These *in vitro* ERTA bioassays could be very effective as part of Tier 1 screening, since the measured EDC activity is based on receptor activation (NIEHS, 2002). Interactions of individual EDCs, especially mixtures in environmental matrices can be very difficult to measure due to their very low individual concentrations. However, the additive effects on ER could prove to be a very sensitive and fast measure of mixtures of estrogenic mimics. When *in vitro* bioassays such as the yeast estrogen screen (YES) (Gaido et al., 1997) and MCF-7 breast tumor cell proliferation (E-Screen) (Soto et al., 1995) are used in isolation, they may yield false negative or positive results (Folmar et

al., 2002). Using several individual assays as lines of evidence, then building a weight of evidence based on additional lines of evidence, may decrease false predictions (NIEHS, 2002).

There are many types of ERTA assays, but none have been standardized for routine monitoring or for use in risk assessments by North American government agencies; although, they are recommended as a part of Tier 1 screening in risk assessments (NIEHS, 2002). The following classifications are used to differentiate ER agonist and antagonist TA assays into three broad groups: yeast reporter gene assays, mammalian reporter gene assays, and mammalian cell proliferation assays. Yeast cells must be transfected with ER and promoter gene systems, since they do not possess endogenous ER (Routledge and Sumpter, 1996). Since mammalian cells possess endogenous ER systems, they do not require transformation. Mammalian reporter gene assays vary, in that some cell lines contain sufficient endogenous ER systems while others require transformation (Soto et al., 1995).

In general, ERTA reporter gene assay responses are measured by quantifying the enzymes produced, typically  $\beta$ -galactosidase ( $\beta$ -gal) or luciferase (Luc). The synthesis of these end-products is controlled by ER binding activity *via* a sequence of DNA called the estrogen-response element (ERE), located within the promoter region for the reporter gene. When an EDC binds to the ER, the ER undergoes a conformational change and interacts with soluble cell factors to form a complex which directly activates the ERE, initiating the expression of the reporter gene and synthesis of its product (ie.  $\beta$ -galactosidase). A color-changing substrate is commonly added to the media for direct

measurement of its color change, indicative of the enzyme concentration, indirectly determining the quantity of ER binding activity. Alternatively, proliferation assays measure ER binding via extent of cell division, which commonly utilize staining techniques or cell counters.

The ERTA endpoints are measured as relative enzyme activity (percent maximal activity), typically using a nominal concentration series of reference estrogen, resulting in a dose-response relationship, where the half-maximal response concentration ( $EC_{50}$ ), slope, and minima/maxima effect levels are calculated. This type of analysis can also provide relative potencies of reference compounds, based on the ratio of  $EC_{50}$  values for the compounds being compared; generally 17 $\beta$ -estradiol is the standard reference estrogen to which other estrogens or EDCs are compared. Some researchers simply state that an effect did or did not occur, but not the extent of the effect (NIEHS, 2002).

Inhibition may be measured by adding the test substance to media containing the reference estrogen, and inhibition of the ERTA assay response is measured in a similar manner as above, via dose-response relationship. By adding the 100% effect concentration of E2, inhibition would be expressed as the decrease from maximal response level in a dose-response manner. Therefore, more inhibition at high concentrations of a test solution is observed as a decrease in response. Cytotoxicity is an important confounding factor, since cell death would result in decreased measures of activity. Common techniques to determine the extent of cytotoxicity may include the use of dyes, such as Trypan Blue, to determine the extent of cytotoxicity in mammalian cell assays; or visual observations of the consistency of yeast plaques.

The responsiveness of each cell line to E2 is determined by the characteristics of the cells themselves, the constructs used, and either the efficiency of the transfection or by the concentration of the transfected/endogenous ER. Mammalian cell lines are preferred, since yeasts contain cell walls making it difficult to extrapolate the access of EDCs and thus their corresponding activity from yeast to mammals (NIEHS, 2002).

Hitherto, there is no consensus among scientists on the best screening methods for determining EDC activities in aquatic samples, although *in vitro* ERTA assays are in high demand as a component of the tiered screening approach recommended by multi-government collaborations and organizations such as the National Toxicology Program (NTP) Interagency Centre for the Evaluation of Alternative Toxicological Methods (NICEATM) (NIEHS, 2002). Validation studies must be completed on one or more standardized ERTA assays, before governments can set minimum performance criteria. This includes comparison of substances between assays, the performance of each assay, and a consideration of advantages and disadvantages pertaining to the cell type and associated assay type. Minimum procedural standards must exist including: dose selection, replicate number, positive and negative controls, acceptability, and proficiency standards (NIEHS, 2002).

In this study, three ERTA assays are compared using a series of reference estrogens, including 17 $\beta$ -estradiol (E2), as part of a validation process, whereby the relative sensitivity of each assay was determined along with repeatability measures (Nelson et al., 2007). The three ERTA assays selected consist of two transfected yeast reporter gene assays (Routledge and Sumpter, 1995; Gaido, 1997) and a mammalian proliferation assay (Soto et al. 2004). A subset of the WWTP samples also underwent

trace organic chemical analysis, to provide a comparison between analytical and ERTA assay analysis (Nelson et al., 2007).

### **1.3 Greater Vancouver Regional District (GVRD)**

The Greater Vancouver Regional District (GVRD) is a regional district comprised of 21 municipalities and one electoral area, in the southwest corner of mainland British Columbia (BC) (Figure 1.3). The GRVD stretches from the U.S. border to Lion's Bay, and from Bowen Island to Langley Township. Municipal boundaries of the GVRD are shown in Figure 1.4. This metropolitan area of Greater Vancouver is home to over 2 million residents and is expected to grow to 2.7 million by 2021 (Statistics Canada, Census 2006).



Figure 1.3. Map of British Columbia (adapted from Magellan Geographix, 1995)

Each day, nearly a billion litres of wastewater, including sewage and storm water runoff from municipal sewerage systems collect in the 600 km of GRVD sewers. From these sewers, 33 pumping stations distribute the wastewater to five wastewater treatment plants; two providing primary treatment and three providing secondary treatment. The treatment plants process 98% (2% lost to overflows during heavy rainstorms) of the



wastewater. Treating wastewater helps to protect fisheries, habitat for wildlife, recreation and quality of life, and public health.

The role of the GRVD is to protect and enhance the quality of life in our region through region-wide essential services. The GVRD works with environmental groups in the river systems. It also works with industry to reduce the volume of wastewater entering the sewer system. The GVRD also manages and maintains natural watercourses in certain parts of the region to ensure that water is effectively conveyed. The GVRD is interested in any potential EDC activity in the region and whether the WWTPs it operates may be releasing them into the environment.

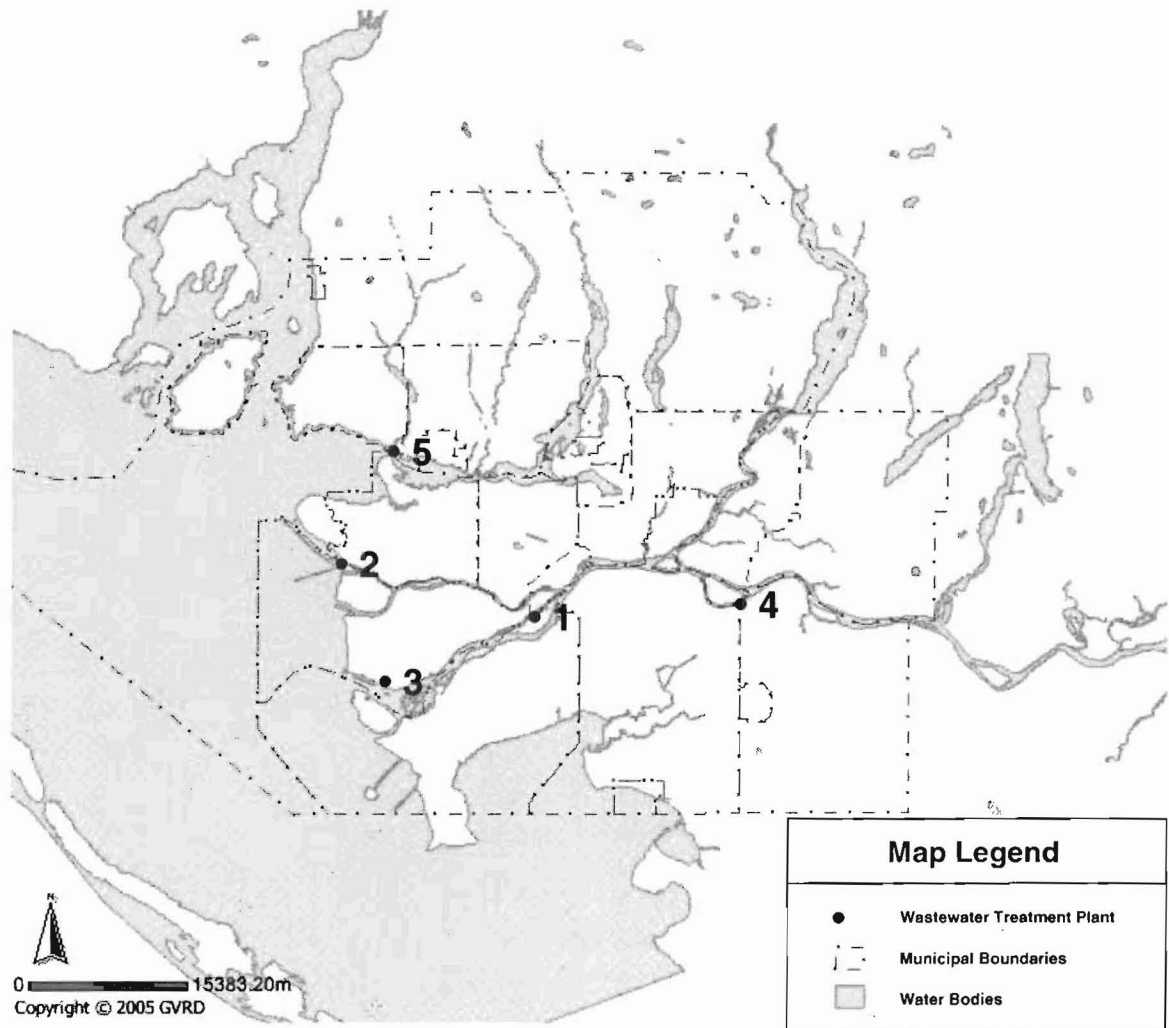
### **1.3.1 Wastewater Treatment**

The dissolved and solid materials suspended in wastewater use oxygen as they break down naturally in the environment. However, the availability of dissolved oxygen in water is the key to the survival of aquatic and marine life. The term used to describe the oxygen used up as organic material decays is biochemical oxygen demand (BOD). Total suspended solids (TSS) describe the suspended material.

The major aim of wastewater treatment is to remove as much of the BOD and TSS as possible before the remaining water, called effluent, is discharged to waterways. Treatment plants can remove various levels of suspended solids and BOD to purify wastewater. The level of treatment chosen depends on how much we need to aid the waterway's natural purifying ability. Primary treatment removes between 30 and 40% of BOD and 50% of TSS. Secondary treatment removes up to 90% of BOD and TSS.

Within the GVRD there are two primary WWTPs that discharge into the marine environment and three secondary WWTPs that discharge into the Fraser River (Figure

1.4). The two primary WWTPs are located in West Vancouver (Lion's Gate) and Richmond (Iona Island); whereas, the three secondary WWTPs are located in Langley (Northwest Langley), Delta (Annacis Island), and Richmond (Lulu Island).



**Figure 1.4. Wastewater Treatment Plants of the Greater Vancouver Regional District (modified from GVRD, 2005). 1 – Annacis Island, 2 – Iona, 3 – Lulu Island, 4 – Northwest Langley, 5 – Lion's Gate.**

Primary treatment is a mainly mechanical process that removes between 30 and 40% of BOD and 50% of the TSS. Secondary treatment includes a biological process that removes up to 90% of BOD and the TSS. Neither primary nor secondary treatment

processes are specifically designed to remove EDCs (e.g., trace organic EDCs such as hormones, various pharmaceuticals and pesticides) that are near analytical detection limits of chemical analytical methods (Folmar et al., 2002; Desbrow et al., 1998).

Even though the concentration of many EDCs in WWTP effluents are near or below the detection limits of chemical analysis, there is still the potential for EDCs to affect the endocrine systems of aquatic animals (Routledge et al., 1998). As a result, using a bioassay that can assess the overall potential endocrine disrupting activity of the whole effluent might be a more meaningful approach than chemical analysis. In addition, it could also assess the potential additive or synergistic effects of EDCs and ultimately provide more information on the potential for effects in the receiving environment.

The GVRD is committed to a receiving environment monitoring (REM) approach to managing its liquid waste discharges in its Stage 2 Liquid Waste Management Plan. This monitoring approach for the receiving environment of the five WWTPs in the GVRD includes the characterization of WWTP effluent to define the nature of the effluent and potential effects (GVRD, 2001).

The larger of the two primary plants, Iona Island, provides primary treatment to wastewater from approximately 600,000 people (in Vancouver, the University Endowment Lands and parts of Burnaby and Richmond) before discharging it through a 7.5 km, deep-sea outfall into the Strait of Georgia. In 2003, the average annual flow was 597 million litres per day (MLD) with average total suspended solids (TSS) and biological oxygen demand (BOD) of 48 mg/L and 76 mg/L, respectively.

The other primary plant, Lions Gate provides treatment to wastewater from approximately 160,000 residents of the District of West Vancouver, the City of North Vancouver, and the District of North Vancouver and discharges to Burrard Inlet under Lions Gate bridge approximately 200 m offshore and 20 m deep. In 2003, the average annual flow was 92 million litres per day (MLD) with average total suspended solids TSS and BOD of 54 mg/L and 89 mg/L, respectively.

The largest of the three secondary plants, Annacis Island, provides treatment to wastewater from approximately 740,000 people in various areas throughout Greater Vancouver (i.e., parts of Burnaby, New Westminster, Port Moody, Port Coquitlam, Coquitlam, Pitt Meadows, Maple Ridge, Surrey, Delta, White Rock, the City of Langley and, the Township of Langley). In 2003, the average annual flow was 485 million litres per day (MLD) with average TSS and BOD of 12 mg/L and 13 mg/L, respectively.

The second largest secondary plant, Lulu Island, provides treatment to wastewater from approximately 120,000 residents who live in the western area of the City of Richmond. In 2003, the average annual flow was 79 million litres per day (MLD) with average TSS and BOD of 8 mg/L and 12 mg/L, respectively.

The other secondary plant, Northwest Langley, provides treatment to wastewater from the residents of the Walnut Grove area in Langley. In 2003, the average annual flow was 9.2 million litres per day (MLD) with average TSS and BOD of <13 mg/L and 11 mg/L, respectively.

## 1.4 Research Objectives

The purposes of this research were: (1) to explore the use of *in vitro* estrogen-receptor transcriptional-activation (ERTA) assays to screen for estrogenic EDCs in aquatic samples, (2) to carry out an investigation on WWTPs as a source of estrogenic EDCs, through discharge into the aquatic environment, and (3) to investigate whether the ERTA bioassays can be applied to monitor EDCs in fish tissues.

## CHAPTER 2 MATERIALS AND METHODS

### 2.1 General Operating Procedures

Permission to use cell lines for the E-Screen (Soto, 1995) and YES (Gaido, 1997; Routledge & Sumpter, 1996) bioassays were obtained from the respective authors. In an effort to treat the bioassays equally for comparison and to make full use of available equipment at SFU, the following modifications were made to the standard operating procedures of the bioassays:

(a) 17 $\beta$ -estradiol (E2) was the reference estrogen used in all bioassays. The E2 standard dilution series consists of 11 dilutions (0.001 nM to 100 nM), plus a solvent (ethanol) blank.

(b) While most chemicals were selected based on the respective author's operating procedures, the plasticware was selected based on a list provided by Soto (2004) which showed no background EDC activity from plasticizers.

(c) Glassware washing included rinsing with water as soon as the solution was emptied, followed by thorough scrubbing with industrial strength detergent, then rinsed three times with water before receiving three methanol rinses, then oven dried (~100 °C) with a drying agent.

All test chemicals were soluble in anhydrous ethanol except dioctyl phthalate. Inhibition and cytotoxicity were assessed by observing the decreased proliferation of samples exposed to the maximal proliferation concentration of E2 (1 nM).

## 2.2 The E-Screen

The human breast cancer (MCF-7 BOS) cell line, used in the E-Screen proliferation ERTA assay, was provided by Ms. Janine Callabro in the Soto/Sonnenschein laboratory at Tufts University. Cell cultures were maintained for one month prior to their use in an assay, to ensure that the number of passages were similar for consistency of the culture (Appendix B). Every two months, new cultures were started from a frozen stock (Appendix B). All chemicals used in this assay were selected based on the standard operating procedures supplied by the Soto laboratory (Appendix B).

The assay was performed in accordance with the standard operating procedures (Appendix B), provided by Ms. Janine Callabro (Soto *et. al.* 2004). A 24-well microplate was seeded with  $20\text{-}30 \times 10^3$  evenly distributed cells per well, using a hemocytometer. As per the operating procedures, dummy plates were placed under experimental plates and a static brush was used to help control static, preventing cell clumping. These plates were incubated for 24 hours to allow the cells to adhere to the surface of the base of each well. Following a 24-hour incubation period, the 1 mL/well of culture media *i.e.* Dulbecco's Modified E Media (DMEM) with phenol red and defined fetal bovine serum (FBS) (Hyclone, Utah, USA), was removed from the plates. Rinsing with Hanks' Balanced Salt Solution (HBSS) was followed by the addition of 1 mL/well of experimental media, *i.e.* DMEM without phenol red and with charcoal dextran stripped defined FBS (Hyclone,

Utah, USA). Phenol red was removed from DMEM since it adsorbed at the same wavelength as the stain used to measure cell density at the end of the experiment. The FBS was treated with charcoal dextran to remove any hormones the serum might contain, potentially interacting with ER and confounding the experiment (Soto et al., 2004).

The reference E2 serial dilution series along with the dilution series of other chemicals were then added to the wells (50  $\mu$ L/well). All reference chemicals were prepared in ethanol. The experimental plates were incubated for 5 days at 37 °C with high humidity. After a 5-day exposure, the cells were observed under a microscope for signs of toxicity or contamination. The experimental solution was removed and 1 mL/well of HBSS was used to rinse the cells prior to the addition of 1 mL/well of 10% trichloroacetic acid (TCA) fixative solution (4 °C). The plates were kept on ice for 30 min and then gently washed with tap water, inverted and dried, prior to staining. Sulforhodamine B (SRB) dye (500  $\mu$ L/well) was used for staining, prepared as a 0.4% solution in 1% acetic acid. A 20-min exposure to the dye was followed by a thorough rinsing of the plates using a 1% acetic acid solution. Plates were then dried in the dark before addition of Tris buffer solution (500  $\mu$ L/well) to solubilize the dye. The 96-well microplates were scanned at 515nm and 650nm (Soto et al., 2004).

### **2.3 The Yeast Estrogen Assays**

The yeast cell lines, *Saccharomyces cerevisiae* for the ERTA reporter gene assays, were obtained and used with permission from their respective authors (Routledge & Sumpter, 1996; Gaido *et. al.*, 1997). The Gaido strain was obtained directly from Dr. Gaido's laboratory in the Chemical Industry Institute of Toxicology (Research Triangle



Park). The Sumpter strain was obtained from Dr. Ikonomou at the Institute of Ocean Sciences (Department of Fisheries and Oceans). These ERTA assays shared many common features; most importantly, both strains had been transfected with a human ER (hER) and a linked reporter gene. Both bioassays used promoters which were dependent on copper to initiate synthesis of the ER, namely the CUP1-MET promoter, while their reporter plasmids contained the ERE and the iso-cytochrome C (CYC1) in a LacZ fusion vector. These were linked to the production of  $\beta$ -galactosidase, which was used as the measure of ER induced TA. The mechanism involved the expression of the reporter gene producing  $\beta$ -galactosidase, which metabolized galactopyranoside to a chromogenic agent, measured at 415 nm for the Gaido YES and 540 nm for the Sumpter YES using a spectrophotometer. The bioassays were performed, respectively in accordance to the procedures of Routledge & Sumpter (1996) and Gaido *et. al.*, (1997).

Cell cultures were verified by running the assay with standards for one month prior to the analysis of test substances. This was to ensure the passage number was similar and the cultures were consistent (Yeast Culturing Protocol, Appendix B). Every week new cultures were started from plates stored at 4 °C. The plates were made each month from frozen stock (Yeast Freezing Protocol, Appendix B) and were never stored longer than one month. The chemicals and solutions used in these assays are described in the standard operating procedures supplied by the respective cell line donors (Appendices A, B and C).

The yeast estrogen screen (YES) bioassays are similar assays in that they had been transformed to include the human estrogen receptor (hER) in their main genome, estrogen response elements (ERE) and a lac-Z gene on a plasmid. Essentially, the

estrogenic substance binds to a hER which polymerizes and binds to the ERE, which controls the expression of the lac-Z gene, resulting in the transcription and subsequent release of  $\beta$ -galactosidase into the medium. However, the two YES bioassays employed different chromogenic substances for visualization: 2-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) was used in the Gaido method, while chlorophenol red- $\beta$ -D-galactopyranoside (CPRG) was used in the Sumpter method. The intensity of the color was then measured by absorbance; these values were then normalized for the background activity (Lorenzen *et. al.*, 2004).

Lorenzen *et al.*, (2004) provided a modified version of the Gaido (1997) procedure allowing for the use of a 96-well plate with spectrophotometric measurements of endpoints (Appendix C). Briefly, on day 0 at 10 a.m., a yeast culture was started by adding a single colony of yeast from a streaked plate into 5 mL selective media and incubated overnight at 30 °C with shaking. On day 1 at 10 a.m., the culture was diluted 10 fold, by adding 45 mL of growth medium. On day 2 at 10 a.m., the culture was then diluted by 50% in growth media (Lorenzen *et. al.*, 2004).

At 1 p.m., aliquots (10  $\mu$ L) of standards or test solutions were transferred in triplicate to the microplate wells and allowed to dry (approximately 30 min). At 2 p.m., copper sulfate (0.1 M, final concentration in yeast culture) is added to the mid-log phase ( $OD_{600nm}$  of 0.8-1.0) yeast culture to induce hER production prior to exposure. This yeast culture is added to the 96-well plate in aliquots of 200  $\mu$ L/well. After mixing for 2 min, the microplates were sealed and incubated overnight at 30 °C, without shaking. At the end of the 20 hr incubation, yeast cells were resuspended and 100  $\mu$ L aliquots were

transferred to a 96-well microplate containing 100  $\mu$ L of assay buffer solution (Appendix III). After 2 min of shaking, the microplates were kept at room temperature for an additional 40 min, until the absorbance was read at 415 nm and 595 nm (Lorenzen *et. al.*, 2004).

Lyticase (instead of Oxalyticase), sodium dodecyl sulfate (SDS),  $\beta$ -mercaptoethanol and all hormone standards were obtained from Sigma Chemical Company (St. Louis, MO) with the exception of methyltrienolone (R1881) which was purchased from New England Nuclear (Perkin-Elmer Life Sciences, Boston, MA).

The Routledge & Sumpter (1996) YES bioassay was performed as previously described. On day 0, a yeast culture, an aliquot (125  $\mu$ L) of yeast stock was added to 50 mL growth medium and incubated overnight at 28 °C on a shaker. On day 1, 1 mL of the yeast culture was transferred to 50 mL growth medium containing 0.5 mL 10 mg/mL chlorophenol red- $\beta$ -d-galactopyranoside. Aliquots (10  $\mu$ L) of standards and environmental extract dilutions were transferred in triplicate to microplate wells and allowed to dry (approximately 30 min). The culture was added to the treated 96-well microplates, shaken for 2 min and incubated for 5 days at 30 °C. On day 6, the microplates were shaken for 2 minutes, allowed to settle for 1 hr prior to reading the absorbance at 540 nm and 620 nm (Routledge & Sumpter, 1996).

## 2.4 Statistics and Calculations

### 2.4.1 Dose-Response Analysis (Non-linear Regression)

Results from the E-screen and YES bioassays were plotted using SigmaPlot (V. 8.02). The ERTA assay E2 equivalent quotient (EEQ) values were generated based on the E2 dose-response curve and used to quantify the unknown samples or reference chemicals based on the measured activity of 17 $\beta$ -estradiol. The curve was generated using the Hill function:

$$\text{Response} = (V_m \times \text{Dose}^\alpha) / (K^\alpha + \text{Dose}^\alpha)$$

$V_m$  is the maximal response,  $K$  is the half-maximum effective concentration and  $\alpha$  is the Hill coefficient (Lorenzen et al. 2004). SigmaPlot's non-linear regression performs least squares analysis to plot the best-fit line based on the above formula for a set of dose-response data. A correlation coefficient of  $R^2 > 0.95$  was acceptable; however, the values were generally 0.99. The only constraints were that the minimum values be greater than 0 and that the maximum values be larger than the minimum.

Other important parameters also determined from the dose-response curves; these include the no observable effect level (NOEL), lowest observable effect level (LOEL), effective concentration in which 50% of the maximal effect occurs ( $EC_{50}$ ), and maximal effect level. The observable effect levels are determined from experimental data points, not the plot. The LOEL is the lowest dose to have an effect significantly greater than the background. The NOEL is the next lowest dose from the LOEL, which is not

significantly lower than the background, using the Student's T-test with. Hypotheses were tested at alpha = 0.05 significance level..

#### **2.4.2 Linear Regression**

SigmaPlot's linear regression performs least squares analysis to plot the best-fit line for a set of data. The correlation coefficient is a measure of how well the line fits the data.

#### **2.4.3 Analysis of Variance and Student's T-test**

Differences in sample 17 $\beta$ -estradiol equivalents were analysed using Microsoft Excel and SigmaPlot. Analysis of variance (ANOVA) was used to compare differences between the different bioassays. It was also used to compare results for samples collected from different locations and dates. Hypotheses were tested at alpha = 0.05 significance level.

#### **2.4.4 17 $\beta$ -Estradiol Equivalent Quotient (EEQ) Value Calculations**

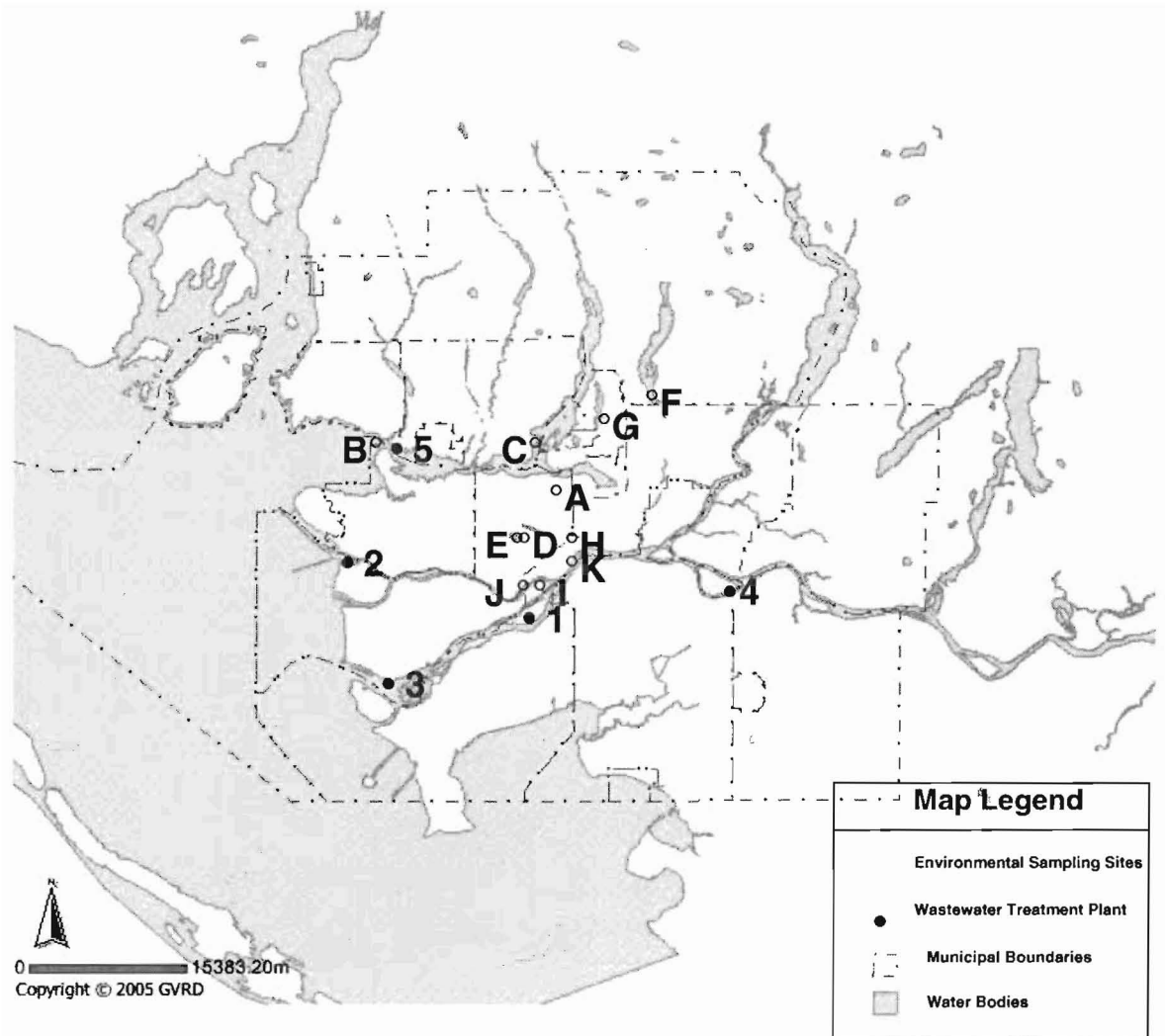
A bioassay-derived EEQ values was determined from the dose-response curve as the equivalent concentration of 17 $\beta$ -estradiol that would elicit the same estrogenic activity as the sample (Soto et al., 2004). On the other hand, a chemically-derived EEQ value of the sample was calculated as the sum of individual chemical concentrations ( $C_i$ ) in the mixture multiplied by their respective estradiol equivalent factor (EEF) (Soto et al., 2004):

$$\text{EEQ of a chemical mixture} = \sum \{(C_i) \times (\text{EEF})\}$$

## **2.5 Aqueous Sample Collection**

Aqueous samples were collected in pre-cleaned 4L glass bottles; the few exceptions were noted otherwise. GVRD water samples consisted of lake, river and ocean environmental grab samples; they were obtained from the shoreline, approximately one to two feet away from the shore, using a pole sampler, which submerged the inverted 4 L glass bottle, then re-inverted it once submerged several inches below the water's surface, allowing it to fill completely.

The map, in figure 2.1, of the GVRD shows all sites sampled in this study. Ocean grab samples were collected at A, Rocky Point, (Port Moody); B, Ambleside Park (North Vancouver); and C, Deep Cove (North Vancouver). Lake grab samples were collected at D, Burnaby Lake (Burnaby); E, Deer Lake (Burnaby); F, Buntzen Lake (Anmore); G, Sasamat Lake (Coquitlam), H, Como Lake (Coquitlam). Fraser River samples were collected at I, J, and K. The five WWTP sites shown in figure 2.1 are listed as numbers and correspond to: 1, Annacis Island (Annacis Island/New Westminster); 2, Iona (Richmond), 3, Lulu Island (Richmond/Delta); 4, Northwest Langley (Langley); and 5, Lion's Gate (North Vancouver).



**Figure 2.1. Map of the GVRD with the five WWTPs numbered 1, 2, 3, 4, and 5. Note: 2 and 5 are the primary WWTPs and 1, 3, and 4 are secondary WWTPs. A - Rocky Point (Port Moody); B - Ambleside Park (North Vancouver); C - Deep Cove (North Vancouver); D - Burnaby Lake (Burnaby); E - Deer Lake (Burnaby); F - Buntzen Lake (Anmore); G - Sasamat Lake (Coquitlam), H - Como Lake (Coquitlam); I, J, and K –Fraser River. WWTP sites shown: 1 - Annacis Island (Annacis Island/New Westminster); 2 - Iona (Richmond), 3 - Lulu Island (Richmond/Delta); 4 - Northwest Langley (Langley); and 5 - Lion's Gate (North Vancouver).**

### 2.5.1 Wastewater Treatment Plant Sample Collection

WWTP raw influent (untreated) and effluent (treated) samples were collected at each of the five WWTPs (Figure 2.1) operated by the GVRD. Sampling consisted of grab or composite samples collected throughout 2004 (Table 2.2). An influent and an effluent sample were collected from each WWTP. The samples were collected in pre-cleaned 1L glass bottles and transferred *via* pre-cleaned stainless steel funnel into pre-cleaned 4L glass bottles.

**Table 2.1. Sample Locations and Dates for the 2004 series of WWTP samples.**

WWTP	Grab-1	Grab-2	Composite-1	Grab-3	Composite-2
Annacis Island	14-Apr	31-May	14-Jun	23-Sep	16-Nov
Iona	19-Apr	31-May	21-Jun	14-Oct	21-Oct
Lulu Island		4-May	14-Jun	14-Oct	16-Nov
Langley		11-May	15-Jun	30-Sep	3-Nov
Lion's Gate		13-May	21-Jun	23-Sep	3-Nov

Since EDC concentrations in grab samples were quite variable, 24-h composite samples were also collected from the WWTPs. Flow proportional SIRCO samplers were used at all but Langley WWTP, where a time proportional ISCO sampler was used. All samplers collected over a 24-h period; from midnight to midnight (1- Ann and 2 - Iona) or 0700 to 0700 h (3 - Lulu, 4 – Langley , 5 - Lion's Gate). Samples were collected in pre-cleaned 4L glass bottles from the samplers.



### **2.5.2 GVRD Environmental Sampling Series**

Lake water sampling involved collection of three water samples from each lake; the three sampling sites were selected randomly along the edge of each lake. Sampling was done on a weekly basis for a span of two months, and repeated 2 weeks after the first series, providing a 2-month period between sampling at one site (Table 2.1). Ocean water sampling involved collection of only one water sample from each site; these were collected at the boat launches in Rocky Point, Deep Cove and Ambleside Marine Parks.

Fraser River water samples were collected near the Annacis Island WWTP. Three samples were collected at three different sites on the same day. The selected sites consisted of two downstream and one upstream of Annacis Island WWTP.

The number of samples that could be processed per week was limited by the time spent on sample extraction (approximately 5 hours per sample) and the accessibility of assay equipment (microplate reader).

**Table 2.2. Lake, Ocean and River water sampling dates.**

SITE	SAMPLING 1	SAMPLING 2
Sasamat Lake	June 4, 2005	Sept 3, 2005
Buntzen Lake	June 11, 2005	Sept 10, 2005
Burnaby Lake	June 18, 2005	Sept 17, 2005
Deer Lake	June 25, 2005	Sept 24, 2005
Como Lake	July 2, 2005	Oct 1, 2005
Rocky Point Park	July 9, 2005	Oct 8, 2005
Ambleside Park	July 16, 2005	Oct 15, 2005
Deep Cove	July 23, 2005	Oct 22, 2005
Fraser River 1, 2, 3	July 30, 2005	Oct 29, 2005

## **2.6 Extraction Procedure for Aqueous Samples**

Water samples were extracted based on a modification of the method used by Soto *et al*, 2004. Samples were extracted on the same day of sample collection. Each 4 L sample was split into 4 aliquots (1 L), and transferred into its own 2 L separatory funnel containing 60 mL of dichloromethane (DCM). The separatory funnel was shaken for 5 min and the layers were allowed to separate for 15 min. The bottom (DCM) layer was removed, and 60 mL of fresh DCM was added to the remaining aqueous solution, which was mixed for another 5 min and allowed to separate for 15 min. The second extraction step was repeated and the extracts from all three extractions were combined and concentrated down to approximately 4 mL using ROTAVAP and N-EVAP evaporators (Organomation Associates, Berlin, MA) at 40 °C. A 2 mL aliquot was removed, dried completely and solvent-exchanged into ethanol (anhydrous). For selected samples, the

remaining DCM (2 mL) was dried completely and stored at -40 °C and sent to the Institute of Ocean Sciences (IOS) (Sidney, BC, CANADA) for HR-GCMS analysis.

After extraction, 17 $\beta$ -estradiol concentration in the extract was usually 10,000 times more concentrated than the original 4 L sample (concentrated from 4 L to 400  $\mu$ L). When YES assay was performed on the extracted samples, 17 $\beta$ -estradiol concentration was diluted 20-fold (10  $\mu$ L of test solution in 200  $\mu$ L of yeast). Therefore, the extract was concentrated 500-fold. If other serial dilution of the samples were to be tested on the yeast assay, the correlated dilution factor was employed.

## **2.7 Sediment Sample Collection and Extraction**

Sediment grab samples were collected from a GVRD boat equipped with a dredge. Samples were taken along the Lion's Gate WWTP outfall area along the initial dilution zone (IDZ). Sediment extraction procedures were conducted according to Lorenzen *et al.* (2002). Homogenization was performed by hand using a large metal stirring rod for 30 sec. Homogenized sample (5 g) was transferred to a 50 mL centrifuge tube and 20 mL ethyl acetate was added. The mixture was shaken for 10 min and centrifuged for 5 min at 200 g. The organic layer was transferred to a round bottom flask and the procedure was repeated two times using 15 mL ethyl acetate. The organic extracts were added to the first extract in the round bottom flask. The extracts were dried using a rotary evaporator, reconstituted in 4 mL ethyl acetate, and transferred to a 6 mL pre-cleaned glass vial where it was dried to completion and stored in -40 °C freezer overnight. The following morning, the sample was reconstituted in 200  $\mu$ L anhydrous ethanol for the assay test solutions (Lorenzen *et al.* 2004).

## **2.8 Tissue Sample Collection, Dosing and Extraction**

Chinook salmon and rainbow trout were purchased from Queen City Seafood on May 7, 2007. The doses consisted of 100  $\mu\text{L}$  of: 0.01M (Positive Control),  $1.00 \times 10^{-8}$  M (Test Dose 1 – high log-phase),  $1.00 \times 10^{-10}$  M (Test Dose 2 – mid log-phase), 99% Ethanol (Vehicle Control), Water (Method Control). These doses were added to 5.0 g of tissue. They were reconstituted in 100  $\mu\text{L}$ . Tissue samples were extracted using the same method as the sediment samples. This was based on the Agriculture Canada biosolid extraction method (Lorenzen et al. 2004). The one difference was that tissue required 2 min homogenization using a model PT 10/35 Polytron homogenizer (Brinkman Company, Rexdale, ON, Canada) and some tissue samples were smaller than 5 g.

## **2.9 Chemical Analysis of WWTP Samples**

Ten composite WWTP sample extracts were sent to Dr. Ikonomou's laboratory in Institute of Ocean Sciences (IOS) for chemical analysis using a GC-HRMS based trace analytical method (Ikonomou et al., submitted for publication). The samples were reconstituted in 1 mL DCM and a 0.5 mL aliquot was removed for analysis. A 50  $\mu\text{L}$  volume of EDC surrogate mix was added to each aliquot before sample clean-up. After Florisil column clean-up, samples were derivatized and spiked with performance standards before gas chromatography-high-resolution spectrometry (GC-HRMS) analysis.

Quantitation was achieved using the isotope dilution approach. A six-point calibration curve was generated for each target analyte. All results were corrected against background levels measured in procedure blanks. Method quality control measures

included: a sample duplicate and lab procedure blank with each batch of 10 samples, instrument blanks, bracketing verification standards, and use of internal standards (i.e., labelled surrogate internal standards and performance standards). Limits of detection (LOD) were based on an amount that would generate an instrument response of  $S/N > 3$ . The average LOD values and the LOD range for each of the target analyzed are presented in Table 2.2. Further details on the methodology used and the criteria used for target analyte identification and quantification are reported elsewhere (Ikonomou et al., submitted for publication).

**Table 2.3. GC-HRMS analytes and their LOD, for the 10 composite samples.**

Analyte	Average LOD (ng/L)	LOD Range (ng/L)
Bisphenol A (BPA)	3.2	2.9 – 3.9
Di(2-ethylhexyl) phthalate (DEHP)	5.2	5.2 – 5.2
Estrone (E1)	5.4	4.7 – 5.9
Equilin	8.9	8.9 – 8.9
17 $\alpha$ -Estradiol	5.3	5.3 – 5.3
17 $\beta$ -Estradiol (E2)	4.9	4.9 – 4.9
19-Norethindrone (NET)	11.1	9.4 – 12.6
Mestranol	6.1	6.1 – 6.1
17 $\alpha$ -Ethinylestradiol (EE2)	6.9	6.8 – 6.9
Norgestrel	7.1	5.8 – 9.7
$\alpha$ -Zearalanol	26.8	4.9 – 52.4
Estriol (E3)	5.9	5.9 – 6.0
$\beta$ -Estradiol-3-benzoate	5.2	5.0 – 5.3
Nonylphenol (NP)	44.2	4.3 – 70.5

## 2.10 Chemical and Biological Estradiol Equivalent Factors (EEF)

Several estrogens were selected from the GC-HRMS list of analytes for analysis using the *in vitro* ERTA assays, to determine their estrogenic activity relative to the standard, E2. The selection was based primarily on whether they were detected in any of

the 10 samples analyzed. The 17 $\beta$ -estradiol equivalency factor (EEF) were calculated by taking the ratio of the ERTA assay EC<sub>50</sub> for the chemical versus the ERTA assay EC<sub>50</sub> of E2. Chemical EEFs were calculated as sum of individual chemical concentrations (C<sub>i</sub>) multiplied by their respective EEF:

$$\Sigma \{(C_i) \times (EEF)\}$$

An E2 equivalence is obtained for samples *via* E2 equivalent effects for the activity in the sample extrapolated from the dose-response curve of E2.

## CHAPTER 3 RESULTS

### 3.1 A Comparison of Three ERTA Bioassays

#### 3.1.1 Dose Response Analysis of 17 $\beta$ -Estradiol

A comparison of the 17 $\beta$ -estradiol dose-response curves shows that the 2 yeast bioassays have very similar detection limits, maximal effect level and EC<sub>50</sub> (Figures 3.1, 3.2, and 3.3). However, the E-Screen assay appeared to be approximately 4-fold more sensitive than the yeast systems, although the E-Screen lacks the relatively high concentration detection limit (Table 3.1).

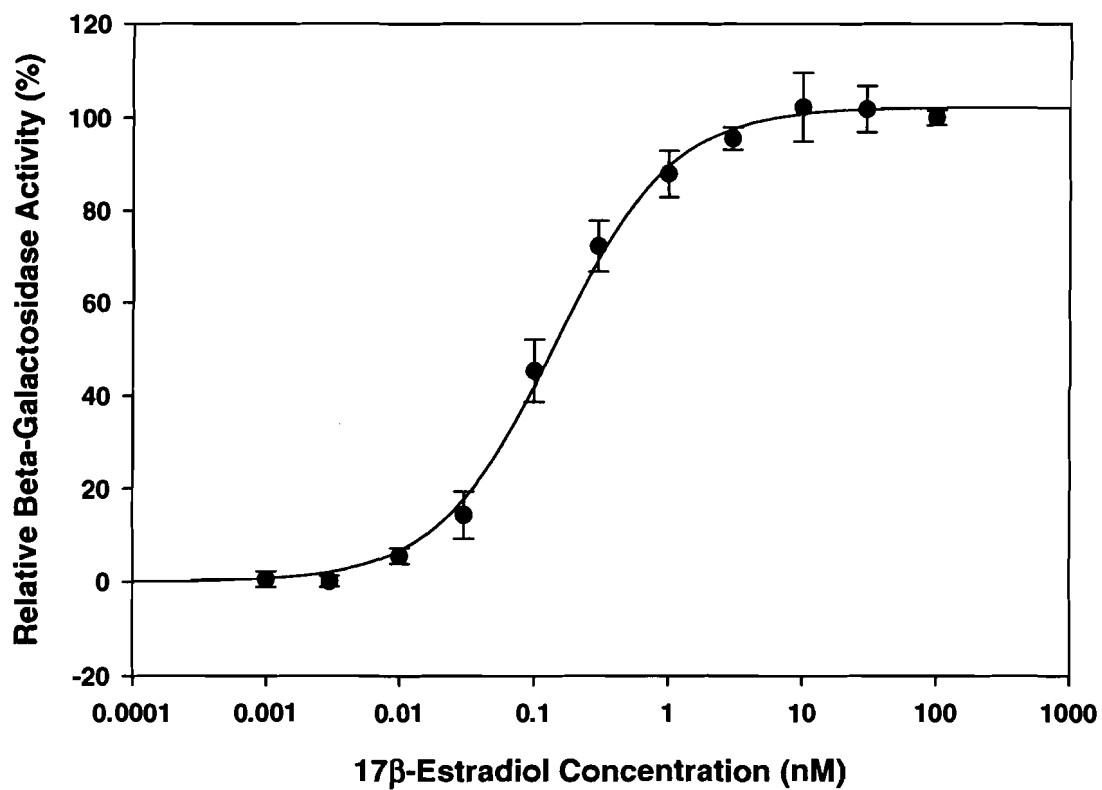
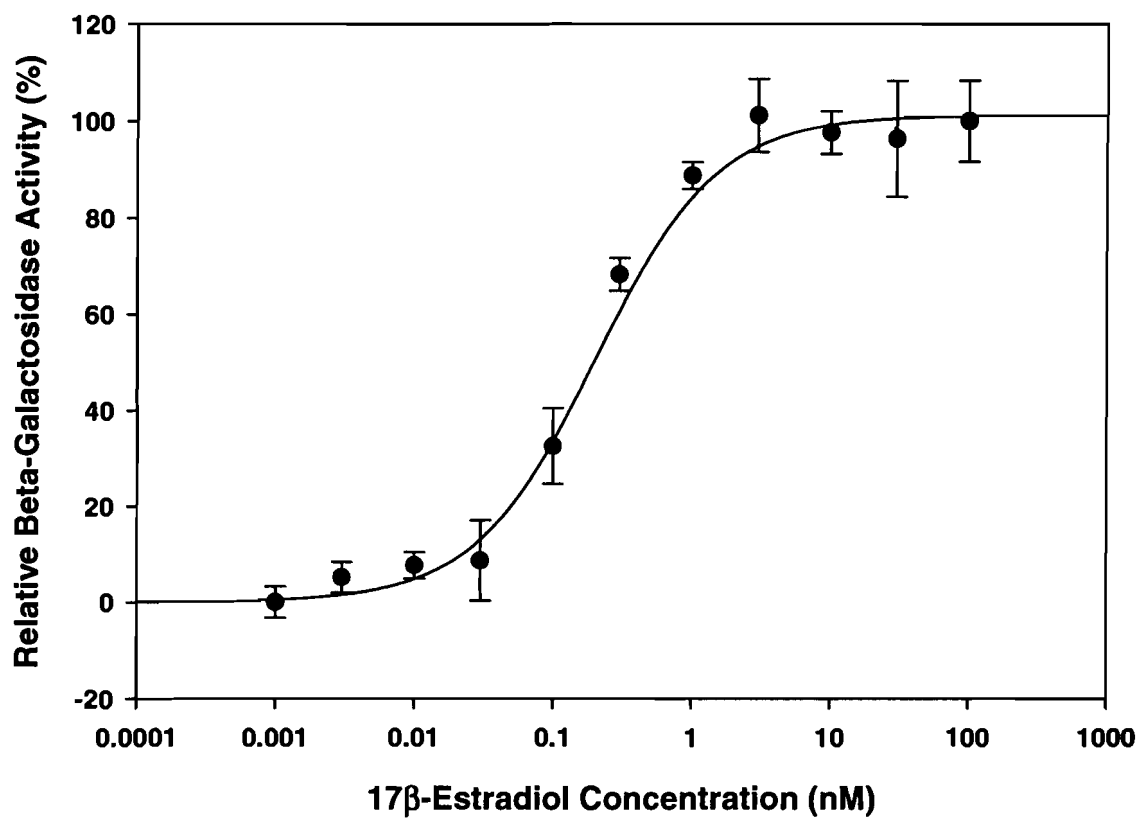


Figure 3.1. Typical E2 dose-response curve obtained using the yeast assay described by Gaido et al., 1997.





**Figure 3.2.** Typical E2 dose-response curve obtained using the yeast assay described by Routledge and Sumpter, 1996.

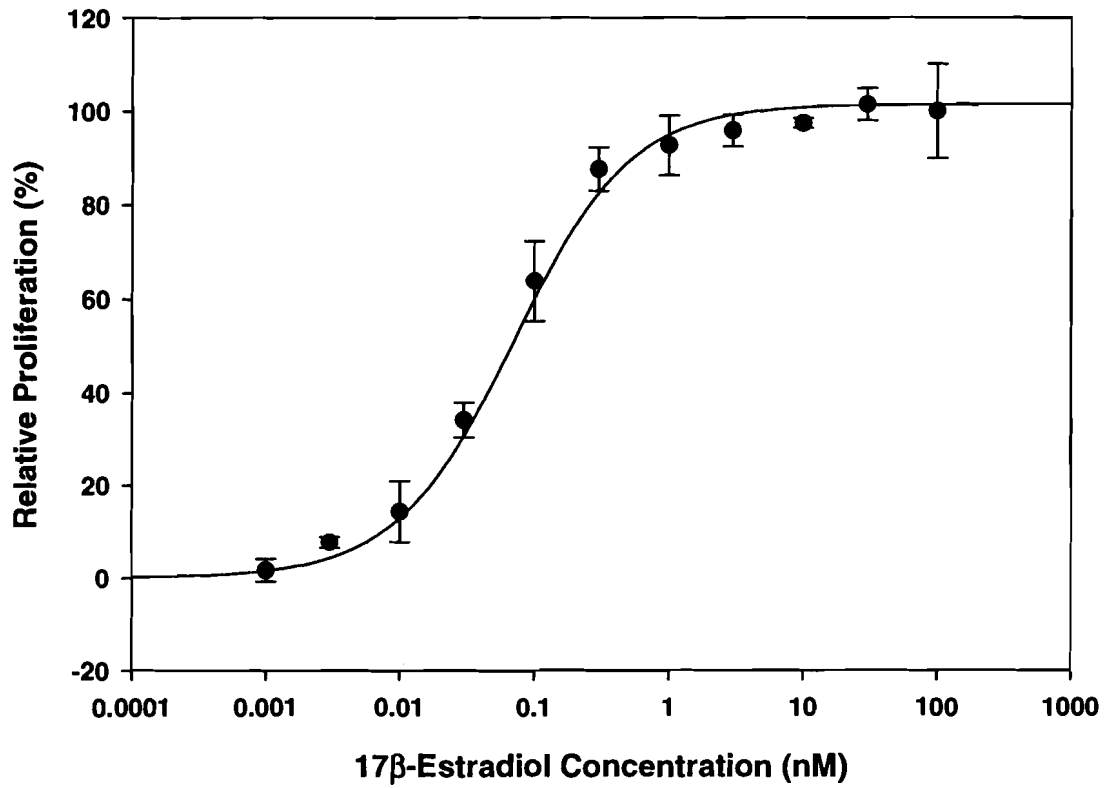


Figure 3.3. Typical E2 dose-response curve obtained using the E-Screen assay described by Soto et al., 1995.

The results from 30 replications are summarized in Table 3.1. They confirmed our initial observations on the assays: while the two yeast bioassays data are similar, the No Observable Effect Level (NOEL) and the Lowest Observable Effect Level (LOEL) of the E-Screen bioassay are ten times lower than those of the yeast assays. However, as noted above, in the dose-response analysis, the maximal effect of the E-Screen bioassay is about 30 times lower than the yeast assays as well.

**Table 3.1. NOEL, LOEL, EC<sub>50</sub> and Maximal Effect Concentration (MAX) values of E2 Dose-Response Curve Analysis for the three assays (n = 30).**

ERTA Assay	EC <sub>50</sub> ± STD	NOEL	LOEL	MAX
E-Screen	53.2 ± 7.2 pM	3 pM	10 pM	1000 pM
Gaido YES	242 ± 28 pM	30 pM	100 pM	30000 pM
Sumpter YES	203 ± 67 pM	30 pM	100 pM	30000 pM

### 3.1.2 Reference Estrogens

A comparison of the dose-response curves of several other estrogenic chemicals provided further evidence on the differences among the bioassays, while yielding valuable inter-assay comparison data for assay validation. The EC<sub>50</sub> values for a battery of reference estrogens are listed in Table 3.2. The variation amongst assays is large but never >5-fold.

**Table 3.2. EC<sub>50</sub> Values (in Molar) for Selected Reference Estrogen Standards from the Three Bioassays (n=4).**

Chemical Name	E-Screen	Gaido YES	Sumpter YES
Estrone (E1)	50 x10 <sup>-10</sup>	10 x10 <sup>-10</sup>	12 x10 <sup>-10</sup>
17α-Estradiol (αE2)	0.5 x10 <sup>-10</sup>	3.0 x10 <sup>-10</sup>	2.5 x10 <sup>-10</sup>
17β-Estradiol (E2)	0.5 x10 <sup>-10</sup>	2.4 x10 <sup>-10</sup>	2.0 x10 <sup>-10</sup>
Estriol (E3)	100 x10 <sup>-10</sup>	50 x10 <sup>-10</sup>	40 x10 <sup>-10</sup>
17α-Ethynylestradiol (EE2)	0.08 x10 <sup>-10</sup>	0.2 x10 <sup>-10</sup>	0.3 x10 <sup>-10</sup>
19-Norethindrone (NET)	3.0 x10 <sup>-12</sup>	30 x10 <sup>-12</sup>	30 x10 <sup>-12</sup>
Bisphenol A (BPA)	3.0 x10 <sup>-12</sup>	30 x10 <sup>-12</sup>	30 x10 <sup>-12</sup>
Diethyl phthalate	3.0 x10 <sup>-12</sup>	30 x10 <sup>-12</sup>	30 x10 <sup>-12</sup>
Methylethienolone (R1881)	3.0 x10 <sup>-12</sup>	30 x10 <sup>-12</sup>	30 x10 <sup>-12</sup>

### 3.1.3 Bioassay Comparisons using a subset of composite WWTP samples.

#### 3.1.3.1 Bioassay Analysis and Bioassay Estradiol Equivalent Quotients (EEQ).

Although the bioassays were able to detect estrogenic activities in most of the subset of 10 WWTP samples, there was variation in results between assays. The EEQ values of the 10 composite WWTP samples, obtained from their respective E2 reference dilutions series were obtained from quadruplicate sample analysis in the each assay (Table 3.3). A subset of these samples also was selected for HR-GCMS chemical analysis.

**Table 3.3. Bioassay EEQ Values (Molar) for Ten Composite WWTP Samples.**

WWTP	Type	E-Screen	Gaido YES	Sumpter YES
Annacis	Influent	$3.15 \times 10^{-11}$	$5.51 \times 10^{-11}$	$5.89 \times 10^{-11}$
Annacis	Effluent	$5.15 \times 10^{-9}$	$5.70 \times 10^{-9}$	$4.81 \times 10^{-9}$
Iona	Influent	$2.51 \times 10^{-10}$	$1.31 \times 10^{-10}$	$3.00 \times 10^{-10}$
Iona	Effluent	$4.60 \times 10^{-10}$	$9.56 \times 10^{-11}$	$3.29 \times 10^{-10}$
Lulu	Influent	$1.28 \times 10^{-10}$	$7.98 \times 10^{-11}$	$1.64 \times 10^{-10}$
Lulu	Effluent	$4.36 \times 10^{-10}$	$5.10 \times 10^{-10}$	$8.14 \times 10^{-10}$
Langley	Influent	$8.48 \times 10^{-11}$	$1.11 \times 10^{-10}$	$2.55 \times 10^{-10}$
Langley	Effluent	$7.50 \times 10^{-11}$	$9.33 \times 10^{-11}$	$1.28 \times 10^{-10}$
Lion's Gate	Influent	$3.08 \times 10^{-10}$	$4.07 \times 10^{-10}$	$3.00 \times 10^{-10}$
Lion's Gate	Effluent	$5.15 \times 10^{-10}$	$2.80 \times 10^{-10}$	$3.29 \times 10^{-10}$

Table 3.3 reveals that the three bioassays have comparable bioassay EEQ values for each sample; however, only mild correlation was observed among the bioassays with the following correlation coefficient,  $R^2$  values: YES (Gaido) EEQ versus YES (Sumpter) EEQ,  $R^2=0.56$ ; YES (Gaido) EEQ versus E-Screen (Soto) EEQ,  $R^2=0.55$ ; YES (Sumpter) versus E-Screen (Soto) EEQ,  $R^2=0.31$ . It is not surprising that these assays did not correlate highly with each other because of the logarithmic plot of the dose-response curve from which EEQ values are derived. As a result, a slight variation in the slope of the dose-response curve may result in an order of magnitude difference in activity, and the corresponding predicted EEQ values.

#### **3.1.3.2 Chemical Analysis of 10 WWTP Samples.**

Only 10 WWTP samples were selected for chemical analysis. The GC-HRMS analytical method (Ikonomou *et al.* 2006) permitted simultaneous determination of 14 free EDCs including the natural and synthetic estrogens (see Table I for target EDCs analysed). Among the 14 analytes measured, 7 were consistently detected in all the samples. They are presented in Table 3.4.

**Table 3.4. Target EDC concentrations measured by GC-HRMS in influent and effluent WWTP samples. Full chemical names provided in Table 3.5 (Note: Several chemicals were not included in this table because they were below the LOD. Mestranol concentrations were below detection in all samples other than WWTP 1 effluent; where it was found to be  $2.10 \times 10^{-11}$  M).**

WWTP	Steroidal EDCs (M)				Industrial EDCs (M)			
	Influent	E1	E2	E3	NET	NPs	BPA	DEHP
	1	$1.21 \times 10^{-11}$	$4.41 \times 10^{-12}$	$3.34 \times 10^{-11}$	$5.58 \times 10^{-11}$	$3.56 \times 10^{-9}$	$1.64 \times 10^{-10}$	$5.19 \times 10^{-9}$
	2	$2.13 \times 10^{-11}$	$6.98 \times 10^{-12}$	$3.38 \times 10^{-11}$	$5.44 \times 10^{-11}$	$2.50 \times 10^{-9}$	$1.54 \times 10^{-10}$	$4.97 \times 10^{-9}$
	3	$1.25 \times 10^{-11}$	$5.51 \times 10^{-12}$	$3.75 \times 10^{-11}$	$4.78 \times 10^{-11}$	$2.38 \times 10^{-9}$	$1.88 \times 10^{-10}$	$3.32 \times 10^{-8}$
	4	$2.09 \times 10^{-11}$	$7.35 \times 10^{-13}$	$4.56 \times 10^{-11}$	$4.89 \times 10^{-11}$	$5.56 \times 10^{-9}$	$2.64 \times 10^{-10}$	$3.50 \times 10^{-8}$
	5	$3.09 \times 10^{-11}$	$6.98 \times 10^{-12}$	$4.30 \times 10^{-11}$	$7.27 \times 10^{-11}$	$2.42 \times 10^{-9}$	$2.47 \times 10^{-10}$	$3.38 \times 10^{-8}$
Effluent	E1	E2	E3	NET	NPs	BPA	DEHP	
	1	$9.99 \times 10^{-11}$	$4.11 \times 10^{-11}$	$1.80 \times 10^{-11}$	$5.18 \times 10^{-11}$	$4.73 \times 10^{-9}$	$2.24 \times 10^{-10}$	$3.57 \times 10^{-9}$
	2	$2.17 \times 10^{-11}$	$7.35 \times 10^{-12}$	$3.27 \times 10^{-11}$	$5.22 \times 10^{-11}$	$2.17 \times 10^{-9}$	$1.67 \times 10^{-10}$	$5.04 \times 10^{-9}$
	3	$8.85 \times 10^{-11}$	$2.57 \times 10^{-12}$	$1.80 \times 10^{-11}$	$3.82 \times 10^{-11}$	$1.15 \times 10^{-9}$	$6.36 \times 10^{-11}$	$8.08 \times 10^{-9}$
	4	$4.78 \times 10^{-12}$	$3.67 \times 10^{-13}$	$1.80 \times 10^{-11}$	$4.00 \times 10^{-11}$	$7.62 \times 10^{-10}$	$1.07 \times 10^{-11}$	$3.42 \times 10^{-8}$
	5	$3.20 \times 10^{-11}$	$6.25 \times 10^{-12}$	$3.01 \times 10^{-11}$	$8.38 \times 10^{-11}$	$2.28 \times 10^{-9}$	$2.81 \times 10^{-10}$	$3.39 \times 10^{-8}$

### 3.1.3.3 Chemical E2 Equivalent Quotients (EEQs)

The EEF is the EC<sub>50</sub> of the test substance divided by the EC<sub>50</sub> of 17β-Estradiol in the same assay. As most of the chemicals found in the WWTP samples were also selected as target reference estrogens, the bioassay EEF values could be derived directly from my experimental results (Chapter 2; Table 2.2); otherwise, data from literature was used to calculate the bioassay EEF for di(2-ethylhexyl) phthalate. Table 3.5 lists the EEF values derived from the E-Screen and Yes bioassays.

**Table 3.5. Bioassay Estradiol Equivalent Factors (EEF).**

Chemical Name	E-Screen	Gaido YES	Sumpter YES	Mean EEF
Estrone (E1)	0.01	0.24	0.17	0.14
17α-Estradiol (αE2)	1.00	0.80	0.80	0.87
17β-Estradiol (E2)	1.00	1.00	1.00	1.00
Estriol (E3)	0.005	0.005	0.05	0.02
17α-Ethynylestradiol (EE2)	6.25	12.00	6.67	8.3
19-Norethindrone (NET)	<0.005	<0.005	<0.005	<0.005
Bisphenol A (BPA)	<0.005	<0.005	<0.005	<0.005
Diethyl phthalate	<0.005	<0.005	<0.005	<0.005
(R1881) methyltrienolone	<0.005	<0.005	<0.005	<0.005
Nonylphenol (NP)	<0.005	<0.005	<0.005	<0.005
Bisphenol A (BPA)	<0.005	<0.005	<0.005	<0.005
di(2-ethylhexyl) phthalate (DEHP)	<0.005	<0.005	<0.005	<0.005



It should be noted that the chemicals listed in table 3.5 were selected based on the Fisheries and Oceans Canada (FOC) chemicals of potential concern list used in the HR-GCMS analysis. Since some reference chemicals were analysed prior to and following the HR-GCMS analyses, chemicals below the LOD of the HR-GCMS method were not evaluated by the bioassays, thus no EEF value was produced.

Relative potency of estrogens varies greatly in the literature; therefore, this data was collected by running all the reference chemicals and compared to those values reported by the authors of our assays. A compiled listing of EEF values based on our reference chemical analyses is provided in Table 3.7. The relative estrogenicity exhibited by reference chemicals, in this study, generally corresponded with those available in the literature (NIEHS, 2002; Appendix D).

**Table 3.6. EEQ Values, in the WWTP samples, were calculated as the sum of individual chemical concentrations multiplied by their respective EEf.**

WWTP		EEQs of Individual Steroids (M)			EEQs of Individual Industrial Chemicals (M)		
Influent	E1	E2	E3	NET	NPs	BPA	DEHP
1	$1.70 \times 10^{-12}$	$4.41 \times 10^{-12}$	$6.69 \times 10^{-13}$	$2.79 \times 10^{-13}$	$1.78 \times 10^{-11}$	$8.19 \times 10^{-13}$	$2.59 \times 10^{-11}$
2	$2.98 \times 10^{-12}$	$6.98 \times 10^{-12}$	$6.76 \times 10^{-13}$	$2.72 \times 10^{-13}$	$1.25 \times 10^{-11}$	$7.70 \times 10^{-13}$	$2.49 \times 10^{-11}$
3	$1.75 \times 10^{-12}$	$5.51 \times 10^{-12}$	$7.49 \times 10^{-13}$	$2.39 \times 10^{-13}$	$1.19 \times 10^{-11}$	$9.40 \times 10^{-13}$	$1.66 \times 10^{-10}$
4	$2.93 \times 10^{-12}$	$7.35 \times 10^{-13}$	$9.11 \times 10^{-13}$	$2.44 \times 10^{-13}$	$2.78 \times 10^{-11}$	$1.32 \times 10^{-12}$	$1.75 \times 10^{-10}$
5	$4.32 \times 10^{-12}$	$6.98 \times 10^{-12}$	$8.60 \times 10^{-13}$	$3.64 \times 10^{-13}$	$1.21 \times 10^{-11}$	$1.23 \times 10^{-12}$	$1.69 \times 10^{-10}$
Effluent	E1	E2	E3	NET	NPs	BPA	DEHP
1	$1.40 \times 10^{-11}$	$4.11 \times 10^{-11}$	$3.60 \times 10^{-13}$	$2.59 \times 10^{-13}$	$2.36 \times 10^{-11}$	$1.12 \times 10^{-12}$	$1.78 \times 10^{-11}$
2	$3.03 \times 10^{-12}$	$7.35 \times 10^{-12}$	$6.54 \times 10^{-13}$	$2.61 \times 10^{-13}$	$1.09 \times 10^{-11}$	$8.34 \times 10^{-13}$	$2.52 \times 10^{-11}$
3	$1.24 \times 10^{-11}$	$2.57 \times 10^{-12}$	$3.60 \times 10^{-13}$	$1.91 \times 10^{-13}$	$5.76 \times 10^{-12}$	$3.18 \times 10^{-13}$	$4.04 \times 10^{-11}$
4	$6.69 \times 10^{-13}$	$3.67 \times 10^{-13}$	$3.60 \times 10^{-13}$	$2.00 \times 10^{-13}$	$3.81 \times 10^{-12}$	$5.33 \times 10^{-14}$	$1.71 \times 10^{-10}$
5	$4.47 \times 10^{-12}$	$6.25 \times 10^{-12}$	$6.02 \times 10^{-13}$	$4.19 \times 10^{-13}$	$1.14 \times 10^{-11}$	$1.40 \times 10^{-12}$	$1.70 \times 10^{-10}$

### 3.1.3.4 Bioassay-derived and chemical analysis-derived EEQ.

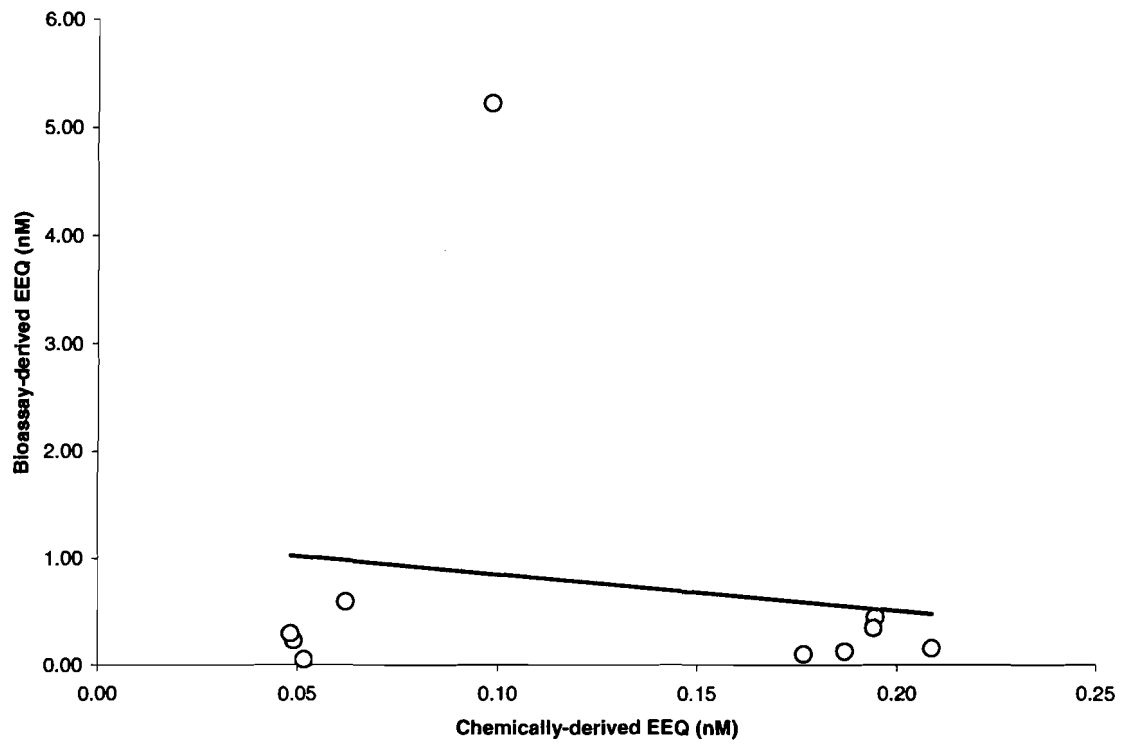
Chemical and bioassay analysis were performed on the same 10 composite WWTP samples to compare the two approaches of EEQ estimation. Estrogen concentrations determined by GC-HRMS, were converted to EEQ using the estrogen equivalent factors (EEF) reported in the literature. The bioassays data were compared with the chemical analysis data using the correlation between the EEQ obtained by a bioassay and the EEQ calculated from the chemical analysis data for the same sample.

**Table 3.7. Total chemically-derived EEQ, steroid-only chemically-derived EEQ and mean bioassay-derived EEQ n = 10).**

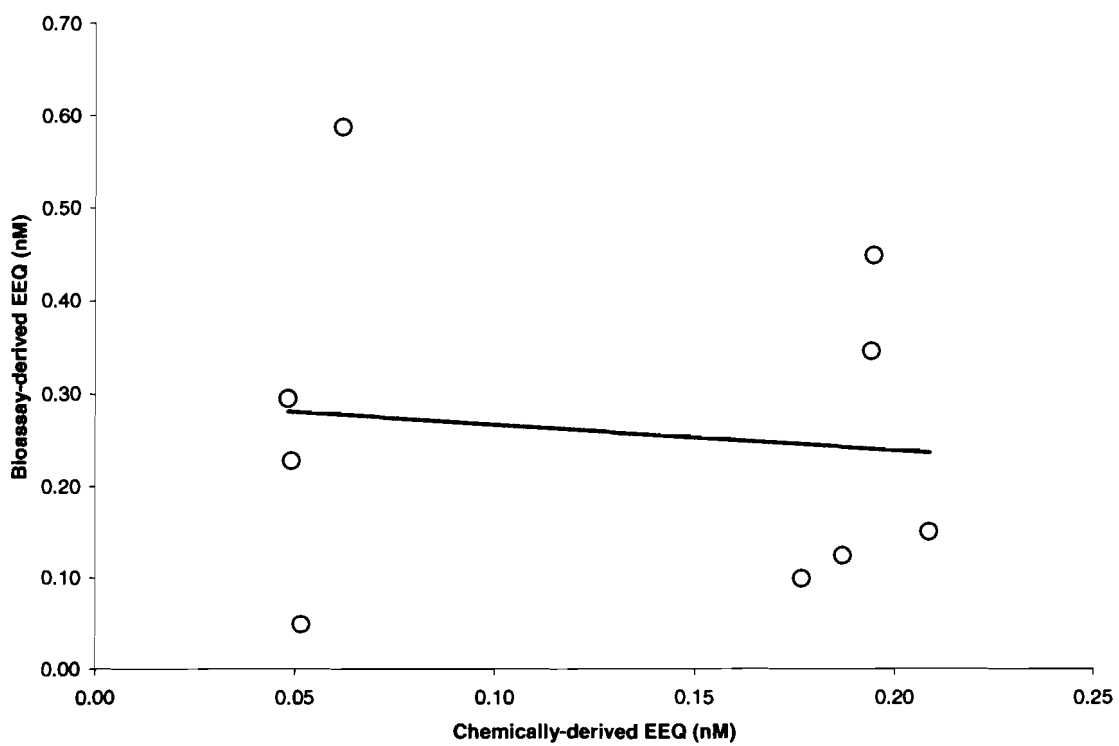
	WWTP	Total Chemical EEQ (M)	Steroid-only EEQ (M)	Mean Bioassay EEQ (M)
<b>Influent</b>	1	$5.16 \times 10^{-11}$	$7.05 \times 10^{-12}$	$4.85 \times 10^{-11}$
	2	$4.90 \times 10^{-11}$	$1.09 \times 10^{-11}$	$2.27 \times 10^{-10}$
	3	$1.87 \times 10^{-10}$	$8.25 \times 10^{-12}$	$1.24 \times 10^{-10}$
	4	$2.09 \times 10^{-10}$	$4.82 \times 10^{-12}$	$1.50 \times 10^{-10}$
	5	$1.95 \times 10^{-10}$	$1.25 \times 10^{-11}$	$4.49 \times 10^{-10}$
<b>Effluent</b>	1	$9.84 \times 10^{-11}$	$5.58 \times 10^{-11}$	$5.22 \times 10^{-9}$
	2	$4.82 \times 10^{-11}$	$1.13 \times 10^{-11}$	$2.95 \times 10^{-10}$
	3	$6.20 \times 10^{-11}$	$1.55 \times 10^{-11}$	$5.87 \times 10^{-10}$
	4	$1.77 \times 10^{-10}$	$1.60 \times 10^{-12}$	$9.89 \times 10^{-11}$
	5	$1.94 \times 10^{-10}$	$1.17 \times 10^{-11}$	$3.45 \times 10^{-10}$

No correlation could be found between the total of EEQs derived from chemical analysis and the EEQs derived from the ERTA bioassay of individual chemicals. The

total EEQ derived from chemical analysis also did not correlate with the mean EEQ derived from the bioassays (Figures 3.4 and 3.5). Industrial EDCs had very low EEQ values; they generally were at or below the detection limits of the bioassays, although the concentrations of industrial EDCs were very high. Indeed, industrial EDCs are four orders of magnitude greater in concentration than any of the steroidal chemicals. Despite applying the assay's limit of detection as the EEQ for the industrial chemicals, the EEQ derived from chemical analysis of the industrial chemical are comparable with the EEQ derived from chemical analysis of the steroidal hormones in certain samples (Table 3.4, 3.5, and 3.6).

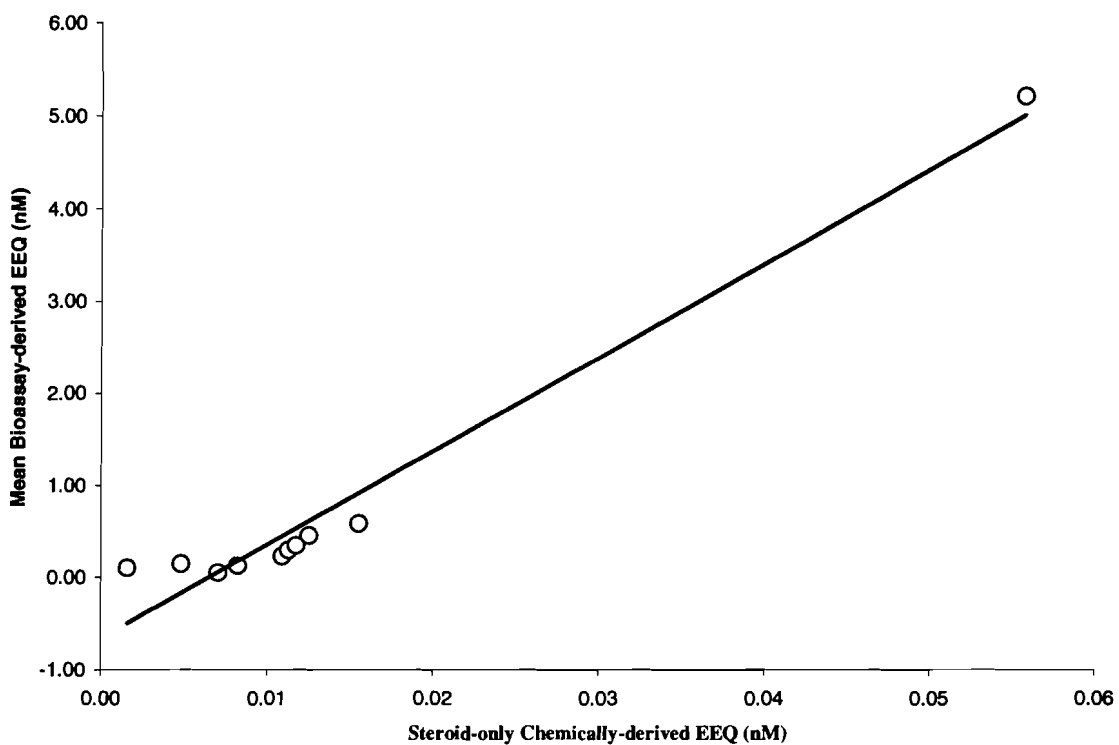


**Figure 3.4. Correlation between bioassay-derived EEQ and chemical analysis derived EEQ (n = 10). (R<sup>2</sup> = 0.02)**

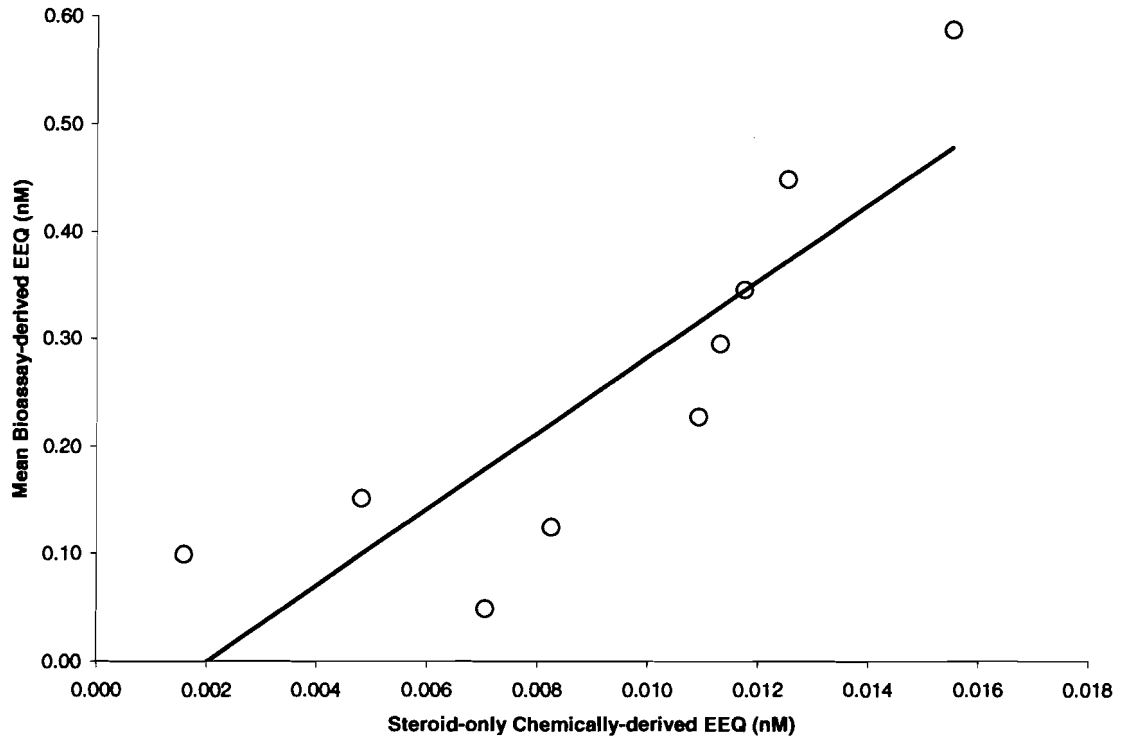


**Figure 3.5. Correlation between bioassay-derived EEQ and chemical analysis derived EEQ. 10 samples, one outlier removed. ( $R^2 = 0.01$ )**

A comparison of the steroid-only chemical-derived EEQ and bioassay-derived EEQ shows good correlation, despite a difference of an order of magnitude in the values. The raw data has a correlation coefficient,  $R^2 = 0.97$ . After removal of an outlier,  $R^2$  is reduced to 0.73 (Figures 3.6 and 3.7).



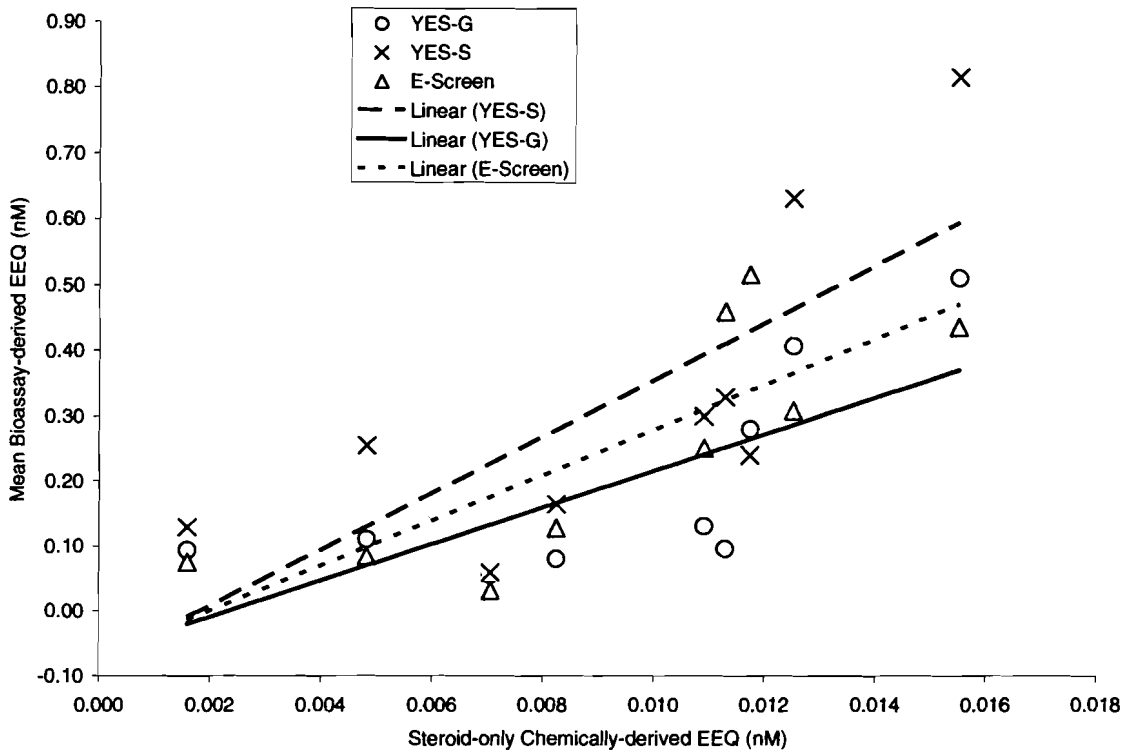
**Figure 3.6. Bioassay-derived EEQ versus Steroid-only Chemically-derived EEQ. ( $R^2 = 0.97$ ;  $n = 10$ )**



**Figure 3.7. Bioassay-derived EEQ versus Steroid-only Chemically-derived EEQ; Outlier removed,  $R^2 = 0.73$ . (n = 9, since an outlier was removed).**

The following linear regression correlations were produced: Chemical analysis-derived EEQ *versus* Gaido's YES EEQ,  $R^2=0.54$ ; Chemical analysis-derived EEQ *versus* Sumpter's YES EEQ,  $R^2=0.58$ ; Chemical analysis-based EEQ *versus* the Soto's YES EEQ,  $R^2=0.65$  (Figure 3.8). Despite a relatively strong correlation between steroid-only chemically-derived EEQ values with the mean bioassay EEQ values, there is approximately one to two-orders of magnitude difference. The mean bioassay-derived EEQ has a better overall fit (regression coefficient,  $R^2 = 0.73$ ) with the total steroid EEQ than the individual assays after the outlier was removed from the plots (Figures 3.6 and 3.7).





**Figure 3.8. Individual Assay EEQ versus Steroid EEQ (n = 9, outlier removed as per figures 3.6 and 3.7). O = Gaido; X = Sumpter; Δ = E-Screen. Gaido  $R^2 = 0.54$ ; Sumpter  $R^2 = 0.56$ ; E-Screen  $R^2 = 0.65$ . Regression lines: Gaido = solid line; Sumpter = broken line; E-Screen = segmented line.**

It is interesting that total steroid EEQ and chemical analysis-based EEQ are consistently about an order of magnitude lower in activity relative to the mean of the bioassay-derived EEQ (Figures 3.9 and 3.10). Inclusion of the industrial chemicals did not significantly change the WWTP sample's chemically-derived EEQ values, and there is no correlation with the bioassay-derived EEQ. The order of magnitude difference between chemically-derived EEQ and bioassay-derived EEQ is reduced with the inclusion of the industrial chemicals' EEQ.

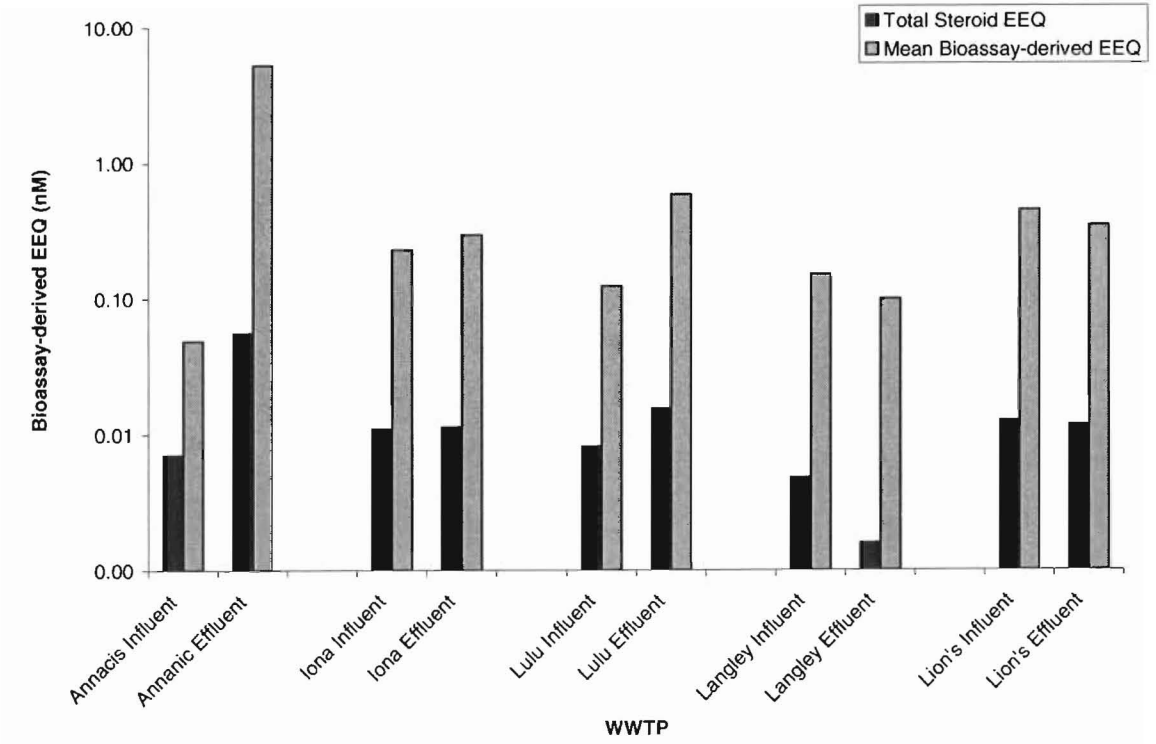


Figure 3.9. Mean bioassay-derived EEQ versus total steroid-only chemically-derived EEQ.

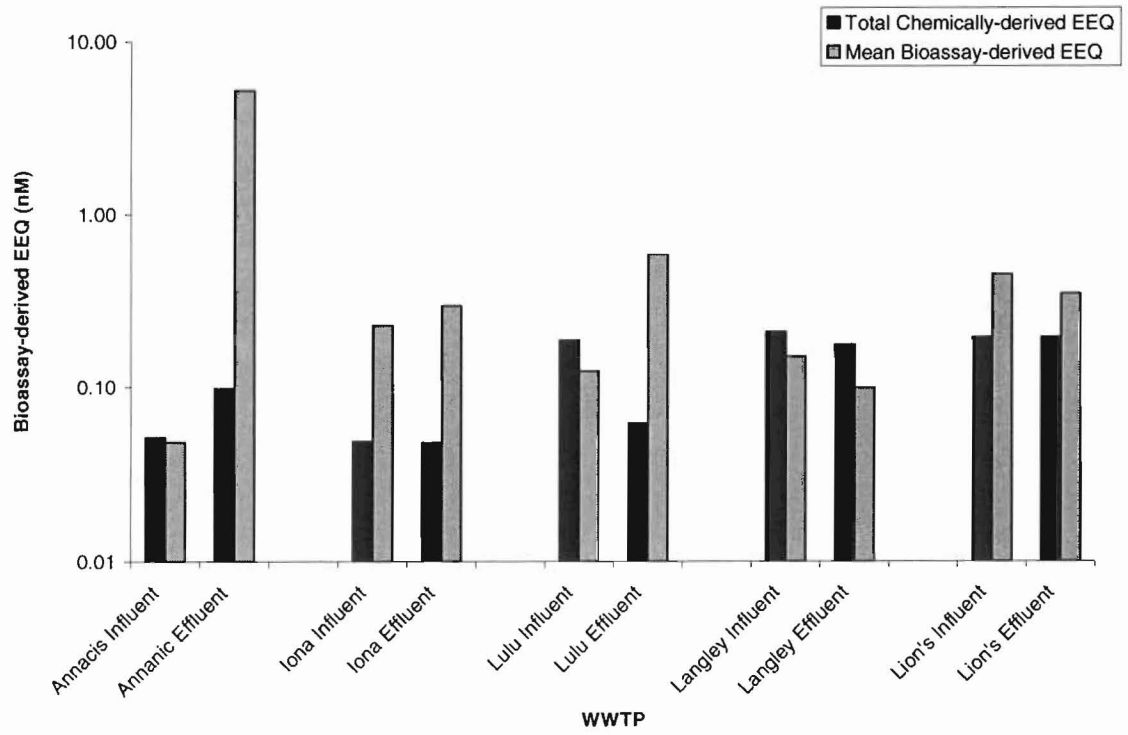


Figure 3.10. Mean bioassay-derived EEQ versus total chemically-derived EEQ.

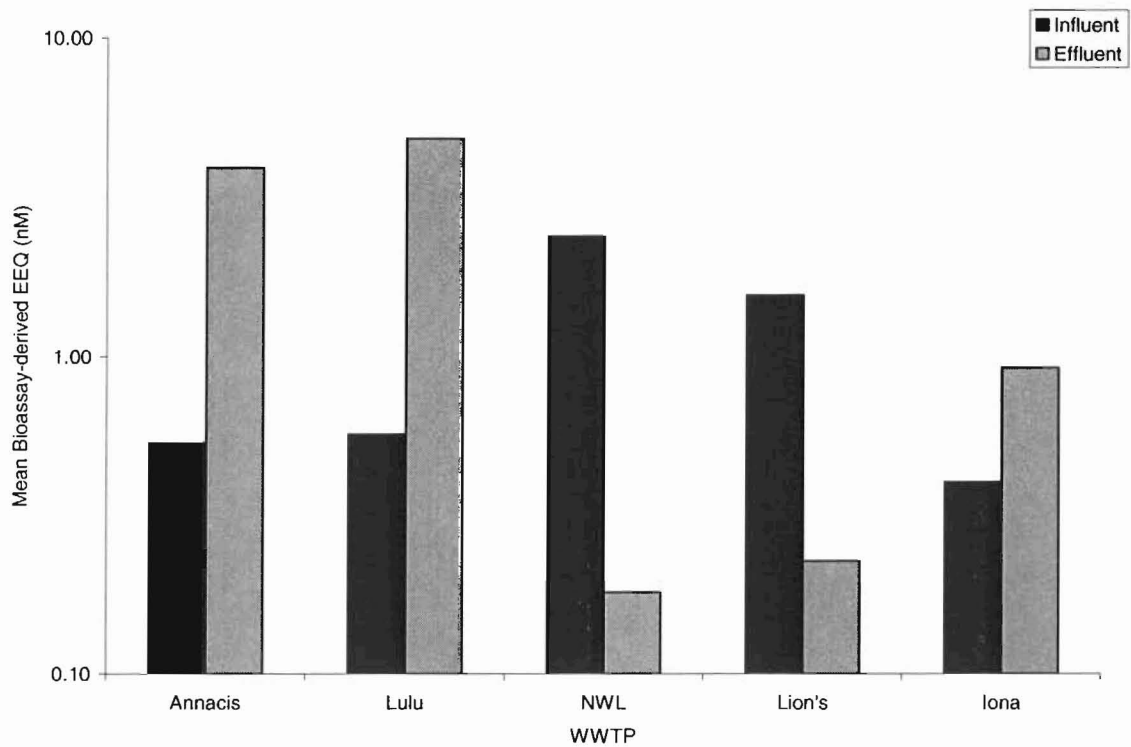
## 3.2 WWTP Assessment

### 3.2.1 Estrogenicity of WWTP samples.

Bioassay-derived EEQ and chemical analysis-derived EEQ also were plotted separately against the WWTP site of sampling (Figures 3.9 and 3.10). Several observations can be made regarding the data: (i) chemical analysis-derived EEQs in almost all cases were much lower than the bioassay-derived EEQs in all WWTP samples tested, (ii) influent EEQ are not consistently higher than effluent EEQ in the WWTP. Indeed, WWTPs 1 (Annacis Island), 2 (Iona) and 3 (Lulu Island) show lower influent EEQs than effluent EEQs in both the GC-HRMS analysis and the bioassays, (iii) the GC-HRMS derived EEQ assumes additivity of the analytes (Figures 3.9 and 3.10). Since the EEQs are calculated by EEF of Korner et al. (1999), the presence of a large concentration of a weak estrogenic compound may not be as important as a small amount of a very potent estrogenic compound, and (iv) the WWTP using secondary treatment (WWTPs 1, 3 and 4) do not always show lower estrogenic activities in the effluent samples with respect to WWTP using primary treatment (WWTPs 2 and 5). Since the sample size was small (N=4), large deviation was observed amongst the results. Moreover, statistical analyses (one way ANOVA) did not reveal any significant differences ( $p < 0.05$ ) among the bioassay-derived EEQs. Based on the results of these studies, WWTPs with secondary treatment are comparable to the WWTPs with primary treatment in removing the EDCs from the influent. Indeed, the EEQ in the wastewater estrogenicity may actually increase after secondary treatment (Figures 3.9 and 3.10).

### 3.2.2 Influent versus Effluent and Treatment Type

Of the two primary WWTPs, Lion's (5) had greater activity in the influent versus the effluent; whereas, Iona (2) had a greater activity in the effluent as compared to the influent (Figure 3.11). In two of the three secondary WWTPs, Annacis and Lulu, the effluent had higher estrogenic activity than the influent (Figure 3.11). In contrast, the influent in the third secondary WWTP, Northwest Langley (NWL), had higher estrogenic activity than the effluent.



**Figure 3.11. Mean Bioassay EEQ values of influent (black bar) and effluent (grey bar) at each WWTP (n =4 for Lulu, NWL, and Lion's; n=5 for Annacis and Iona). Error bars represent the 95% Confidence Interval.**

### 3.2.3 Seasonal Variation at Individual WWTPs

The WWTPs were sampled twice in a year: (a) a subset of composite WWTP samples which were collected to investigate the applicability of the three ERTA assays WWTP influents and effluents and to conduct HR-GCMS analysis, and (b) the WWTPs were sampled again for four times in 2004. Annacis Island and Iona WWTPs were sampled five times.

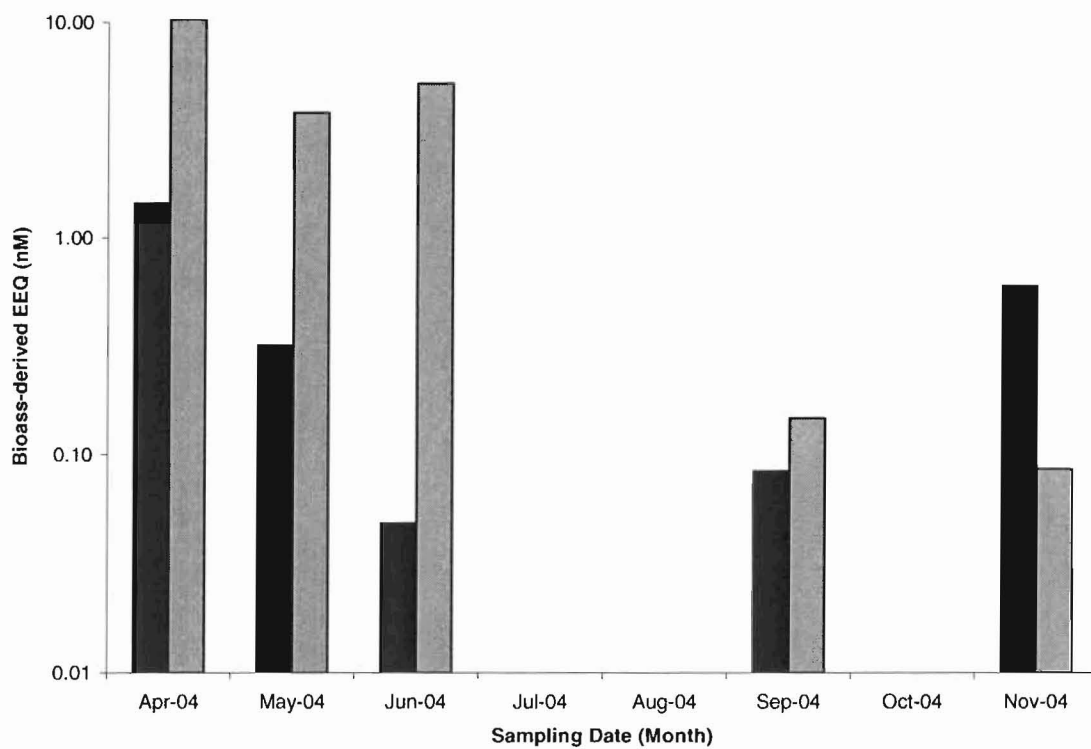
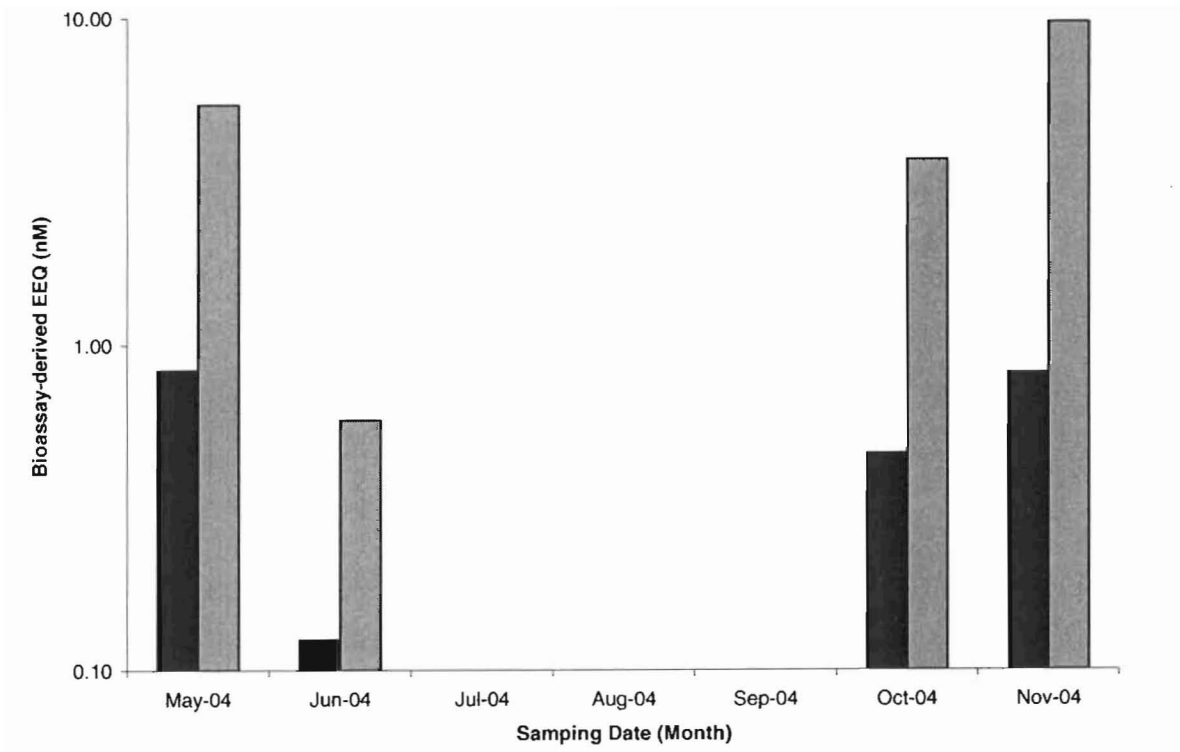


Figure 3.12. Annacis Island WWTP. Influent = Black bar; Effluent = Grey bar.



**Figure 3.13. Lulu Island WWTP. Influent = Black bar; Effluent = Grey bar.**

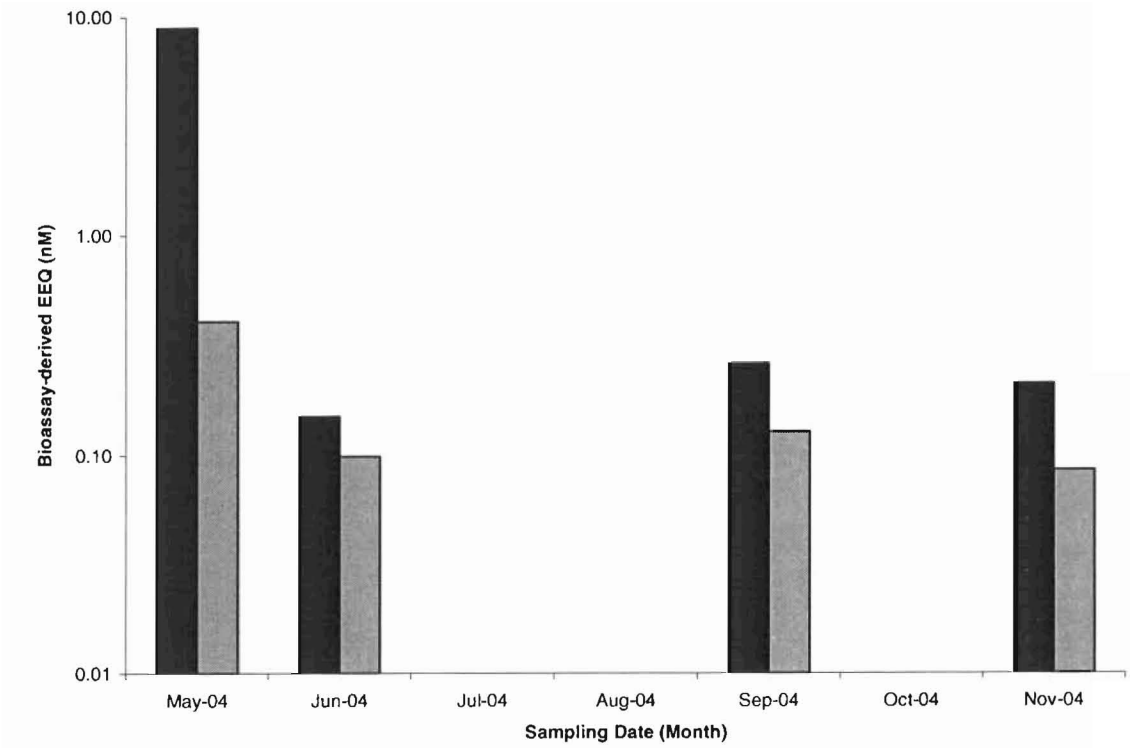
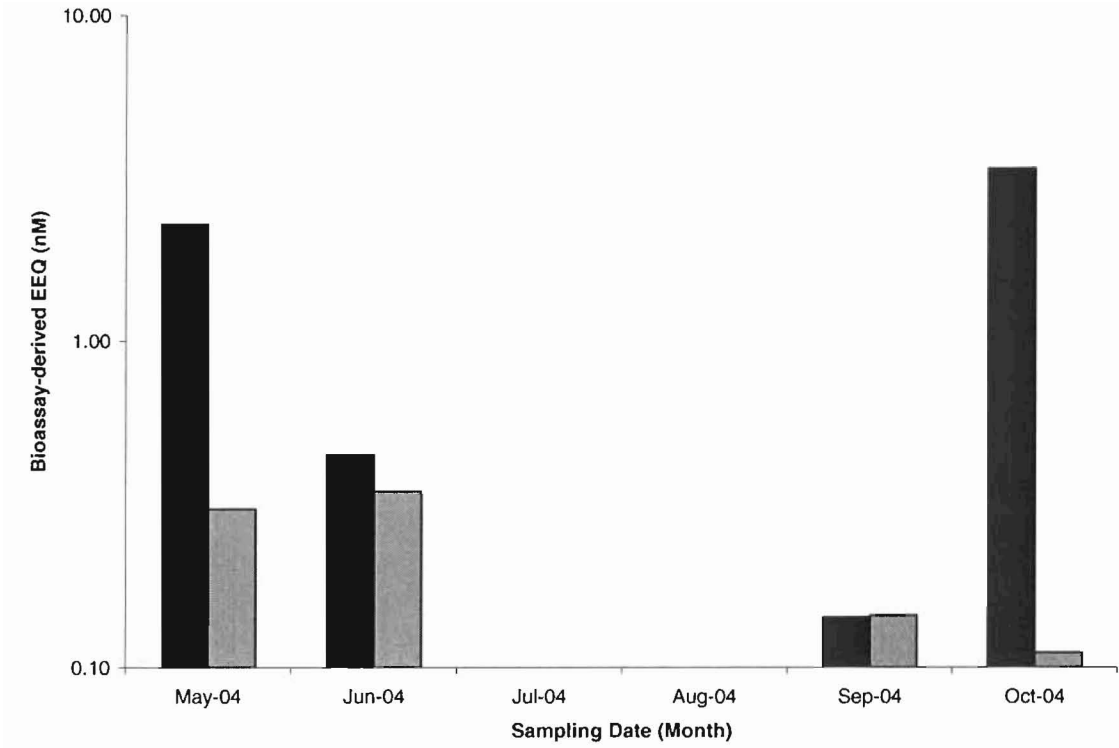


Figure 3.14. Northwest Langley WWTP. Influent = Black bar; Effluent = Grey bar.





**Figure 3.15. Lion's Gate WWTP. Influent = Black bar; Effluent = Grey bar.**

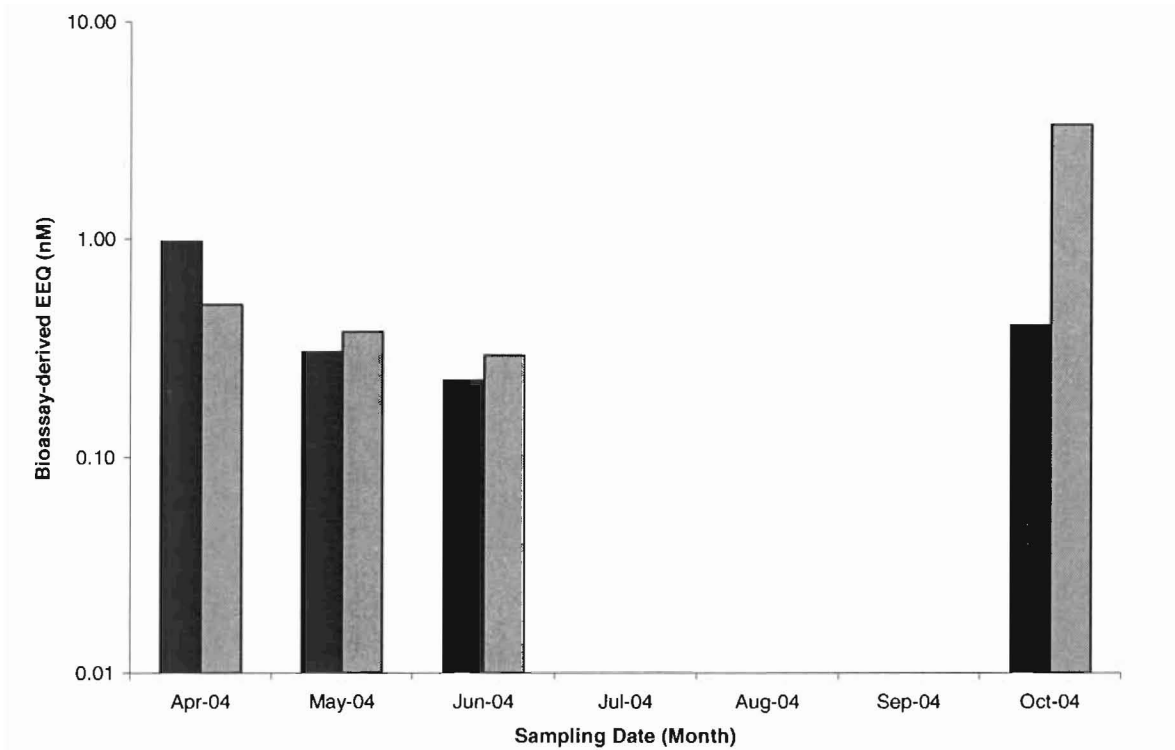


Figure 3.16. Iona WWTP. Influent = Black bar; Effluent = Grey bar.

### 3.3 Degradation Study

The samples were stored at  $-4^{\circ}\text{C}$  and re-analysed each week for 8 weeks, then monthly for 3 months, to ensure accuracy and consistency of sample measurements. All 3 bioassays were used in this investigation, the mean bioassay-derived EEQ was used in the following plots. While using all 3 assays may have increased the variation, the use of the mean bioassay-derived EEQ has been shown to be a more reliable measure of the estrogenicity in environmental samples.

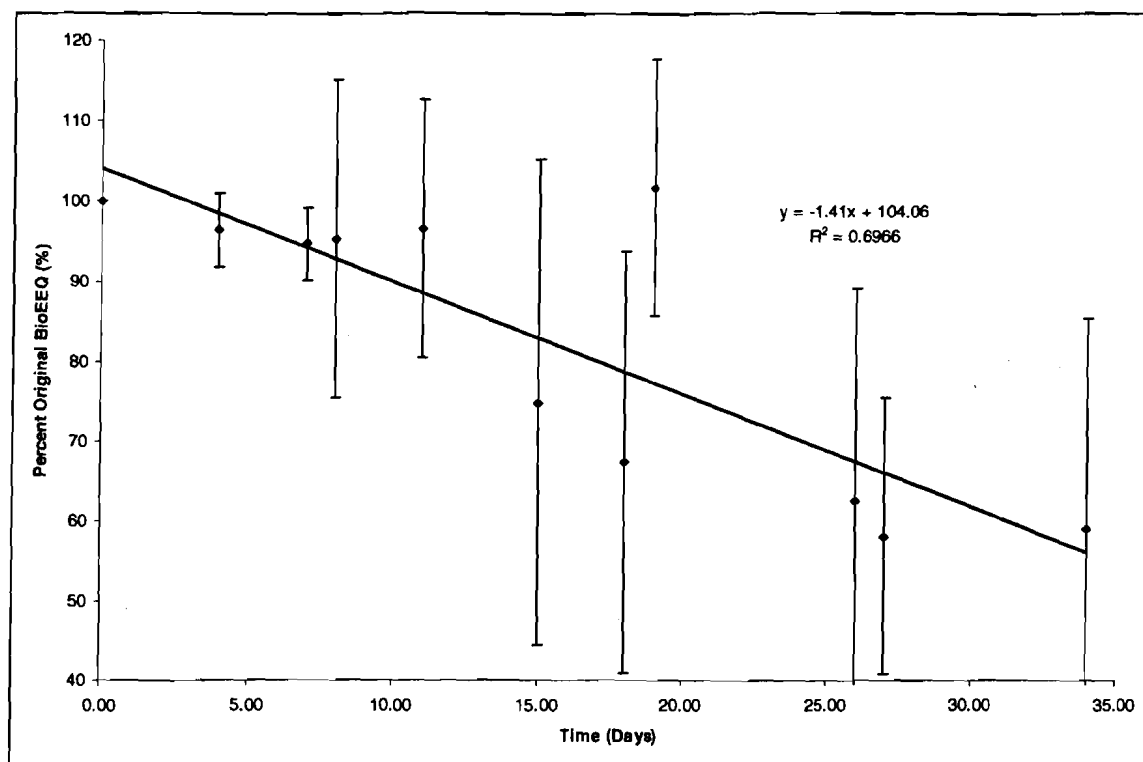


Figure 3.17. Degradation of samples, measured each week.

Handling the sample each week likely had an effect on the degradation. However, some samples experienced much more rapid decay than others and handling was consistent between samples. The following series of plots illustrates the variation between sample degradation; each plot is the degradation of both influent and effluent samples taken from a WWTP, some variation in time span exists due to initial extraction dates and limitations on number of samples per assay and number of assays per day/week. These samples belong to the same subset, which had HR-GCMS analysis.

After two weeks of storage, the EEQ of Langley effluent decreased to almost 20% of its original estrogenicity. Most samples decreased to approximately 60% of their initial estrogenicity after 30 days. Despite large variations amongst samples, the trend is still

strong, with an overall correlation coefficient,  $R^2 = 0.697$  (Figure 3.19). Some samples reach <0.1% of their initial estrogenicity after 2 months (Figure 3.21).

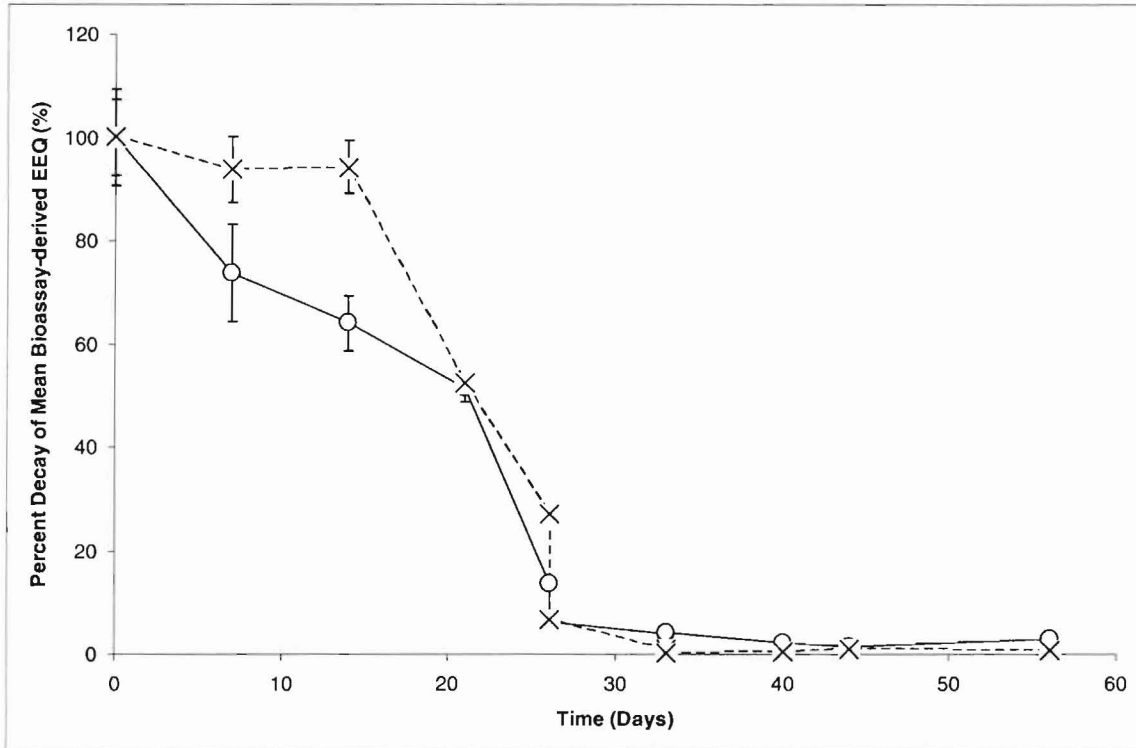


Figure 3.18. This plot shows the decrease of bioassay-derived EEQ for a single grab sample, monitored each week over a span of 2 months (X = Influent; O = Effluent; n = 4).

## 3.4 Environmental Assessment

### 3.4.1 WWTP Receiving Environment

The yeast bioassay showed that there was cytotoxicity in all 5 sediment samples that were collected around the 5 WWTPs. Due to the cytotoxicity to the yeast on the YES assays, accurate absorbance reading could not be obtained. Therefore, the estradiol equivalent concentrations in the sediments were not estimated. These samples showed

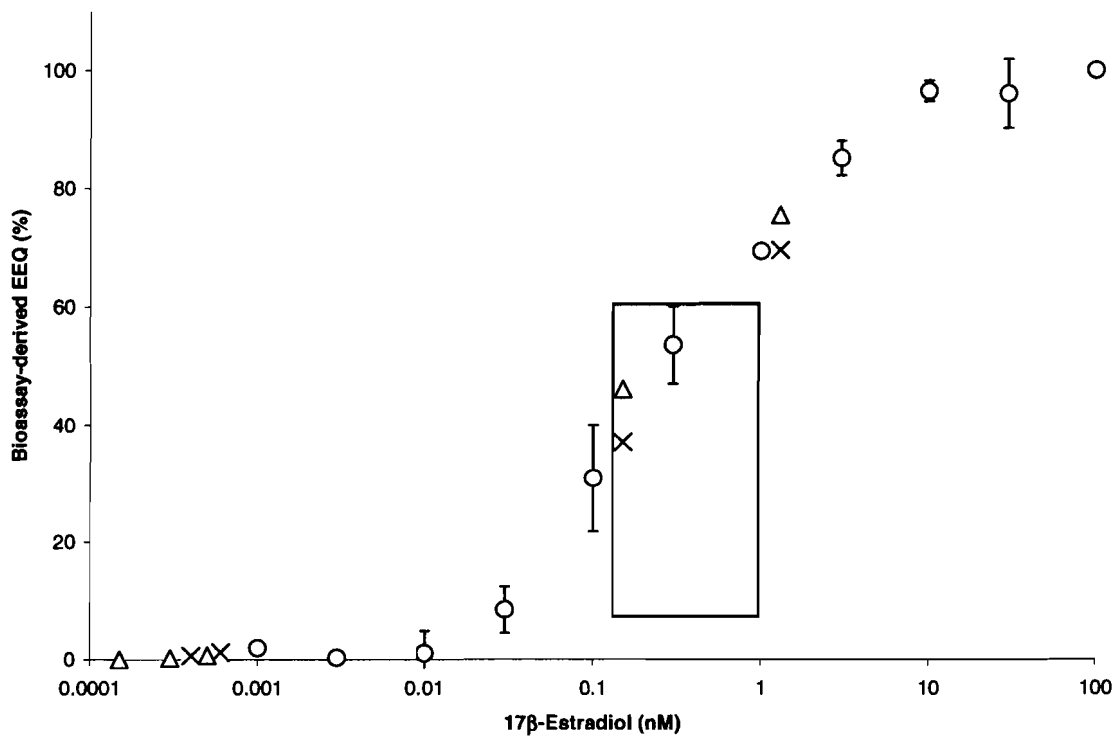
signs of cytotoxicity in the 10-fold dilution. Ten aqueous samples collected from the outfall of WWTP 5 were below detection limit of the assays; although, some samples showed trace activity when concentrated 10-fold.

### **3.4.2 Local Lakes, River and Ocean Environmental Samples**

Measurements on all assays consistently showed that environmental samples had either no activity or undetectable activity. The values were all at or near the lowest detectable concentration of estradiol. Cytotoxicity was present in the samples collected at Rocky Point. No activity was present even when samples were concentrated 10-fold.

## **3.5 Tissue Assessment**

Fish and mussel samples injected with different concentrations of  $17\beta$ -estradiol showed corresponding dose-related estrogenic EEQ values (Figure 3.19). Moreover, the bioassay-derived EEQ values decreased with each consequent dilution. The chinook salmon and rainbow trout appeared to share similar EEQ values for the same doses. The starting concentration for test dose 1 (high log-phase) dose was 10 nM (indicated by the box in figure 3.19); a decrease from 10 nM to 0.1-0.3 nM represents an approximate recovery of 1-3%. Test dose 2 (low log-phase) dose was 1 nM; only background activity levels were produced at this dose. The positive control dose had greater estrogenic activity than the test doses; while the negative controls both had background activity levels.



**Figure 3.19.** 17β-Estradiol equivalent values from injected tissue (O = 17β-Estradiol; X = Chinook salmon; Δ = Rainbow trout).

## **CHAPTER 4 DISCUSSION**

### **4.1 A Comparison of Estrogen-Receptor Transcriptional-Activation (ERTA) Bioassays**

#### **4.1.1 Dose-Response Analysis of Reference Estrogens**

Method validation consists of several key aspects of the assay including the precision or repeatability, and the accuracy or proximity to the true value. The purpose of method validation is to demonstrate that the method is reliable and relevant for the purpose it has been designed. The relevance of the ERTAs lie in their mechanisms, in that they detect EDC activity by means of physical chemical interactions at the ER, resulting in signal transduction pathways which ultimately produces a measurable endpoint. The ERTAs used in the present study are specifically designed to provide a measure of total estrogenic EDC binding. As such, they provide insight as to how much estrogenic activity is present in a sample, but they do not indicate what individual EDC concentrations are or the likelihood of these EDCs entering the bloodstream and tissues of an organism. The mammalian system may be more relevant in interpreting the EDC EEQ values for vertebrate exposures because mammalian cells do not possess cell walls found in yeast (NIEHS, 2002). The primary purpose of these assays, in this research, is to provide reliable information regarding what organisms are exposed to, but not necessarily how the EDCs may interact with organisms.

The first aspect of validation for the three ERTAs is precision *i.e.*, the degree of concurrence within the replicates. This also included repeated measures of identical nominal standards of the E2 dilution series between assays. The dose-response curves of the standard reference chemical, 17 $\beta$ -estradiol (E2) of the 3 assays are found to be highly reproducible (Figures 3.1, 3.2, and 3.3; Table 3.1) and are consistent with those reported in the assay protocols (Soto et al., 1995; Routledge and Sumpter, 1996; Gaido et al., 1997). The individual assays appear to provide reliable inter-laboratory results, provided the laboratories adhere to the same protocol.

The next aspect of the validation is the accuracy of the ERTAs *i.e.*, how close the assays correlate with one another on the dilution series of E2 standard and the resulting dose-response curves. The accuracy of an assay also refers to the agreement between the test result and a reference, which are the NOEL, LOEL, and EC<sub>50</sub> of the E2 dose-response curves. The degree of accuracy between the yeast assays is high, since they have the same NOEL, LOEL and EC<sub>50</sub> values ( $p < 0.05$ ) (see page 48). The yeast assays share the same mechanism and cell line, the only differences are the end-products and the laboratories which first transfected them. The E-Screen, however, does not have the same dose-response curve as the yeast assays ( $p > 0.05$ ). This is not surprising, since the E-Screen uses mammalian cell line with its own cellular machinery and the endpoint measured is natural characteristic of the cell line, growth.

In general, the detection limits of the ERTAs are low and provide a means to detect low levels of EDCs in aquatic organisms. Moreover, these are concentration levels that chemicals analysis is hard pressed to detect. Also, these detection limits are derived



from the total ER binding activities of a mixture of EDCs and they are usually higher than those derived by the chemical methods.

The E-Screen assay appears to be the most sensitive among the ERTAs since its dose-response curve shifts down the concentration range by an order of magnitude, relative to the dose-response curves of the YES assays (Figure 3.1, 3.2, and 3.3). Detection of estrogen activity by the E-screen assay is as low as 10 pM (3 ng/L) for E2 (Table 3.1). The NOEL and LOEL values are 10 times less than those of the YES assays, confirming the E-Screen assay is the most sensitive among the bioassays studied. Also, the EC<sub>50</sub> value of the E-screen assay is 5 times lower than those of the YES assays (Table 3.1). However, the E-Screen assay shows a maximal activity 30 times lower than those of the YES assays, thus limiting the range of estrogenicity prediction by this assay (Table 3.1). It should be noted that the maximal effect level of the E-Screen assay is approximately 3 µg/µL, which is an order of magnitude higher than the E2 concentration that can cause endocrine disruption effects in wildlife.

A dose-response analysis of non-E2 reference estrogens is also performed to further compare the three ERTA assays used in the present study. Also, this serves to compare results with other laboratories in assessing the reliability and accuracy of these assays. These are collaborative studies with Dr. Ikonou at the Institute of Ocean Sciences, Fisheries and Oceans Canada (formerly Department of Fisheries and Oceans), whose laboratory performed the HR-GCMS analysis. Therefore, the 6 estrogenic chemicals selected for the bioassays to obtain the EEf values were based on the results of Ikonou et al. (2006). The concordance of the ERTA assay results were quite consistent with variation of less than 5-fold in predicted estrogenicity (Table 3.2). This inter-assay

variation was also shown to be the case in a comparison of estrogenic activity in environmental samples using two reporter gene effects assays, yeast estrogen screen (YES) and the ER-mediated chemically activated luciferase gene expression (ER-CALUX) assay, as well as the estrogen receptor-binding assay (Murk et al., 2002). The ER-CALUX and YES assays predicted lower estrogenicity compared to the estrogen receptor-binding assay (Murk et al., 2002).

The yeast assays yield similar EEQ values, since they are essentially the same system. In contrast, the E-Screen assay would be expected to vary from the yeast assays consistently. This is likely due to biological differences between the yeast and mammalian cell lines; yeast cells have an additional barrier, cell walls, compared to mammalian cells of the E-Screen. As a result, some EDCs may not be absorbed as readily by yeast (NIEHS, 2002).

Although the ERTA assay results for reference estrogens are consistent, previous studies have shown that they are orders of magnitude different when different procedures, cell lines and constructs are used (NIEHS, 2002). In contrast, the present ERTA assay reference chemical values are quite consistent when using the respective cell line and similar procedures in their studies (Soto, 1995, Gaido et al., 1997; Routledge & Sumpter, 1996). While the  $EC_{50}$  values for different reference estrogens may vary amongst laboratories, the relative potencies (EEF) are consistent with those presented in this study (Table 3.5); EE2 is generally comparable with E2, E1 is slightly less potent than E2, E3 and NET are considerably less potent than E2 and industrial EDCs are generally not detectable or near the limit of detection (NIEHS, 2002). Bioassay-derived EEQ values provide a means to estimate the total endocrine disrupting activity of a complex

environmental mixture, since they may account for the additive and synergistic effects amongst the mixture constituents, thereby providing a meaningful prediction of the estrogenicity of the sample. However, inter-laboratory variation exists, since no ERTA assays mechanisms or standard operating procedures are currently validated for the purpose of environmental screening of estrogenic EDCs (NIEHS, 2002). This study provides information to compare on reference estrogens and unknown EDCs mixture, which helps to further understand the use of these ERTA bioassays in screening for estrogenic EDCs in environmental samples.

#### **4.1.2 Bioassay EEQ versus Chemical EEQ Study**

Wastewater treatment plant influent and effluent samples are complex mixtures, which not only provide a source of estrogenic activity from hormones, but also from industrial and domestic chemicals which cause endocrine disrupting activities. ERTA assay analysis of a subset of composite wastewater treatment plant samples was performed for inter-assay comparison of complex environmental mixtures. The three assays produce similar EEQ values for their respective samples; however, some variation may be up to 5-fold between assays for the same sample (Table 3.3). Due to this variation in results, correlation between assays is weak ( $R^2 = 0.31$  – E-Screen versus Sumpter,  $R^2 = 0.55$  – E-Screen versus Gaido and  $R^2 = 0.56$  – Gaido versus Sumpter). The weak correlations observed may be due to the inherent nature of the dose-response curve relationship since minute changes in concentration may exhibit large changes in activity along the slope of the curve.

The inter-assay variation makes it difficult to determine which assay is predicting the most accurate activity level. Upon comparing arithmetic mean of the bioassay EEQ values, it was observed that the mean bioassay EEQ values versus steroid-only chemically-derived EEQ had a correlation coefficient of 0.73; whereas the individual bioassay EEQ values versus steroid-only chemically-derived EEQ values had correlation coefficients of 0.54 (Gaido), 0.56 (Sumpter), and 0.65 (E-Screen) (Figure 3.7 and 3.8). Apparently, this approach decreases the variation in EEQ value predictions which may be due to assay variation or the method of obtaining the EEQ values *via* the dose-response curve, thus providing more reliable bioassay EEQ values.

Many researchers focus on steroid chemical analysis and seem to ignore the weakly estrogenic industrial EDCs, such as NPs, BPA and DEHP which are typically found in relatively high concentrations (Servos et al., 2004). The 10 WWTP samples examined contained high concentrations of these industrial EDCs, relative to the low concentrations of steroids (Table 3.4). However, bioassay EEQs did not correlate well with the chemically-derived EEQs ( $R^2 = 0.02$ ) based on both industrial EDCs and steroids (Figure 3.5). This may be due to the fact that several samples have very high concentrations of industrial chemicals and despite having EEQ values equal to the limit of detection on the assay, the EEQ may be inaccurately representing these chemicals and skewing some samples EEQ values.

Due to the possible skewing effect of the industrial EDCs, the steroid chemically-derived EEQ values were isolated and produced a very high correlation ( $R^2 = 0.98$ ) with the mean bioassay EEQ values; however, there may be an outlier in this comparison, which decreased the correlation ( $R^2 = 0.73$ ) upon removing it (Figure 3.9). While a good

correlation exists between the steroid-only chemically derived EEQ values and the mean bioassay EEQ values, there's approximately one to two orders of magnitude difference between steroid-only chemically-derived EEQ and mean bioassay-derived EEQ; whereas the total chemically-derived EEQ are generally much closer in EEQ (Figures 3.9 and 3.10). The high correlation between steroids and bioassay EEQ indicate that perhaps the steroids are the main cause of estrogenicity and that they are either exhibiting non-additive effects, or perhaps other chemicals not detected are responsible for the differences. Despite the closer proximity of the total chemically-derived EEQ and bioassay-derived EEQ, the correlation is very poor.

## **4.2 WWTP Assessment**

### **4.2.1 Complexity of WWTP samples**

Results of the present study show that the environmental fate of EDC in the WWTP is complex and no clear patterns associated with the treatment process could be identified. Among the five WWTPs operated by the GVRD, there are two primary and three secondary treatment WWTPs (GVRD, 2004) that discharge, respectively into the marine environment and the Fraser River (Figure 1.3). The primary treatment process is essentially a mechanical process that removes 30-40% of biological oxygen demand (BOD) and 50% of the total suspended solids (TSS). The secondary treatment process includes a biological process that removes up to 90% of BOD and the TSS. Neither the primary nor the secondary treatment processes are specifically designed to remove EDCs or trace organics such as hormones, pharmaceuticals and pesticides.

Estrogens are potent chemicals of which the estrogenic effects on fish can be observed in laboratory studies at 1 ng/L (Routledge et al., 1998). Therefore, although the concentrations of estrogenic chemical in the WWTP samples are low when compared to the non-estrogenic EDCs (Table 3.4), about 34.5%, 49.2%, and 11.4% of the total EEQ values are derived from E1, E2 and E3, respectively. It should be noted that biological conversion of E2 to E1 (Johnson and Sumpter, 2001) also may explain why a relatively high E1 level is found in the effluent samples (Table 3.4). The chemical analysis method employed detects only free or unconjugated estrogens and accounts for most of the estrogenicity in the WWTP samples, but the conjugated estrogens are much less estrogenic than those of the unconjugated forms. Matsui et al., (2000) have reported that E1, E2, E2-3-sulfate, and E2-17- $\beta$ -D-glucuronide show respectively 0.21,  $1.3 \times 10^{-3}$ ,  $5.3 \times 10^{-5}$ , and  $5.9 \times 10^{-7}$  of the activity of E2.

In my study the chemical analysis-derived EEQ are consistently lower than the bioassay-derived EEQ by one to two-orders of magnitude in the same sample. A plausible explanation may be that there are synergistic effects in the bioassays, resulting in higher EEQ measures. Other possible explanation may be that some non-estrogenic EDCs in the sample are not included in the GC-HRMS analysis. In contrast, the bioassays measure the total estrogenicity in the sample. Nevertheless, our results from the analysis of the 10 WWTP samples are in direct contrast to those reported by Korner et al (2001) who report that the EEQ of the effluent samples determined by the E-Screen assay are consistently lower than those of the chemical analysis. An explanation for the discrepancy in results between these two studies is not readily available but probably is related to the number and/or types of EDCs selected for the chemical analysis.

#### **4.2.2 Absence of EE2 in the WWTP samples**

EE2 was not detected in the HR-GCMS analysis of the WWTP samples. EE2 is a synthetic estrogen, which is more potent than E2. Purdom et al. (1994) have reported that an EE2 concentration of 0.1 ng/L can affect the reproductive function of male rainbow trout. The absence of EE2 from our WWTP samples may be related to the low ng/L EE2 concentrations in most of the WWTP effluent samples (Desbrow et al., 1998; Belfroid et al., 1999) because EE2 is not biodegradable in WWTP (Ternes et al., 1999). Baronti et al. (2000) have shown that mean EE2 concentrations in WWTP influent and effluent are 3 ng/L and 0.4 ng/L, respectively. Layton et al. (2000) also have reported that as much as 80% of the EE2 may be bound to the sewage sludge and thus removed from the aqueous phase. Therefore, the absence of EE2 in our WWTP samples most likely is related to the greater hydrophobicity of EE2 which makes it more susceptible to the process of sorption than the other estrogens (Lai et al., 2000).

#### **4.2.3 EEQs in influent and effluent samples**

The bioassay-derived estrogenicity of the influent samples is higher than those in the effluent samples for WWTPs 4 and 5 (Figure 3.11) because primary or secondary treatment can remove some of the EDCs from the influent albeit these treatment processes are not very effective (D'Ascenzo et al., 2003). In contrast, the estrogenic activity of the influent samples in WWTPs 1, 2, and 3 was found to be lower than those in the effluent samples (Figure 3.11). Apparently, active estrogens have been generated during water treatment and similar observations have been reported by Servos et al. (2004) and D'Ascenzo et al. (2003). Indeed, E2 concentrations were found to increase

from raw sewage to primary effluent in a Japanese WWTP before decreasing during biological treatment (Nasu et al., 2001). Baronti et al. (2000) also have shown that E2 levels were higher in effluent than influent.

The high estrogenicity measured in the effluent samples of Annacis Island (1) and Lulu Island (3) WWTPs (Figure 3.11) may be related to a high level of E1 as a result of E2 metabolism or deconjugation of E2 conjugates. This is consistent with the observations that about 18% and 81% of the EEQ in WWTP 1 is due to E1 and E2, respectively, and about 87% and 11.5% of the EEQ in WWTP 3 is due to E1 and E2, respectively. A high level of E1 in the effluent samples also has been attributed to E1-3-sulfate deconjugation during activated sludge treatment (Johnson and Sumpter, 2001). Orme et al. (1983) have shown that most of the estrogenic materials excreted in the human urine are biologically less active conjugates which enter the WWTP. Therefore, our results of a higher EEQ in the effluent samples as compared to influent samples can be explained by the conversion of estrogenic conjugates back to the free or unconjugated forms (D'Ascenzo et al., 2003). Deconjugation is likely conducted by *Escherichia coli*, in the fecal matter, which are known to produce large amounts of  $\beta$ -glucuronidases which in turn contributes to the enzymatic de-conjugation of conjugated EDCs (Dray et al., 1972).

Steroid-only chemically-derived EEQs correlated well with bioassay-derived EEQs; whereas, the total chemically-derived EEQ did not correlate at all with the bioassay-derived EEQs. These results confirm that EEQ calculation (with E2 as the reference) is valid only for chemical isomers in a chemical mixture. Therefore, industrial chemicals which are not isomers of estrogens do not give good correlation whereas E1, E2, and E3 are chemical isomers, and they correlate well.



#### **4.2.4 Seasonal Variation at Individual WWTPs**

Despite what appear to be differences in (Figures 3.12, 3.13, 3.14, 3.15, and 3.16) bioassay EEQ values between samples collected in the months of April to June versus September to October, no significant differences were calculated between sampling dates. This could be improved with increased replicates and focus on individual WWTP samples.

#### **4.3 Degradation Study**

The variation of assay-derived EEQ is large over time (Figure 3.17). This may be caused by assay variation, handling of samples for each assay, and degradation. The effects of assay variation are minimal since a reference set of E2 is run each time with the assay. Also, the dose-response curve of the assay must be consistent with that of a specific assay. Each assay has to meet a minimum acceptable standard where the EC<sub>50</sub> should be within a 5-fold range (Personal communication, Dr. Angela Lorenzen, Dr. Kevin Gaido's laboratory). Therefore, the handling effects are minimized. Moreover, the samples are kept at -40 °C between uses and are above this temperature range for only less than 1 min. Thus, sample degradation seems the most likely explanation for the decrease in activity over time. Results of my studies indicated that it is unwise to allow a sample to sit for longer than 2 weeks as seen in a single grab sample (Figure 3.18). Therefore, all samples in this study are processed the next day if not the same day to ensure sample integrity.

#### **4.4 Environmental Assessment**

None of the environmental samples collected had detectable estrogenic activity, indicating that WWTP effluent dilution is considerably efficient in reducing the levels of EDCs in the aquatic environment. Several environmental samples, especially the sediment samples from GVRD, do show toxicity in the assays. However, the water samples from Burrard Inlet at Rocky Point Park, Burrard Inlet at Deep Cove and Ambleside exhibit no toxicity in the assays.

#### **4.5 Tissue Study**

Estradiol fortified filets from chinook and rainbow trout were analyzed using the YES assays of Gaido et al. (1997). The homogenates of the fish tissues produced very similar bioassay-derived EEQ values; future analyses should be performed only on whole fish. The three doses seemed to correlate well with their corresponding bioassay-derived EEQ; although the recovery of the E2 is difficult to determine (Figure 3.19). Based on the 17 $\beta$ -Estradiol Dose response analysis and consequent bioassay-derived EEQ, the recovery was approximately 1-3% based on activity for both chinook and rainbow trout for the medium dose (Figure 3.19).

Future studies involving invertebrates would be highly valuable in determining if the high fat content of the fish muscle may affect the recovery of E2. The potential to apply ERTA assays to well-developed environmental monitoring programs like Mussel Watch would provide invaluable insights into total estrogenicity which is difficult, if not impossible, to detect using chemical analyses.

## 4.6 Conclusions

The individual ERTA assays provide relevant, reproducible and consistent results with laboratories using the respective methods and cell lines. The yeast based ERTA assays provided very similar 17 $\beta$ -estradiol activity ( $p < 0.05$ ); while the E-Screen appeared to be approximately one order of magnitude more sensitive than the yeast based ERTA assays. Reference estrogen comparisons and WWTP samples revealed that the ERTA assays EEQ predictions may vary by up to 5-fold. This variation resulted in poor correlation between ERTA assays; correlation coefficients of 0.3 for E-Screen versus Sumpter, 0.6 for Gaido versus Sumpter, and 0.6 for Gaido versus E-Screen.

The estrogenicity determined by the bioassays usually is higher than the analytical chemistry-derived estrogenicity because not all EDCs present in the environmental samples are quantifiable by the chemical analytical procedure. The total chemically-derived EEQ values did not correlate well with the bioassay EEQs (0.02); whereas, the steroid chemically-derived EEQs correlated much more closely with the mean of the bioassay EEQs (0.73), than any individual bioassay EEQs (0.54 - Gaido, 0.56 - Sumpter, and 0.65 - E-Screen).

The environmental fates of estrogens are very complex and there is no universally accepted bioassay or chemical technique to quantify EDCs in the aquatic environment. Chemical analysis of EDCs is sensitive and specific but has limitations because only target substances are analyzed. *In vitro* bioassays which are based on the interaction between EDCs and estrogenic receptors can be very useful in determining the total estrogenic activity of EDCs in a mixture. Results of my studies indicate that the use of a

suite of bioassays and chemical analysis methods appears to be the best strategy in analyzing WWTP samples for estrogens or EDCs because it may improve the accuracy of the results. My results also show that E1 and E2 are the dominant environmental estrogens in the waste water samples of GVRD WWTPs.

In the present study, all environmental samples are at or below the detection limits of the bioassays. Not even the water samples collected near the outfalls of WWTPs show detectable EDC activity. The bioassays seem to be a viable approach of detecting EDC activity in fish tissue samples; however, the recovery is quite low 1-3%. Future work should focus on improvements to the extraction technique. Further investigation in this direction may prove to be highly valuable in using the bioassays as monitoring tools.



## II. E-SCREEN PROTOCOLS

### I. DETAILED LIST OF MATERIALS

Corning canted neck tissue culture flasks with plug seal caps: 25 cm <sup>2</sup> (Corning # 430-168)	Fisher # 10-126-30 ( <a href="http://www.fishersci.com">http://www.fishersci.com</a> )
75 cm <sup>2</sup> (# 430-720)	Fisher # 10-126-31
150 cm <sup>2</sup> (# 430-823)	Fisher # 10-126-32
Corning 1cc Stripettes (Corning # 4011)	Fisher # 07-200-2
5ml (Corning # 4051)	Fisher # 07-200-9
10ml (Corning # 4101)	Fisher # 07-200-12
25ml (Corning # 4251)	Fisher # 07-200-15
Falcon 15 ml polystyrene centrifuge tubes (Becton Dickinson # 35-2095)	Fisher # 05-527-90
Falcon 96 well assay plates (BD # 35-3915)	Fisher # 08-772-5A
Linbro 24 well tissue culture plates (76-033-05)	ICN Biomedicals # 76-03305
Dulbecco's modified eagle's medium (DMEM) with phenol. red & L-Glutamine, without NaHCO <sub>3</sub>	ICN # 10-331-22
Deficient DME (4500mg/L glucose, without L-Glutamine, Na Pyruvate, or phenol red)	Irvine Scientific # 9587
Hy-Clone Defined Fetal Bovine Serum (FBS)	HyClone # SH30070-03
Gibco-BRL Trypsin (1:250) powder, 25g	Gibco-BRL (Invitrogen) # 27250-042
Sulforhodamine B (a.k.a SRB)	Sigma # S-9012
17-β-Estradiol	Calbiochem # 3301
Methyltrienolone (a.k.a. R1881)	New England Nuclear (PerkinElmer Life Sciences) # NLP-005

## ii. E-SCREEN SUBCULTURE PROTOCOL

The cells are grown in Corning 25 cm<sup>2</sup> canted neck tissue culture flasks (with Plug Seal Cap, Corning cat. # 430-168; available from Fisher (800-766-7000) Fisher cat. # 10-126-30).

The cells are grown in Dulbecco's modified eagle's medium (DMEM) with phenol red & L-Glutamine, without NaHCO<sub>3</sub>, (ICN Biomedicals (800-854-0530) cat. # 10-331-22), with fetal bovine serum (FBS) 5%. (Defined Fetal Bovine Serum from HyClone (800-492-5663) cat# SH30070-03).

**TO MAKE DMEM (10L):** Add powder from 1 (10L) packet of medium to 9L of double-distilled water while stirring constantly. Rinse out the packet with more DD water to get all of the powder into solution. After powder is in solution, add 22.5g NaHCO<sub>3</sub> and stir until dissolved. Bring volume to 10L by adding DD water and adjust pH to 7.1. Pour solution into appropriately sized pressure tank. Filter-sterilize with a 0.2-micron Millipore membrane filter. Aliquot into **glass** bottles for storage. Keep medium in an incubator at 37°C for 48 hours to determine whether or not the medium is contaminated. Before adding FBS to medium, heat inactivate it in a 57°C water bath for 30 minutes and filter it through a 0.2-micron filter.

The cells grow at 37°C in 94% air/6% CO<sub>2</sub>. The caps of the flasks should be loose enough to allow air to circulate (you should be able to gently jiggle them). Aspirate and replace the DMEM-FBS 5% (4mL) every 3-4 days. As phenol red is a pH indicator, the color of the medium will alert you if the cells' environment is too acidic (orange) or too alkaline (pinkish-purple). When the cells are becoming somewhat confluent and starting to look crowded in some areas, you will need to subculture them (usually every 6-8 days.) The cells do not grow in a complete monolayer, so be sure to subculture them before they start to grow over each other. Aspirate the medium and wash with 1.5ml of trypsin-0.53mM EDTA solution. Quickly aspirate off the wash and add 1.5mL of fresh trypsin-EDTA solution. Let the cells sit in the trypsin for a couple of minutes (generally 1-2 minutes at room temperature). When cells are detached (you may have to gently shake the flask or knock it against the palm of your hand), add 3mL DMEM-FBS 5% to neutralize the trypsin. Pipette the cells up and down to break up any clumps and add 1 or 2 drops of cells to two brand new T-25 flasks (approximately 100µl of cell suspension). Add 4mL fresh medium to each flask. Gently shake flasks to disperse cells across the bottom surface. Take care not to seed new flasks too heavily. (Again, they should start to reach confluency in 6-8 days.)

**TO MAKE TRYPSIN-EDTA (10L):** Dissolve 10.0g Glucose, 0.6g HK<sub>2</sub>PO<sub>4</sub>, 0.4768g Na<sub>2</sub>HPO<sub>4</sub>, 4.0g KCl and 80.0g NaCl in 9L **chilled** DD water. Add 2.0g EDTA, 5.0g Trypsin (1:250) (Gibco-BRL cat.# 27250-042) and 3.5g NaHCO<sub>3</sub>. Bring volume to 10L by adding DD water.

Adjust pH to 7.1. Store at 4C in **glass** bottles. (Final concentrations are: Trypsin 0.025% & EDTA 0.01%.)

When cells are grown successfully for a few passages, they may be seeded for experiments. The day before seeding experiments, change the cells' medium so that they are fully nourished. Take one or two confluent T25 flasks and wash with 1.5mL trypsin. Immediately aspirate the wash and add another 1.5mL. Let the cells trypsinize and add 3-5mL of media to neutralize the trypsin when cells have become detached. Pipette the trypsin/media/cell mixture into a Falcon blue-capped 15mL centrifuge tube (Catalogue # 2095 –Falcon

### **iii. STORAGE PROTOCOL**

#### Cell Freezing Protocol

1. Prepare cells as if subculturing by trypsinizing and neutralizing with medium.
2. Place cell suspension in a 15 ml Falcon tube (#2095).
3. Centrifuge the suspension at 1000 rpm for about 1 minute to get a cell pellet.
4. Make up a 7% DMSO/ 93% medium mixture in a separate 15 ml tube.
5. Aspirate the medium supernatant, leaving the cell pellet.
6. Add the DMSO/medium mixture to the pellet and resuspend. Add 1.5 ml of the mixture per cryo-tube. (The number of cryo-tubes you use will depend on the size of the pellet.) Generally 1 confluent 25 cm<sup>2</sup> flask will give you a pellet that can be split into 3 cryo-tubes.
7. Be sure to label each vial with the cell line, passage number and date of freeze.
8. Place vials in the neck of a liquid nitrogen container for about 3 hours OR put into a negative 80 °C for that amount of time. **DO NOT PLACE DIRECTLY INTO THE LIQUID NITROGEN!!!**
9. After about 3 hours, the cells can either remain in the -80 °C for storage or placed into a liquid nitrogen freezer.

#### Cell Thawing Protocol

1. Place about 5 ml of medium into a 15 ml Falcon tube.
2. Remove cryo-tube from the freezer and immediately place in a water bath to quickly



thaw the cells.

3. Add the contents of the cryo-tube to the medium in the Falcon tube as soon as it has thawed.
4. Centrifuge the tube at 1000rpm for about 1 minute to get a cell pellet.
5. Aspirate the medium supernatant.
6. Resuspend the cell pellet in DMEM with FBS 5% and seed into 25 cm<sup>2</sup> flasks. Resuspend in 4 mL of medium per flask. These flasks should be seeded heavier than usual, as you will lose a lot of cells to the freezing and thawing processes.
7. Cells will need to be subcultured sooner than usual, but should then be seeded in the normal density. To maintain your stocks of young, frozen cells, refreeze cells after carrying them for a couple of passages. Try to freeze 3 ampules of cells for every one that you thaw.

### **III. YEAST ESTROGEN ASSAY PROTOCOLS**

#### **i. Materials**

- a. Yeast Nitrogen Base without Amino Acids (Difco 233520 – 100 g)
- b. D-(+)-Glucose (Dextrose) (EM Science DX0145-3 – 2.5 kg)
- c. Copper Sulfate Pentahydrate (BD ACS261 – 500 g)
- d. 2-Mercaptoethanol (Sigma M6250 – 500 mL)
- e. Lyticase (Sigma L2524 – 10000units)
- f. Di-sodium phosphate (Na<sub>2</sub>HPO<sub>4</sub>) (EM Science SX0720-1 – 500 g)
- g. Monosodium phosphate (NaH<sub>2</sub>PO<sub>4</sub>) (EM Science SX0320-1 – 500 g)
- h. Potassium Chloride (KCl) (EM Science B10198-34 – 500 g)
- i. Magnesium Sulfate (MgSO<sub>4</sub>) (Caledon 5350-1 – 1 L)
- j. O-Nitrophenyl β-D-Galacto-Pyranoside (ONPG): (SIGMA #N1127)
- k. Lauryl Sulfate (Sodium dodecyl sulfate) (Sigma L4390 – 10 g)
- l. Sodium Chloride (NaCl) (Caledon 7560-1 – 500 g)
- m. L-Lysine-HCl (Sigma L5626 – 100 g)
- n. L-Histidine-HCl (Sigma H8125 – 100 g)
- o. glycerol (Caledon 5350-1 – 1 L)

#### **ii. Reagent Preparation**

1. **10X Yeast Nitrogen Base without Amino Acids (YNB)**
  - a. Weigh out 67 g Yeast Nitrogen Base without Amino Acids.
  - b. Place in 1000 ml graduated cylinder.

- c. Bring up to 1000 ml with distilled water.
- d. Mix with magnetic stir bar on stir plate.
- e. Filter sterilize with 1000 ml 0.2  $\mu\text{m}$  filter unit. Transfer to 1000 ml sterile glass bottle.

**2. 20% Dextrose Stock**

- a. In 1000 ml beaker, dispense 800 ml distilled water, add magnetic stir bar, and place on magnetic stirrer.
- b. Weigh out 200g Dextrose
- c. Add Dextrose slowly to vigorously stirring distilled water.
- d. Filter sterilize with 1000 ml-0.2  $\mu\text{m}$  filter unit. Transfer to 1000 ml sterile glass bottle.

**3. 10 mM Copper Sulfate**

- a. Weigh out 0.25g Copper Sulfate pentahydrate. Place in 100 ml graduated cylinder.
- b. Bring up to 100 ml with distilled water.
- c. Filter sterilize with 100 ml-0.2  $\mu\text{m}$  filter unit. Transfer to 100 ml sterile glass bottle.

**4. 10% SDS**

- a. Weigh out 10 g Lauryl Sulfate. Place in 100 ml graduated cylinder.
- b. Bring up to 100 ml with distilled water. Mix well.
- c. Transfer to 100 ml sterile glass bottle.

**5. 1 M Sodium Chloride**

- a. Weigh out 58.44 g NaCl. Place in 1000 ml graduated cylinder.
- b. Bring to 1000 ml with distilled water. Mix well.
- c. Filter sterilize with 1000 ml-0.2  $\mu\text{m}$  filter unit. Transfer to 1000 ml sterile glass bottle.

**6. 50% Glycerol with 100 mM NaCl**

- a. Put 50 ml glycerol into 100 ml graduated cylinder.
- b. Add 10 ml of 1M NaCl solution.
- c. Bring up to 100 ml with distilled water. Mix well.
- d. Transfer into 100 ml sterile glass bottle.

**7. Lyticase - 10,000 units**

- a. Prepare the solution by adding 1.25 ml of 0.1 M NaCl/50% Glycerol solution to vial
- b. Mix well. Store at 4°C.

**8. Z Buffer**

- a. Weigh out:
 

16.1 g	Na <sub>2</sub> HPO <sub>4</sub>
5.5 g	NaH <sub>2</sub> PO <sub>4</sub>
0.75 g	KCl

0.25 g MgSO<sub>4</sub>

- b. Place in 1000 ml graduated cylinder.
- c. Bring up to 800 ml with distilled water.
- d. Adjust pH to 7.0 while stirring with stir bar on stir plate.
- e. Bring up to 1000 ml with distilled water.
- f. Filter sterilize with 1000 ml-0.2 µm filter unit. Transfer to 1000 ml sterile glass bottle.

**9. Amino Acids**

- a. **LYS**-1.8g L-lysine-HCl in 500 ml of distilled water. Autoclave.
- b. **HIS**-1.2 g L-histidine-HCl in 500 ml of distilled water. Sterilize with 500 ml-0.2 µm filter unit.

**10. 0.1 M Sodium Phosphate Buffer, pH 6.8**

weigh: 6.90 g monbasic sodium phosphate  
7.10 g dibasic sodium phosphate

- dissolve in 500 ml milli-Q water, check pH, filter sterilize and store at 4 °C

**11. Growth Media for Gaido ER Transformed Yeast - buffered**

- a. Measure out: 50 ml 10X YNB  
50 ml 20% Dextrose  
5 ml Lysine  
5 ml Histidine  
250 ml 0.1 M sodium phosphate buffer, pH 6.8
- b. Bring up to 500 ml with distilled water. Mix well.
- c. Filter sterilize with 500 ml-0.2 µm filter unit. Transfer to 500 ml sterile glass bottle.
- d. Store at room temperature.

**12. Growth Media for Sumpter ER Transformed Yeast - buffered**

- a. Measure out: 5 mL 20% w/v glucose solution,  
1.25 mL 4 mg/mL l-aspartic acid solution  
0.5 mL vitamin solution  
0.4 mL 24 mg/mL l-threonine solution  
0.125 mL 20 mM copper (II) sulfate solution to 45 mL  
single strength minimal medium 50 ml 10X YNB
- b. Filter sterilize with 500 ml-0.2 µm filter unit. Transfer to 500 ml sterile glass bottle.
- c. Store at room temperature.

### iii. Growth, Selection and Freezing Yeast

BJ3505 hER 2ERE from K.W. Gaido

MAT  $\alpha$ , pep4::His3, prb1- $\Delta$ 1,6R, his3- $\Delta$ 200, lys2-801, trp1- $\Delta$ 101, ura3-52(can)  
[Sigma genotype pep: HIS prb- $\Delta$ 1.6HIS lys2-801 trp1-  $\Delta$ 101 ura3-52 gal2 can1]

Contains 2 plasmids:

Plasmid #1: Contains the estrogen receptor with a CUP1 metallothionein promoter which is inducible with CuSO<sub>4</sub> at the time of yeast exposure to substances. ERtrp(YePtrpER)

Plasmid #2: Is a reporter plasmid containing two ERE or estrogen responding elements and the structural gene for  $\beta$ - galactosidase. E2.ura (YRpE2ura)

- for BJ3505 hER 2ERE selection media is buffered Minimal Media (MM) plus HIS and LYS .

#### SELECTION PLATES - for 200 ml (makes ~12 100 mm plates):

- a. Measure 20 ml 10X YNB  
4 g bactoagar  
156 ml water into a 1l media bottle. (Note large bottle to avoid loss due to boiling/overflow while being autoclaved)
- b. Mix for ~2 minutes to dissolve.
- c. Autoclave.
- d. Allow to cool until container can be handled without difficulty.
- e. Add: 2 ml LYS (from stock)  
2 ml HIS (from stock)  
20 mls 20% DEX
- f. Swirl to mix. Pour (pipet to avoid bubbles) 15 ml per 100 mm plate.
- g. Let stand at RT to harden for several hours to minimize condensation.  
Store at 4°C (sealed).

#### GOLD MEDIA

For 600 ml:

60 ml 20% Dextrose (SIGMA, G-5400, CAS# 50-99-7)  
60 ml 10X Yeast Nitrogen Base without amino acids (SIGMA, Y-0626)  
110 ml Gold Concentrate Stock Solution (see below)  
370 ml H<sub>2</sub>O  
Filter Sterilize (0.2  $\mu$ m) and store at 4°C.

**Gold Concentrate Stock Solution:**

Individual Amino Acid Stock Solutions (in H <sub>2</sub> O)			Storage	Stock (ml)
*1. Adenine Sulfate	0.6g/ 500 ml	Autoclave	RT	10
*2. L-Lysine-HCl	1.8g/ 500 ml	Autoclave	4°C	5
*3. L-Tryptophan	2.4g/ 500 ml	Filter	4°C	5
*4. Uracil	1.2g/ 500 ml	Autoclave	RT	5
* these solutions are also used for other media				
5. L-Histidine-HCl	0.24/ 100 ml	Autoclave	4°C	5
6. L-Arginine-HCl	0.24g/ 100 ml	Autoclave	4°C	5
7. L-Methionine	0.24g/ 100 ml	Autoclave	4°C	5
8. L-Tyrosine	0.09g/ 100 ml	Autoclave	RT	20
9. L-Isoleucine	0.36g/ 100 ml	Autoclave	4°C	5
10. L-Phenylalanine	0.3g/ 100 ml	Autoclave	RT	10
11. L-Glutamic Acid	0.6g/ 100 ml	Autoclave	RT	10
12. L-Aspartic Acid	0.4g/ 100 ml	Autoclave	RT	15
13. L-Valine	1.8g/ 100 ml	Autoclave	4°C	5
14. L-Threonine	2.4g/ 100 ml	Autoclave	4°C	5
15. L-Serine	4.5g/ 100 ml	Autoclave	4°C	5
16. L-Leucine	0.36/ 100 ml	Autoclave	RT	5
				120 ml

Filter and store Gold Concentrate Solution at 4°C or add immediately to Gold Media and filter (0.2 μm) complete solution.

## **GROWTH OF YEAST**

1. Take a small scraping of yeast from frozen culture and streak onto selective medium plate. If this does not work, thaw a 100  $\mu$ l aliquot of stock yeast and add 50  $\mu$ l to 5 ml Gold media (non-selective) in a 50 ml polypropylene tube. Incubate at 30C at 300 rpm until culture becomes cloudy - usually overnight . Inoculate a selective media plate with a loop full of culture. Spread in quadrants so that yeast become sufficiently dispersed to produce well separated colonies.
2. Place the plate at 30°C and allow to grow until colonies are visible. (2-3 days)
3. Once colonies are visible, seal plate and store at 4 °C

### **Preservation of yeast ER Cultures (method used by Burnison lab)**

1. inoculate 10 ml of ER selective medium with a single colony
2. incubate o/n with shaking at 30C
3. centrifuge at 2000 g for 2 minutes to pellet yeast
4. remove supernatant and resuspend yeast in 5 ml of medium containing 15% glycerol
5. pipet 1 ml of the cell suspension into cryovials
6. cool slowly by placing vials at 4 °C for 30 min, -20 °C for 30 min then transfer to -70 °C freezer

## ASSAY:

### DAY 0: colony into selective media

- select a single colony of yeast from a streaked plate (less than one month old) and put it in 5 ml SELECTIVE MEDIA in 50 ml polypropylene tube.
- grow at 30°C with shaking (300 RPM) overnight.

### DAY 1: 1:10 dilution (2 pm)

- make a 1:10 dilution of log-phase culture of yeast by adding 5 ml to 50 ml growth medium.

### DAY 2: 1:1 (10 am) dilution, dose plates (~ 1 pm), and exposure (2 pm)

- dilute the overnight culture of yeast in the morning by half in growth media (**add 50 ml media**)
- start the assay in the afternoon - mid-log phase of growth (OD 600 between 0.8 and 1)
- weigh ONPG into 50 ml tubes and store at -7 °C.

1. dispense 10 µl of test solutions into flat bottom microplates and evaporate to dryness. (~30 minutes; final volume will be 200 µl).

- run test solutions in triplicate = 20 hours if 2 pm - 10 am.

2. aliquot **20 ml/plate** of yeast culture (each plate requires 19.2 ml yeast solution at 200 µl/well) into a sterile tubes or bottle.

3. add **10 µl/ml** 10 mM copper sulphate to yeast culture to achieve a 100 µM final concentration (200 µl/20 ml = enough for 1 plate).

4. dispense 200 µl of yeast culture to each treated well - shake for 2 minutes on microplate shaker.

5. incubate overnight in humidified tupperware container at 30 °C in walk-in incubator - no shaking.

### DAY 3: (9 am) assay

1. dissolve ONPG in Z-buffer:

	<b>1 plate</b>	<b>2 plates</b>	<b>3 plates</b>	<b>4 plates</b>
<b>2 mg/ml ONPG</b>	22 mg	44 mg	66 mg	88 mg
<b>Z-Buffer</b>	10.9 ml	21.8 ml	32.7 ml	43.6 ml

- to dissolve, vortex and put in shaking 30 °C incubator for ~15 min.
- ensure all ONPG is in solution (dissolved) - vortex further if necessary.
- ONPG must be in solution before adding other assay buffer components.

2. place cryovial containing 10% SDS in beaker of warm water to re-dissolve.

3. while ONPG is dissolving check bottom of plate for cytotoxicity - note wells that have cell lysis.

4. resuspend yeast cultures in treatment plate by pipeting and aliquot 100 ul of yeast from treatment plate to assay plate with multichannel pipet.

5. when ONPG is completely dissolved complete preparation of assay buffer.  
Add:

	<b>1 plate</b>	<b>2 plates</b>	<b>3 plates</b>
<b>50 mM 2-Mercaptoethanol</b>	29.7 $\mu$ l	59.4 $\mu$ l	89.1 $\mu$ l
<b>200 U/ml oxalyticase</b>	11 $\mu$ l	22 $\mu$ l	33 $\mu$ l
- mix tube then add:			
<b>10% SDS</b>	110 $\mu$ l	220 $\mu$ l	330 $\mu$ l

- invert gently to mix - do not vortex - foams!

- assay buffer is stable for 1 hour, use immediately after preparation.

6. Using repeater pipet with pipet tip on repeater tip (to avoid bubbles) add 100  $\mu$ l assay buffer to each well - start timer, shake for 2 minutes, stagger plates by at least 2 min.

- can also use multichannel pipet for substrate addition.

7. read plates after 40 minutes at 415 nm and 595 nm.



#### IV. EEF DATA (LITERATURE)

EDC	YES (LC <sub>50</sub> /LC <sub>50</sub> )	REFERENCE
Ethinylestradiol (EE2)	88.8/70/50/90	Cold, 1997; Folmar, 2002; Tanaka, 2001; Vander, 2003
Estrone (E1)	0.67/9.6/30/30/40	Routledge, 1995; Cold, 1997; Tamanaka, 2001; Tanaka, 2001; Vader, 2003
Estriol (E3)	0.004/0.025/0.63/0.2	Gaido 1997; Routledge 1995; Cold, 1997; Tanak, 2001
Nonylphenol (NP)	0.0002/0.002/0.0022/ 7.2x10 <sup>-5</sup> /0.10.1/0.01	Gaido, 1997; Routledge 1995; Cold, 1997; Folmar, 2002; Tamanaka, 2001; Tanaka, 2001; Vander, 2003
Bisphenol A (BPA)	0.00007/0.0003/0.006/0.006	Gaido, 1997; Routledge, 1995; Tamanaka, 2001; Tanaka, 2001
17 $\alpha$ -Estradiol	0.015	Gaido, 1997
4-n-octylphenol	0.0005	Tamanaka, 2001
Testosterone	0.000005/0/0.001	Gaido, 1997; Routledge, 1995; Cold, 1997
Mestranol	7.3	Cold, 1997
EDC	E-Screen	REFERENCE
Ethinylestradiol (EE2)	91/125/100/115	Korner, 2001; Gutenberg, 2001; Soto, 1995; Cargouet, 2004
Estrone (E1)	9.6/1/1/25	Korner, 2001; Gutenberg, 2001; Soto, 1995; Cargouet, 2004
Estriol (E3)	7.1/10/8.33	Gutenberg, 2001; Soto, 1995; Cargouet, 2004
Nonylphenol (NP)	0.01/0.0013	Korner, 2001, Gutenberg, 2001
Bisphenol A (BPA)	0.0053/0.0025	Korner, 2001/Gutenberg, 2001
17 $\alpha$ -Estradiol	10	Soto, 1995
4-n-octylphenol	0.01/0.003	Gutenberg, 2001; Soto, 1995

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