A SURVEY OF ESTROGENIC CHEMICAL CONTENTS IN WATER/BIOSOLID SAMPLES FROM CANADIAN WASTEWATER TREATMENT PLANTS

by
Ben H.H. Shieh
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Name: Ben H.H. Shieh

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Title of Thesis: A Survey of Estrogenic Potencies in Waste Water and Biosolid Samples From Canadian Waste Water Treatment Plants

Examining Committee:

Chair: **Dr. Arne Moores,** Professor
Department of Biological Sciences, S.F.U.

Dr. F. Law, Professor
Department of Biological Sciences, S.F.U.
Senior Supervisor

Dr. C. Kennedy, Professor
Department of Biological Sciences, S.F.U
Supervisor

Dr. R. Nicholson, Associate Professor
Department of Biological Sciences, S.F.U
Public Examiner

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ABSTRACT

Many estrogenic chemicals are released into the surface waters by municipal wastewater treatment plants (WWTPs) and livestock farms. Estrogenic chemicals, a sub-class of chemicals collectively called the endocrine disruption chemicals (EDCs), are able to bind with the estrogenic receptor and cause adverse health effects to aquatic species. The objectives of this study were: (a) to use the *in vitro* Yeast Estrogen Screen (YES) bioassay in determining the total estrogenic activities in effluent/biosolid samples from the WWTPs across Canada, and (b) to identify the most efficient treatment method(s) of removing estrogenic chemicals from the raw influents of the WWTPs. Results of our study showed that the estrogenic activities in the final effluents ranged from 0.00057 to 0.390 ng estradiol equivalents (EEQs)/ml whereas the estrogenic activities in the biosolids ranged from 3.6 to 24.6 ng EEQs/g dry weight. The advanced biological nutrient removal method appeared to be the most efficient procedure in removing the estrogenic chemicals from the raw influents; it could reduce the EDC contents in the raw influents by >30 folds. This is the first survey study in which the estrogenic activities in raw influents, final effluents and biosolids of the different WWTPs in Canada are monitored. Future studies should focus on the chemical identities of the estrogenic compounds in the sample extracts and the improvement of treatment methods use in removing the estrogenic chemicals from the raw influents.

DEDICATION

To my family and Juliana Vandergugten

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CHAPTER 1 INTRODUCTION

1.1 Environmental estrogens and endocrine disrupting chemicals

A number of environmental contaminants are capable of altering the physiological functions of fish and mammals by blocking/enhancing hormonal releases (Holbrook et al. 2002 and Soto et al. 1995). These chemicals collectively are termed endocrine disruption chemicals (EDCs). The European Union Commission (EU Commission, 1996) defines EDCs as " an exogenous substance which causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine functions". Some EDCs are formed naturally by living organisms while others are synthesized chemically in the laboratory (Garcia-Morales et al. 1994, Tan et al. 2007, Sarmah et al. 2006). The EDCs in the environment usually are in the form of a complex chemical mixture instead of a single pure compound (Sanderson and Giesy, 1998). Estrogenic chemicals are the most studied chemicals among the EDCs because of their potency and potential harmful effects to the aquatic species.

Estrogenic chemicals may enter the aquatic environment through human and animal wastes which contain estrogens such as 17-β-estradiol (E_2) (Desbrow et al. 1998, Routledge et al. 1998 and Larsen et al. 2008). As wastewater treatment plants (WWTPs) collect human wastes, they release the hormones into the aquatic environment along with the final effluents (Nelson et al. 2007). The widespread use of contraceptive pills by humans also contributes significantly the amount of 17-α-ethynylestradiol (EE_2), a potent synthetic estrogen discharged into the aquatic environment. Livestock and poultry farms are other sources of estrogenic chemical

contamination of the aquatic environment (Furuichi et al. 2006). The effluents released from the livestock and poultry farms also contain E_2 and other hormones. According to Raman et al. (2004), about 10-30 kg of E_2-like chemicals is released by diary and chicken farms into the rivers and lakes daily in the USA. Industrial chemicals and pesticides also are another source of estrogenic chemical discharge into the aquatic environment (Soto et al, 2004, and Metcalfe et al. 2001).

The estrogenic chemicals in the aquatic environment may attach themselves to the sediments or are dissolved in the surface waters depending on their water solubility (Barnabea et al. 2009, Kumar et al. 2010, and Mohapatra et al. 2010). Previous studies have shown that exposure of fish to E_2 in the laboratory elicits adverse health effects including feminization and courtship behaviour change at a level as low as 1.0 ng/L (Terns et al. 1999). However, the adverse effects of estrogenic chemicals on feral fish remain controversial and have been a subject of ongoing debates (Hoffmann et. al. 2010, Pedemonte et al. 2008, and Saaristoa et al. 2000). This study focuses on the assessment of estrogenic chemical contents in the wastewater and biosolid samples of Canadian WWTPs using the *in vitro* Yeast Estrogen Screen (YES) bioassay.

1.2 Examples of estrogenic chemicals

By definition, estrogenic chemicals include all compounds which have similar physiological effects as E_2 or the chemicals that can bind onto the estrogen receptors of the living organisms (Soto et al. 1995). Examples of natural estrogenic chemicals include the major female hormones such as E_2, estrone, and estriol, and the phytoestrogens such as genistein and daizein.

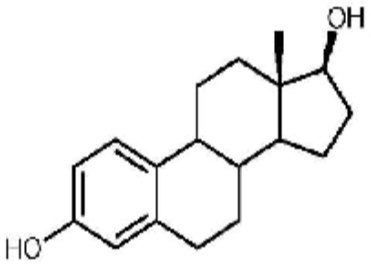

Figure 1.1. Structure of 17-*β*-estradiol

E_2 (Figure 1.1) is produced by the ovaries and testicles; it plays an important role in maintaining the female reproductive organs and secondary sexual characteristics of mammals. E_2 also is the most potent naturally occurring estrogenic chemical in living organisms (Hertz, 1985). Exposure of male fish to E_2 has been shown to induce vitellogenin production (egg yolk precursor) and feminization of male sex organs. It also causes certain fish species such as the *Oryzias latipes* to hatch only with female and intersex males (Beresford et al. 2004, Jobling et al. 2003, and Metcalfe et al. 2001). Also, an excess of E_2 in humans has been linked to the development of breast cancer and prostate cancer as well as apoptosis of male germ cells (Pentikainen et al. 2006).

Phytoestrogens is a general name given to a class of naturally occurring estrogenic chemicals (Figure 1.2) that are produced by plants to defence against animals and insects (Hughes, 1988 and Korkina, 2007). Phytoestrogen-rich plants include soybeans and whole grain products (Adlercreutz et al. 1982).

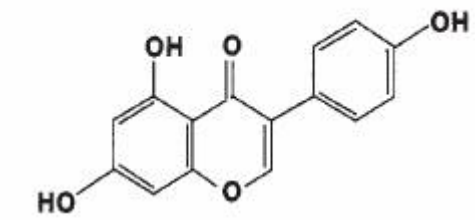

Figure 1.2. Genistein, an example of a phytoestrogen

Phytoestrogens enter human or animal bodies mainly through ingestion of plant materials. Previous studies have shown that diets rich in phytoestrogens can cause infertility, endocrine disruption and liver malfunctions in animals (Setchell et al. 1987, and Shutt et al. 1976). In addition, allowing rats to consume phytoestrogens at natural dietary levels causes a significant increase in uterine weight and inhibition of the binding of natural estrogenic hormones to the estrogen receptor (Whitten et al. 1992). Phytoestrogens are also known to induce the growth of human breast carcinoma cells suggesting that plant estrogenic chemicals behave similarly to human estrogens (Hsu et al. 1999).

17-α-Ethynylestradiol (EE_2) (Figure 1.3) is a synthetic estrogen which is the major active component of the oral contraceptive pills (Larsen et al. 2008).

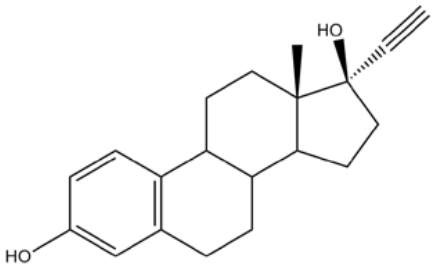

Figure 1.3. Structure of 17-*α*-ethynylestradiol

EE_2 is metabolized by phase II conjugation pathways in human. However, it can be de-conjugated back into its active form by microorganisms in the WWTPs (Royledge, 1998). EE_2 shares a similar chemical structure as E_2. However, the binding affinity of EE_2 to the estrogen receptor is much stronger than that of E_2; about 2 and 5 times stronger than E_2 in human and fish species, respectively (Nash et al. 2004, Thorpe, 2003, and Tilton et al. 2005). Thus, EE_2 is considered to be an estrogenic chemical which poses a high risk to aquatic species. EE_2, just like E_2, has been shown to induce vitellogenin production and cause feminization in rainbow trout (Verslycke, 2002). EE_2 also can cause behavioural changes in male zebra fish because courtship to female fish and aggression towards other male fish are greatly reduced (Colman et al. 2008).

Bisphenol A (BPA) (Figure 1.4) is a synthetic chemical used in the manufacturing of plastics (Calafat et al. 2005, and Kang et al. 2006).

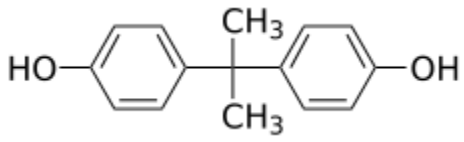

Figure 1.4. Structure of BPA

Polycarbonate plastic food containers, utensils, and water pipes are some of the household items where BPA can be found (Calafat et al. 2005, and Kang et al. 2006). BPA has a tendency to leach into the environment from plastic ware. Thus human contact with BPA is common and widespread (Staples et al. 1998). BPA has endocrine disruption properties similar to the natural estrogens and can interact with the estrogen receptor (Gould et al. 1998, Matthews et al. 2001, and Vivacqua et al. 2003). In addition to mimicking estrogenic activity, BPA is also known to up regulate the production of estrogen receptors in exposed individuals (Ramos et al. 2003). A previous study reveals that BPA is able to induce abnormalities in oocyte development in female rats (Hunt et al. 2003). In male rats, exposure to BPA causes a decrease in sperm production and fertility success (VomSaal et al 1998, and Al-Hiyasat et al. 2002).

1.3 Detection methods for estrogenic chemicals

Estrogenic chemicals are ubiquitous in the aquatic environment. They are routinely detected and quantified using analytical instrumentation. Although chemical analysis is a very useful tool to identify and/or quantify individual estrogenic chemicals in water or biosolid samples, it is unable to provide an estimate of the total estrogenic chemicals in a mixture because chemical analysis does not detect estrogenic chemicals that are at concentrations less than the analytical detection limits. Other disadvantages of chemical analysis include the need to develop an analytical method and the availability of an analytical standard (Sanderson and Giesy, 1998). These disadvantages can be overcome by using the *in vivo* bioassays such as the uterotrophic assay which takes into account of the pharmacokinetics of the estrogens, is based on the growth of the uterus in the rodent (Shelby et al., 1996; Odum et al., 1997). However, the uterotrophic assay is costly and time-consuming to perform and the results often vary with the administration

route of the estrogenic chemicals and the biological endpoint measured (Milligan et al., 1998). In the present study, we propose to use the relatively quick and inexpensive Yeast Estrogen Screen (YES) bioassay which expresses the human estrogen receptors (Routledge and Sumpter, 1996; Gaido et al., 1997; and Peck et al. 2007) to monitor the total contents of estrogenic chemicals in the wastewater and biosolid samples collected from the WWTPs across Canada. Because the yeast cells do not contain any endogenous estrogens and there is little or no estrogenic chemical biotransformation takes place, the YES bioassay also can eliminate the concern of false positive/negative results (Gaido et al. 1997, Petit et al. 1997, and Shelby et al. 1996).

The YES bioassay relies on a strain of genetically modified yeast cells which contain the genes that encode the human estrogen receptor (hER). The yeast cells also possess plasmids which encode the human estrogen response element (ERE) (Gaido et al. 1997). When an exogenous estrogenic compound is absorbed by the yeast cells and binds to hER, an estrogen chemical-receptor complex is formed, signalling the ERE to initiate the release of an enzyme called β-galactosidase (Gaido et al. 1997). The amount of β-galactosidase released is proportional to the exogenous estrogenic chemicals bound to hER (Gaido et al. 1997, and Lorenzen et al. 2004). After the addition of ortho-nitrophenol β-1-D galactopyranoside (ONPG), a substrate of the β-galactosidase, the β-galactosidase released in response to the estrogenic chemicals in the sample is quantified. This enzymatic reaction results in the development of a bright yellow color (Gaido et al. 1997, and Lorenzen et al. 2004) of which the optical density can be quantified with a UV/VIS spectrophotometer. After measuring the optical density in each

sample, a dose-response curve is constructed to determine estrogenic chemical content (Lorenzen et al. 2004).

1.4 Research objectives and study area

The objective of my study were: to use the YES bioassay in quantifying the concentrations/potencies of the estrogenic chemicals in the wastewater and biosolid samples collected from different WWTPs across Canada, and to identify the most efficient treatment methods for the removal of estrogenic chemicals from the raw influents. Sample collection was made possible with the assistance of Environment Canada and Metro Vancouver. The type of samples we received included raw influents, primary effluents, final effluents and de-watered biosolids. These samples were collected from a total of 13 different WWTPs across Canada. The specific details of the WWTPs are shown in Table 1.1. The samples collected from the WWTPs were sent directly by courier services to Dr. Francis Law's laboratory at Simon Fraser University. The samples were extracted immediately upon arrival and the YES bioassay was conducted within 24 hr after extraction.

With this study we hope to gain a better understanding of the extent of estrogenic chemical contamination in Canadian WWTPs. The data obtained from this study also can be used to help improve the treatment methods currently used in Canadian WWTPs.

WWTP Code/ Sampling season	Liquid treatment	Solids treatment
A/both	Trickling filter/solids contact	Thermophilic anaerobic digestion, dewatering
B/both	Biological nutrient removal	Mesophilic anaerobic digestion
C/cold	Activated sludge	Biopasteurization, mesophilic anaerobic digestion, dewatering
D/both	Aerated lagoon	None
F/cold	Activated sludge	Dewatering
J/summer	Facultative lagoon	None
L/summer	Biological aerated filter	Mesophilic anaerobic digestion, dewatering
M/cold	Chemically-assisted primary	Dewatering
N/both	Chemically-assisted primary	Mesophilic anaerobic digestion, dewatering
P/summer	Activated sludge	Mesophilic anaerobic digestion, dewatering
R/both	Aerated lagoon with primary treatment	Mesophilic anaerobic digestion, dewatering, primary sludge only
W/summer	HPO, activated sludge	Mesophilic anaerobic digestion, dewatering
X/both	Facultative lagoon	None

Table 1.1. Treatment information of selected Canadian WWTPs in the current study

CHAPTER 2 MATERIALS AND METHODS

2.1 Sample extraction procedures

2.1.1 Wastewater extraction

Influent and effluent samples were extracted using the solid phase extraction method reported by Huang and Sedlak (2001) with modification. Briefly, these wastewater samples were extracted by the C-18 solid phase extraction filter discs (Empore, 3M) previously conditioned with methanol and distilled water. About 300 ml aliquots of the influent or effluent samples were filtered through the C-18 extraction disc under vacuum. The C-18 extraction disc was rinsed with 10 ml methanol which was combined with the filtrate. The combined filtrate was evaporated to dryness under a gentle stream of nitrogen. The residues were re-dissolved in 500 μl of ethanol. For sites where sample collection was conducted for three days, the sample from each day was extracted separately before being combined. All wastewater sample extracts were stored in a -30 ^{o}C freezer for the YES bioassay.

2.1.2 Biosolids extraction

Biosolids were extracted using the liquid-solid phase extraction method reported by Ternes et al. (2002) with modification. Briefly, five grams of de-watered biosolids was mixed with 10 ml of ethyl acetate in a glass centrifuge tubes equipped with a stopper. The centrifuge tube was mixed initially by hand for 20 sec before being shaken vigorously in an automatic shaker for an additional 10 min. The mixture was centrifuged at 2000 g for 10 min to separate the layers. The supernatant was removed and put into a new test tube. This extraction procedure

was repeated twice. The supernatants were combined and evaporated to dryness under a gentle stream of nitrogen. The residues were reconstituted in 500 μl of ethanol. For sites where sample collection was conducted for three days, the sample from each day was extracted separately before being combined. The final extract was stored in a -30 ^{o}C for later use.

2.2 The YES Bioassay

2.2.1 Preparation of the yeast cells

The method of preparing the yeast cells for the YES bioassay was modified from the procedures of Gaido et al. (1997) and Lorenzen et al. (2004). Yeast cells were inoculated onto a selective agar plate from a previously frozen liquid culture. The inoculated plate was put into a 30 ^{o}C room for 3-4 days depending on the growth rate of the yeast cells. The agar plate containing the yeast colonies could be stored in a 4 ^{o}C refrigerator and used for up to 2 weeks before preparing a new agar plate. After sufficient yeast growth was seen on the agar plate, one to two colonies of the yeast cells was transferred to a 50-ml polyethylene tube containing 5 ml of selective media. The liquid culture was placed on a mechanical shaker and incubated at 30 ^{o}C for approximately 24 hr. On the second day, the liquid culture was diluted 1:10 by adding 45 ml of selective media into the original 5 ml culture. The liquid culture was incubated for an additional 20 hr. On the third day, the culture was diluted 1:1 by transferring 25 ml of the culture into another 50 ml polyethylene tube containing 25 ml of fresh selective media. The diluted culture was put back into the incubation room and allowed to grow for an additional 4 hr before being used for the YES bioassay.

2.2.2 Standard dilution series

The standard dilution series consisted of 11 E_2 standard concentrations. A stock solution of E_2 standard was prepared by dissolving 27.24 mg of E_2 in 10 ml ethanol. A sub-stock solution was prepared by taking an aliquot (20 µl) of the stock solution and mixing it with 980 µl of ethanol. The dilution series was prepared by using the sub-stock as the highest concentration of the series. Table 2.1 shows the details of the 11 dilution series of E_2:

Dilution NO.	**1**	**2**	**3**	**4**	**5**	**6**
Concentration (ng/ml)	2.59E+01	7.78E+00	2.59E+00	2.56E+00	7.78E-01	5.19E-01
Dilution NO.	**7**	**8**	**9**	**10**	**11**	
Concentration (ng/ml)	3.11E-01	1.56E-01	1.04E-01	1.56E-02	1.04E-02	

Table 2.1. Dilution series scheme of pure E_2 standard solution

2.2.3 Dilution of sample extract

As the E_2 equivalents (EEQs) in the sample extracts remained unknown before conducting the YES bioassay, a dilution series with different dilution factors were prepared from each sample extract. Table 2.2 shows the 6 different extract concentrations in the present study:

Dilution Number	**1**	**2**	**3**	**4**	**5**	**6**
Dilution Factor	1.00E+00	2.00E-01	4.00E-02	8.00E-03	8.00E-04	8.00E-05

Table 2.2. Dilution scheme for wastewater and biosolid sample extracts

2.2.4 Media Preparation

The selective media was prepared by combing the required reagents together (Refer to appendix II – 7 for media ingredients). The reagents were mixed with 490 ml of distilled water and autoclaved for 1 hr. The autoclaved media could be stored in the fridge (4 ^{o}C) for later use. In general, the media could be kept for about 2 weeks.

2.2.5 Yeast Preservation

The yeast cells used in the YES bioassay could be stored for a long period in a -75 ^{o}C freezer. The preservation procedure was performed by inoculating at least 2 yeast colonies from the agar plate into a 50-ml polyethylene tube containing 5 ml of selective media a 50-ml polyethylene tube containing 5 ml of selective media. This yeast liquid culture was placed on a shaker and allowed to grow for at least 12 hr at 30 ^{o}C. At the conclusion of the incubation, the liquid culture was centrifuged at 2000 g for 2 min to separate the layers. The supernatant was slowly removed and the yeast cells were mixed with 5 ml of 15 percent glycerol-media solution which was prepared by mixing 0.75 ml of glycerol with 4.25 ml of the selective media. A 1 ml aliquot of the yeast-glycerol-medium solution was transferred to a cryogenic vial. These vials were placed in a 4 ^{o}C fridge for 30 min before being transferred to a -20 ^{o}C freezer for an additional 30 min. Finally, the vials containing yeast cells were kept in a -75 ^{o}C freezer.

2.3 Sample and data analysis

2.3.1 Distilled water spiked with a known amount of E_2

A distilled water sample spiked with a known amount of E_2 was prepared and diluted to the following concentrations: 0.518, 5.18 and 51.8 ng of E_2 per ml. The E_2-spiked water samples were extracted by the same procedure as described for the wastewater samples.

2.3.2 Wastewater sample extract storage test

Wastewater sample extracts were stored in the laboratory freezer at -30 ^{o}C for different duration to assess the effects of storage on the sample estrogenic activities. A raw influent and a final effluent samples were chosen at random for this test. The sample extracts were analysed using the YES bioassay after being stored for a period of 0, 3, 5 month(s) (where 0 month means without any storage time). The EEQ of raw influent and final effluent extracts were compared at the end of each storage period.

2.3.3 Biosolid sample extract storage test

The biosolid samples were provided by Metro Vancouver. A total of 4 different biosolid samples were used in this experiment. These samples were primarily thickened screened primary sludge (TSPS), thickened wasted secondary sludge (TWSS), digested sludge (DSST), and biosolids (BSD) (*i.e.*, de-watered biosolids). After extraction, the extracts were stored in a -30 ^{o}C freezer and analyzed by the YES assay every 2 weeks over a period of two months. The EEQ results were compared at the end of each storage period.

2.3.4 Wastewater treatment plant sample analysis

Ten µl aliquots from each of the standard dilution series and the sample extract dilution series were pipetted into separate wells of a 96-well cell culture plate. The standard dilution series were examined in duplicates whereas the sample dilution series were studied in triplicates. About 200 µl of the yeast cell culture mixed with 100 µl of copper sulphate solution was added using a multi channel pipette. The copper sulphate solution was used to stimulate the production

of hER in the yeast cells. The entire 96-well plate was wrapped by a parafilm and placed in the incubation chamber at 30 ^{o}C over night for about 20 hr. On the second day, the mixture in each well was mixed using a pipette. This was followed by transferring 100 µl aliquot of the mixture from each well into a new 96-well plate. A 100 µl aliquot of the substrate reaction buffer mixture (containing 110 µl of 10% SDS, 10.9 ml of Z-buffer, 29.7 µl of beta mercaptoethanol, 0.022 g of ONPG and 11 µl of oxalyticase) was added to the newly prepared 96-well plate. About 40 min after adding the substrate reaction buffer, the 96-well plate was analyzed with an UV/VIS spectrophotometer at a wavelength of 415 nm (colorimetric change) and 595 nm (turbidity caused due to yeast growth). The optical density was documented for data analysis.

2.3.5 Data analysis

The yellow color generated in the YES bioassay was a result of the substrate-enzyme reaction. The optical density of the yellow color was proportional to the EEQ of our samples. To correct for cell turbidity, the optical density at 415 nm was divided by that of 595 nm. We constructed the dose-response curves as follows: The E_2 standard dose-response curve was constructed by plotting the optical density (Y-axis) against the E_2 concentrations (or dilution factors) (X-axis). The sample dose-response curve was normalized by the maximal optical density of the E_2 standard. The EC_{50}s were determined for the standard and sample curves. The sample EC_{30}s and EC_{20}s were also determined because the sample and the E_2 standard dose-response curves differed in slopes and maxima. The formula (Prism: Graphpad.com, 2011) used for EC_{30} and EC_{20} conversions was:

$$ECX = [(X/100\text{-}X)\ \hat{}\ 1/H]*EC_{50}, \quad (1)$$

where X represents EC_{20} or EC_{30}, and H is the Hill slope of the dose response curve. We calculated the E_2 equivalents (EEQs) in our samples based on the average values of the EC_{20}, EC_{30} and EC_{50} (Hilscherova et al. 2002). The detection limit (LOD) of the bioassay was arbitrarily set to the EC_{20} of the E2 standard, ($\sum EC_{20}$)/n, (n = 23)

The EEQs of our samples were calculated based on the relative potency of the sample EC_{ave} and the pure E_2 standard. The equation for EEQ calculation was reported by Lorenzen et al. (2004):

EEQ = [EC_{ave} (ng/ml)/Sample EC_{ave}]*[Volume of assay medium (ml)/Volume of sample extract tested (ml)]*[Volume of stock extract (ml)/Weight (g) or Volume (ml) of the sample]

where EC_{ave} is the average of EC_{20}, EC_{30} and EC_{50}, volume of assay medium is the final incubation volume (0.21 ml), volume of sample extract tested (0.1 ml), and volume of stock extract is the volume of extract added to the incubation (500 μl).

Statistical calculations were conducted using the Prism 4 program (GraphPad Software, La Jolla, CA, USA). The EEQs in each sample was reported as the mean ± S.E.M. of three separate experiments. The Students't-test was used for statistical comparison: the level of significance was chosen at a *p*-value of < 0.05, < 0.001, or < 0.0001 depending on the dataset.

CHAPTER 3 RESULTS

3.1 Assay reproducibility and effects of storage on extracts

3.1.1 Reproducibility of YES bioassay

The measured results of the E_2-spiked samples were 0.48 ng/ml, 4.6 ng/, and 46.5 ng/ml respectively (see the Y-axis of Fig. 3.1); they were very close to the spiked concentrations in the samples (see the X-axis of Fig. 3.1). To determine the precision of the YES bioassay, we calculated the percent coefficient of variation (CV%) of each experiment; they were 20.10 %, 15.99 %, and 10.35 % for the 0.52, 5.18, and 51.8 ng/ml spiked concentrations, respectively.

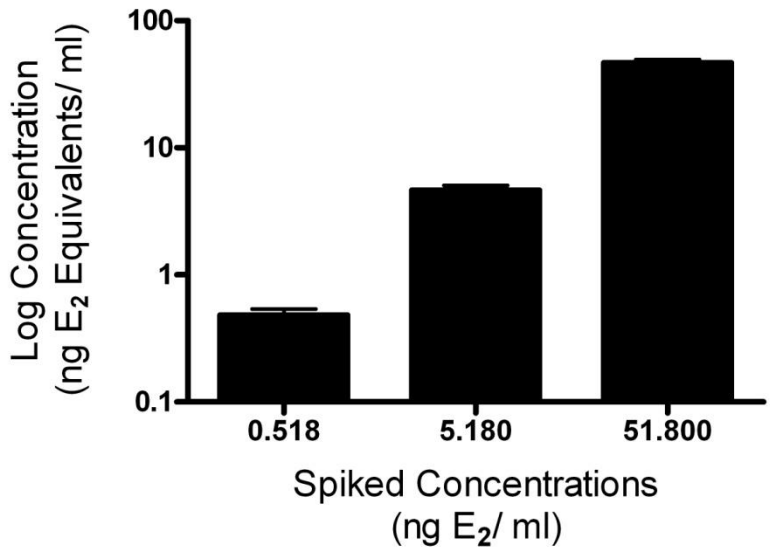

Figure 3.1. Bioassay results of distilled water spiked with known amounts of E_2.

3.1.2 Wastewater Extract Storage Test

Figure 3.2 shows that raw influent extracts may be stored up to 6 months without significant changes in the EEQ value. In contrast, the extracts of the final effluent showed dramatic increases in the EEQ level after a 5-month storage period.

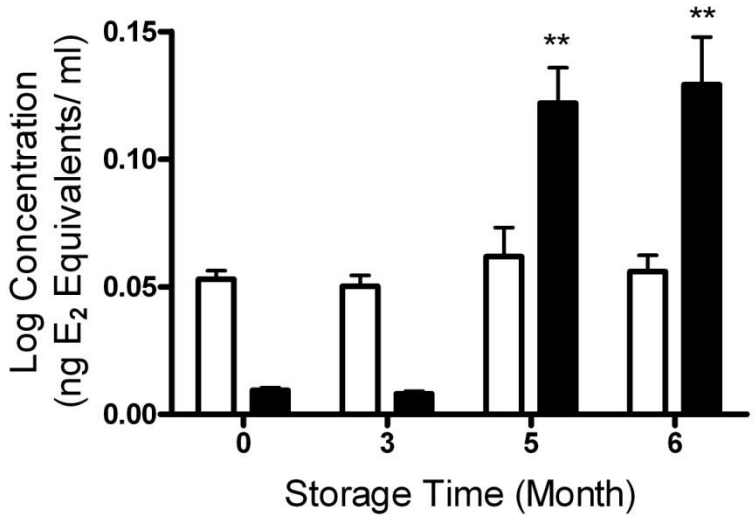

Figure 3.2. Bioassay results of wastewater extract storage
□, Raw Influent Extracts; ■, Final Effluent Extracts.
**** $p < 0.001$, significantly different to the extracts without storage.**

3.1.3 Storage of biosolid extracts

Four different kinds of biosolids were examined in this study: TSPS, TWSS, DSST, and BSD. The EEQs of TSPS and BSD remained relatively unchanged up to 2 weeks of storage. In contrast, the EEQs in the TWSS and DSST extracts were significantly changed only after 1 week of storage (Figure 3.3).

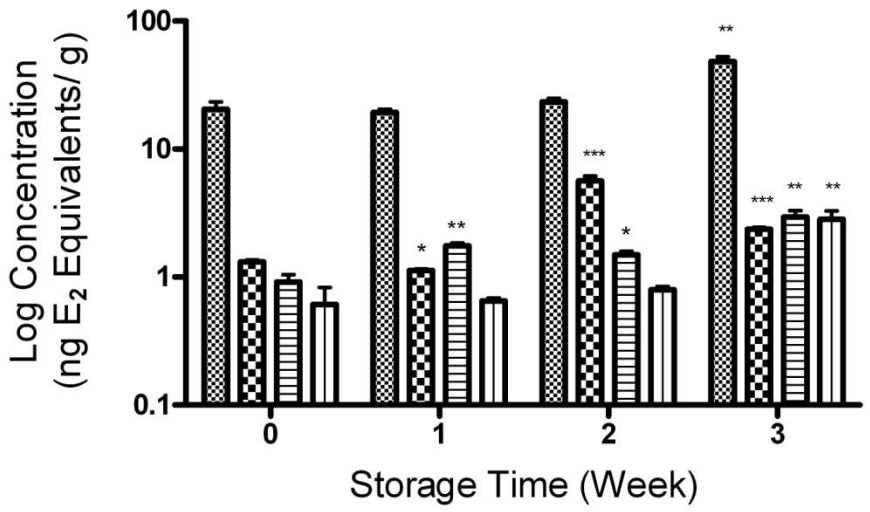

Figure 3.3. Bioassay results of biosolid extract storage:
, TSPS Extracts; , TWSS Extracts; , DSST Extracts; , BSD Extracts.
***p* < 0.05, significantly different to the extracts without storage.**
****p* < 0.001, significantly different to the extracts without storage.**
*****p* < 0.0001, significantly different to the extracts without storage.**

3.2 Wastewater analysis results

3.2.1 EEQs of raw influents

Figure 3.4 shows a comparison of the EEQ levels among the raw influents from different WWTPs. Site W had the lowest level of EEQ at 8.18E-4 ng/ml. Site A, at 7.39E-3 ng/ml, had the second lowest EEQ level among all sites. Both sites W and A had very low EEQ levels when compared to the raw influents of the other sites. In contrast, site D had the highest EEQ levels followed by site B; they were 3.84E-1 ng/ml and 1.53E-1 ng/ml, respectively.

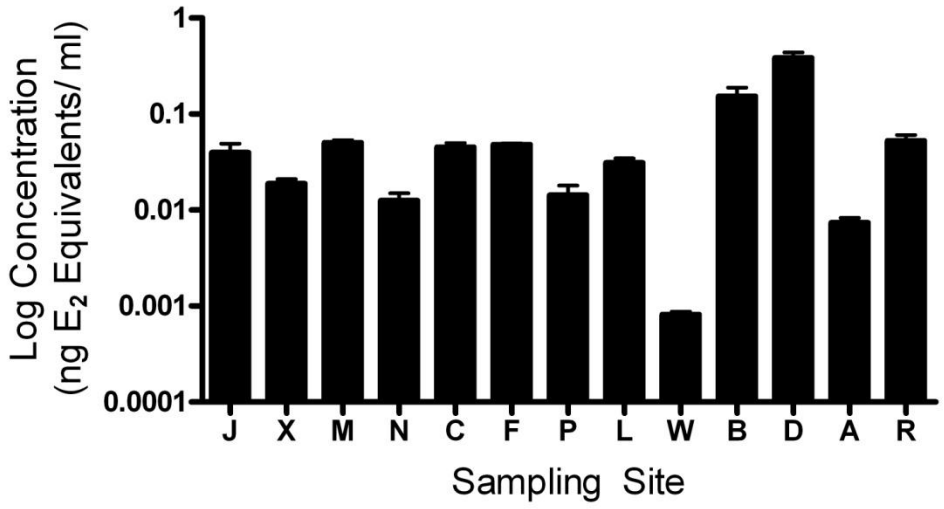

Figure 3.4. EEQs of raw influent samples from Canadian WWTPs

Figure 3.5 shows the seasonal variation of EEQs in raw influents. We received both winter and summer influent samples from only 6 sampling sites. Our results showed that site B and D did not show any seasonal variation in their EEQ levels. In contrast, all other sites including X, N, A, and R showed significantly different EEQs in the summer and winter samples. It was interesting to note that while the EEQs of summer samples at sites X and N were higher than those of the winter samples, the opposite was observed at sites A and R.

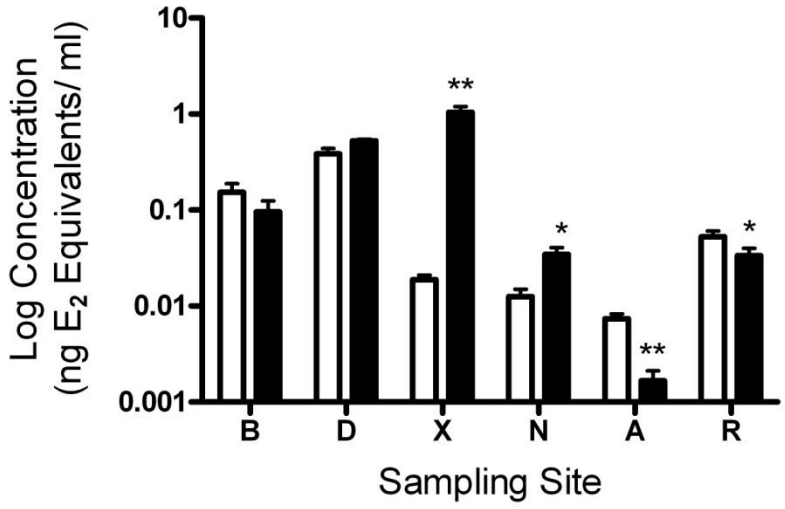

Figure 3.5. Seasonal variations of raw influent samples at different WWTPs
▭ , Winter Samples; ▬, Summer Samples.
***p < 0.05, significantly different to winter samples.**
****p < 0.001, significantly different to winter samples.**

3.2.2 EEQ levels of primary effluent samples from different WWTPs:

Only 7 primary effluent samples were received from the 13 WWTPs selected for the present study. A comparison of the EEQ levels in the primary effluent samples is shown in Figure 3.6. The primary effluent from site W had the lowest level of EEQs (6.41E-3 ng/ml). In contrast, site L had the highest EEQs (6.64E-2 ng/ml).

The EEQs in the primary effluents also were compared with the EEQs in the respective raw influent samples (Figure 3.6). The treatments at sites C, F, P, and R were able to lower the EEQs in the primary effluents. However, only site C and R showed a significant decrease in the EEQ level. In contrast, sites L, W, and A showed significant increases of EEQ levels in the primary effluent samples. An increase of the primary effluent EEQ concentration instead of a

decrease was unexpected because WWTP treatment was supposed to remove estrogenic chemicals from the raw influent.

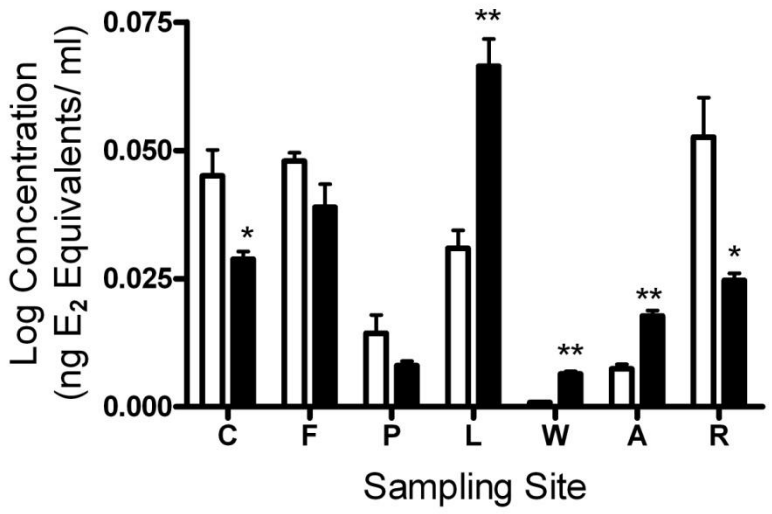

Figure 3.6. EEQs of primary effluent versus raw influent from different WWTPs: ▭, Raw Influent Samples; ▬, Primary Effluent Samples.
***p* < 0.05, significantly different to raw influent EEQs.**
****p* < 0.001, significantly different to raw influent EEQs.**

We also examined the seasonal variation of the EEQs in the primary effluent (Figure 3.7). Our results showed that summer samples from site A had significantly lower EEQ levels than the winter samples. However the opposite was observed for site R.

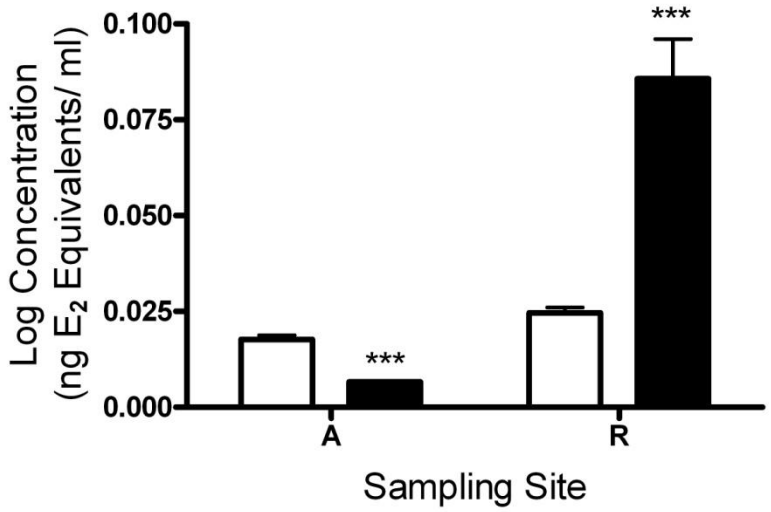

Figure 3.7. Seasonal variations of primary effluent from Canadian WWTPs: ▭, Winter Samples; ▬, Summer Samples.
*****p < 0.0001, significantly different to winter samples.**

3.2.3 EEQ levels in the final effluent from different WWTPs in Canada:

Figure 3.8 summarizes the EEQs in the final effluents of Canadian WWTPs. Site J had the highest EEQ level amongst all the sampling sites in Canada. Surprisingly, the final effluent of site J had a 10 fold increase in the EEQ level when compare to the raw influent. On the other hand, the final effluent at site B showed the lowest EEQ level compare to the other Canadian WWTPs. There is a 30 fold decrease of EEQ level in the final effluent of site B compared to the raw influent.

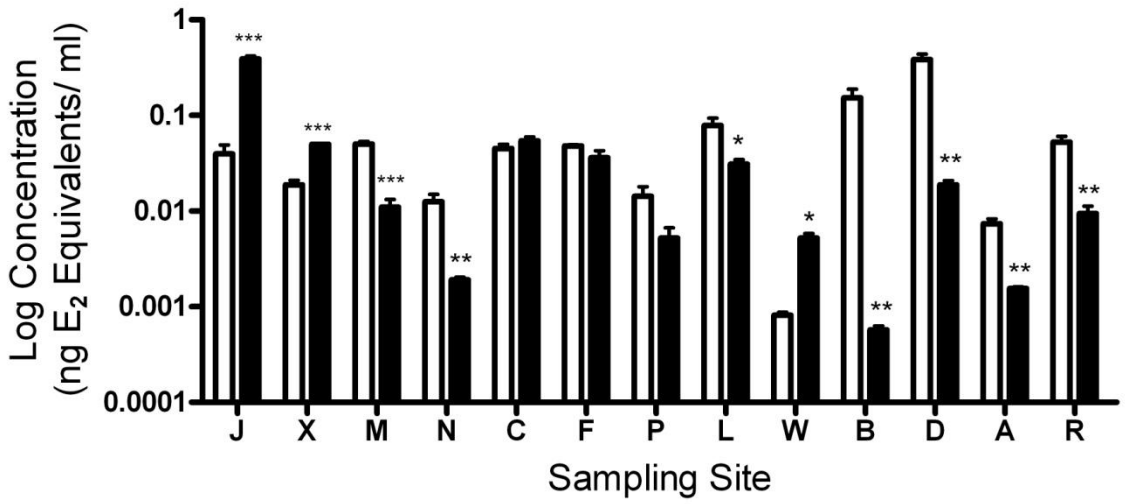

Figure 3.8. EEQs of final effluent versus raw influent in Canadian WWTPs:
▭ , Raw Influent samples; ▬, Final Effluent Samples.
***p* < 0.05, significantly different to the EEQs in raw influent.**
****p* < 0.001, significantly different to the EEQs in raw influent.**
*****p* < 0.0001, significantly different to the EEQs in raw influent.**

Seasonal variation of EEQ levels in the final effluent appeared to be quite consistent; summer EEQs were significantly higher than the winter EEQs with the exception of site B (Figure 3.9). The summer and winter EEQs at site B differed only by about 2 %, seasonal changes did not appear to affect the EEQ levels at this site.

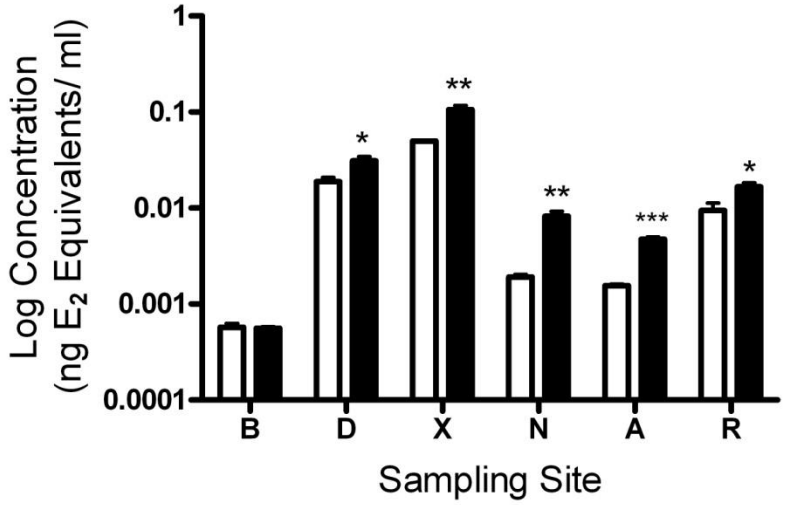

Figure 3.9. Seasonal variations of final effluent from different WWTPs:
, Winter samples; , Summer Samples.
***p* < 0.05, significantly higher than winter samples.**
****p* < 0.001, significantly higher than winter samples.**
*****p* < 0.0001, significantly higher than winter samples.**

3.3 Biosolid EEQ results

Figure 3.10 shows the biosolid EEQ results. Sites W and B had low EEQ levels; they were 1.57 and 1.67 ng/g, respectively. In contrast, site L had the highest EEQ level (24.6 ng/g) among all the WWTP sites

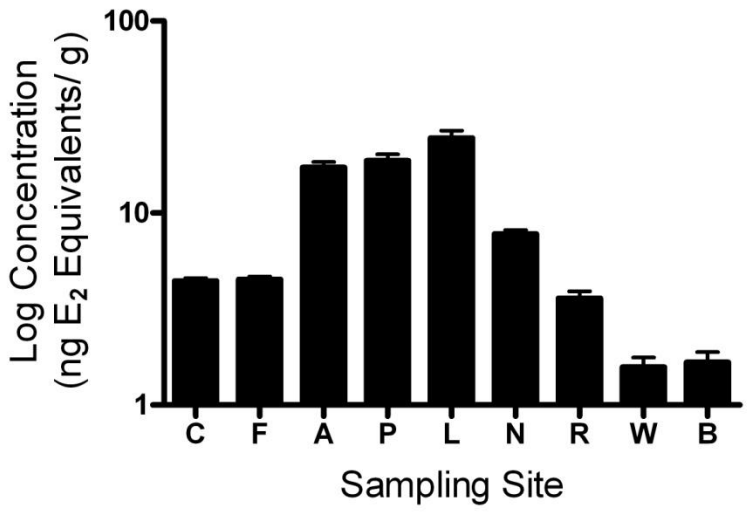

Figure 3.10. Bioassay results of biosolid samples from different WWTPs.

Figure 3.11 shows the seasonal variation of estrogenic activity in the biosolid samples. Summer sample from sites R had significantly lower estrogenic activities than the winter sample. In contrast, summer sample from sites A showed significantly higher estrogenic activities in the summer sample.

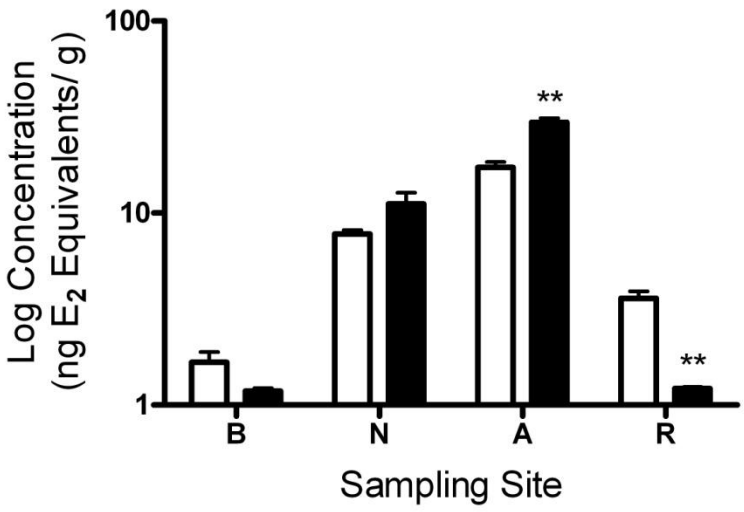

Figure 3.11. Seasonal variations of biosolid samples from different WWTPs:

, Winter Samples; **, Summer Samples.**

****p* < 0.001, significantly different to the winter samples.**

CHAPTER 4 DISCUSSION

4.1 Reproducibility of the YES bioassay and the effects of storage on extract activities

The limit of detection (LOD) for E_2 in the YES bioassay is assumed to be 8.7E-3 ng/ml, the EC_{20} of the E_2 standard. The LOD reported for E_2 by the YES bioassay range from 1.0E-5-1.5E-2 ng/ml (Wozei and Hermanowicz, 2006). Our results are in agreement with the findings of these studies. The accuracy and precision of the YES bioassay for E_2 are studied using distilled water samples spiked with the E_2 standard. The accuracy of the YES bioassay is >90% because the measured EEQs generally are 10% lower than the actual amounts of E_2 added to the samples (Figure 3.1). The CV% of the E2-spiked samples is used to determine the precision of the YES bioassay; it is about 10% (Figure 3.1). Therefore, the YES bioassay is an accurate and precise method for determining E_2 in distilled water. However, the

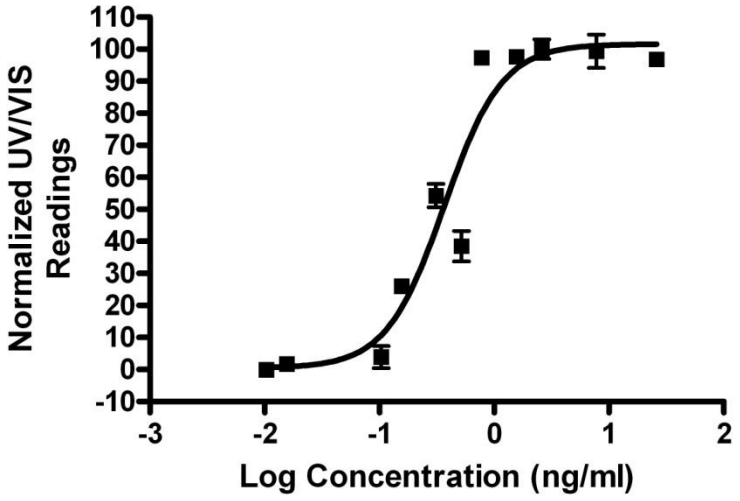

Figure 4.1. A typical pure E_2 standard curve of the YES bioassay

distilled water samples are not the ideal surrogates of the WWTP sample. For example, although the distilled water sample yields a dose-response curve very similar to that of the E_2 standard (Figure 4.1), the wastewater samples show a dose-response curve with different slope and maximum (Figure 4.2). The distilled water and wastewater samples give a different dose-

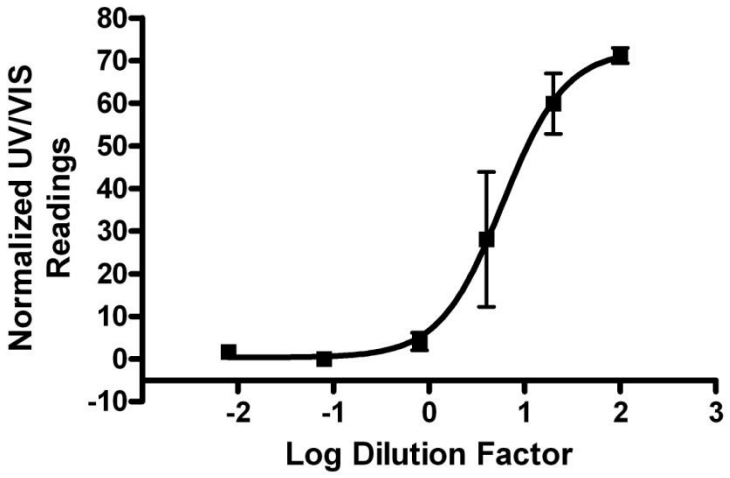

Figure 4.2. A typical WWTP sample dose response curve of the YES bioassay

response curve probably because of the interaction effects of the estrogenic chemicals. Thus the estrogenic chemicals in wastewaters and biosolids may interact pharmacokinetically resulting in an increase or inhibition of the total estrogenic activity (Villeneuve et al. 2000). Because of the interaction, the EEQs of the WWTP samples are calculated using the average values of $EC_{20,}$ EC_{30} and EC_{50} (Hilscherova et al. 2002).

4.1.1 Effects of storage on EEQs

Because the WWTP samples are stored for a few days before analysis, the concern is that the estrogenic chemicals in the samples may degrade over time, yielding erroneous results. Figures 3.2 and 3.3 show the total estrogenic activity in the extracts of the WWTP samples increase only after they are stored for a prolonged time period. Surprisingly, the estrogenic activities of the samples are increased rather than decreased. An explanation for an increased EEQ value in the stored extracts is not readily available but is probably related to the non-enzymatic as well as enzymatic de-conjugation of the conjugated estrogenic metabolites. Other plausible explanation may be that the chemical composition of the extracts is changed after a prolonged storage period and the estrogenic chemicals interact synergistically in the bioassay. Figure 3.2 shows that the EEQs in the raw influent extracts remain unchanged after a 6-month storage period but the EEQs in the final effluents increase dramatically after a 5-month storage period. In comparison, the EEQs in the biosolid extracts remain unchanged only for 2 or 3 week duration. An exception being the TWSS (thickened wasted secondary sludge) extract of which the EEQs increase by almost 5 folds only after a 2-week storage period (Figure 3.3). Apparently, wastewater extracts can be stored for a longer period than biosolid extracts. An explanation for the different storage effects on wastewater and biosolid extracts is not available but may be due to the high estrogenic chemical contents (Figure 3.10) and microorganism counts in the biosolids. As a result, de-conjugation of estrogenic metabolites and synergistic interactions may occur simultaneously in the biosolid extracts and shorten the storage time of these extracts.

4.2 Interpretation of water/biosolid analysis results:

4.2.1 Raw influents

Our results showed that the total estrogenic activities in raw influents do not always reflect the number of people living around the WWTPs. Although the raw influent from site D has the highest EEQs, the population in the area is small (about 4,800). In contrast, although sites W and A have the lowest EEQs in their raw influents, the population counts at sites W and A are about 52,400 and 1 million respectively (Environment Canada, 2011). This comparison indicates that, in addition to human wastes, pharmaceutical and household product wastes also may contribute to the EEQ levels of raw influents. As mentioned in the "Introduction", estrogenic chemicals include all substances that can bind to the estrogen receptor. Thus the estrogenic contaminants may come from different sources although human wastes remain the primary contributor of estrogenic chemicals in WWTPs.

4.2.2 Final effluent

We also compare the EEQs in the final effluents with those reported in other countries (Table 4.1). Our results show that the highest EEQ site in Canada has an EEQ level very similar to that of the Lafayette County, USA which in turn is about 10 fold higher than the other sites in the world with the exception of SEQ, Australia. The lowest Canadian EEQ site is close to Ria de Aveiro, Portugal. Together these results show that the EEQs in the present study are consistent with those reported elsewhere in the world. Our final effluent results (Figure 3.8) show that

Location	EEQ (ng/ml)	Source
Canadian WWTPs	5.73E-04 - 3.90E-01	Current Study
Ria de Aveiro Protugal	5.0E-04 - 8.4E-02	Sousa et al. 2010
SEQ, Australia	2.46E+00 - 6.58E+00	Ying et al. 2009
Rhine Neckar Triangle Germany	3.24E-02 - 5.14E-02	Pawlowski et al. 2004
Lafayette County, USA	2.10E-02 - 1.47E-01	Tilton et al. 2002

Table 4.1. A comparison of EEQ levels in the final effluent of Canada with other countries

treatment methods in Canadian WWTPs may increase or decrease the estrogenic activities in the raw influents. For instance, sites M, N, B, D, A, and R all show significant reduction of estrogenic activities in the final effluent whereas sites J, X, and W show significant increases in estrogenic activities.

4.2.2.1. Sites with a decreased final effluent EEQs

4.2.2.1.1. Both site M and N (Table 1.1) use the primary chemical assist method to treat the wastewater. This treatment method involves the addition of a coagulant to improve the efficiency of dirt and debris settling in the wastewater (Westerhoff, 2003, and Kanokkantapong et al. 2008)**.** The coagulant added is also able to trap lipophilic chemicals along with the dirt and debris (Westerhoff, 2003, and Kanokkantapong et al. 2008). Because the majority of the estrogenic chemicals are lipophilic chemicals; the coagulant is able to lower the estrogenic activities of the wastewater. Up to 20% of the estrogenic activities in the raw influents can be removed by the coagulant (Westerhoff, 2003).

4,2,2,1,2. Microorganisms such as bacteria and fungi used by the WWTPs to remove nutrients are also known to metabolize a variety of EDCs in wastewater (Liu et al. 2008, and

Shokrollahzadeh et al. 2007). It has been shown that the microbial digestion process is able to remove about 30 – 70% of the estrogenic activities from wastewater (Johnson et al. 2007). Site B (Table 1.1) uses an advanced biological nutrient removal method which relies heavily on microorganisms to remove nutrients from the wastewater. This treatment method is able to metabolize various estrogenic chemicals to a less potent compound and may explain why it is able to drastically reduce the estrogenic activity of the raw influent (Figure 3.8). Sites D, R, and A (Table 1.1) all apply artificially circulating air into the wastewater during the treatment process to enhance microbial growth (Metro Vancouver, 2011). This may explain why the estrogenic activities in the wastewater are reduced. Site L uses a secondary, enhanced aerated filter which allows the wastewater to pass through a biological filter for nutrient removal (Metro Vancouver, 2011); this probably contributes to the reduction of estrogenic activity observed in the final effluents (about 60.0% removal). Overall, the advanced biological removal treatment method for site B appear to be the most efficient treatment by which the estrogenic activity in the final effluent can be lowered by >30 fold.

4.2.2.2 Sites with an increased final effluent EEQs

4.2.2.2.1. High levels of dissolved organic carbons (DOCs) generally are associated with the wastewater before treatment. Because the estrogenic chemicals are lipophilic, they can bind to the DOCs and thus are not bioavailable. The lagoon facultative treatment method used by sites J and X (Table 1.1) can remove the DOCs in the wastewater (Metro Vancouver, 2011) and release the bound estrogenic chemicals to free chemicals making them more bioavailable to the YES bioassay. Although all lagoon treatment methods effectively reduce the DOCs in the wastewater, there is no artificial circulation of air into the wastewater to promote microorganism growth in

this treatment method. Thus a lack of enhanced microbial growth in combination with the release of free estrogenic chemicals from DOCs may explain why there is an increase in the estrogenic activity in the wastewater of the lagoon facultative method (Figure 3.8).

4.2.2.2.2 The secondary, high purity oxygen (HPO) activated sludge treatment method used by site W (Table 1.1) also increases the estrogenic activities in the wastewater. This treatment method uses highly purified oxygen to oxygenate the wastewater before allowing the wastewater to enter the activated sludge tank for nutrient digestion (Metro Vancouver, 2011)**.** The addition of high purity oxygen to wastewater is different from circulating air into the wastewater as air contains other gasses besides oxygen. The high purity oxygen may eliminate certain microbes that are not suited to living in a condition of high level oxygen. Thus an increased estrogenic activity in this treatment method (Figure 3.8) may be explained by the interaction between high purity oxygen and the estrogenic chemicals in the wastewater.

4.2.3 Biosolids

The estrogenic activities found in the biosolids of WWTPs using the mesophilic anaerobic digestion method are different (Figure 3.10). For instance, the EEQs in the biosolids of site W and B are extremely low whereas the EEQs in the biosolids of sites L and P are very high. These results probably can be explained by the uneven distribution of estrogenic chemicals in the storage tanks rather than the treatment efficiency of the WWTPs. Thus it is important to improve the sampling protocols in the future by collecting multiple samples from different areas of the storage tank and combining them into a composite sample. The proposed sampling protocols should provide a more representative or less bias sampling strategy than the one currently used.

Other plausible explanation for the different biosolid EEQ results may be related to the operating conditions of the WWTPs *e.g.*, temperature, duration, etc. However, this is highly unlikely because of the large difference in EEQ values between the biosolid samples.

4.2.4 Seasonal variations of the sample

Seasonal variation of estrogenic activity is observed in the raw influent samples (Figure 3.5). This may be due to environmental factors such as population fluctuation, temperature effects, microorganism growth and other unknown anthropogenic effects. In contrast, nearly all final effluent samples show a higher or slightly higher EEQ level in the summer than the winter (Figure 3.9). An exception is site B which shows almost no seasonal variation. These results are explainable by the enzymatic activities of the microorganisms which de-conjugate the phase II metabolite(s) of the estrogenic chemicals in the final effluents (Lee, et al. 2004); because while some microorganisms metabolize the estrogenic chemicals by conjugation other microorganisms may facilitate the de-conjugation reaction (Lee, et. al. 2004). It should be noted that while conjugated estrogenic chemicals are not bioavailable to the yeast cells, de-conjugated or free metabolites are bioavailable and can be absorbed by the yeast cells. Perhaps, due to a rise in ambient temperature in the summer, de-conjugation reaction in the microorganism is enhanced releasing more free estrogenic chemicals into the final effluent. Since the exact composition of the microorganisms in the final effluent samples remains unknown, we can only assume that more chemicals undergo de-conjugation reaction than conjugation reaction. Fernandez et al. (2008) and Sousa et al. (2009) have shown that the total estrogenic activities in wastewater samples are increased at high temperature. Our results are in agreement with their observation. With respect to the absence of EEQ seasonal variation at site B, we can only assume that the

level of conjugated estrogenic chemicals in this site is low. The biosoild samples also show seasonal variation in estrogenic activities (Figure 3.11). However, it is hard to explain why the estrogenic activity at site R is lower in the summer. Perhaps, the seasonal effects observed are due to the uneven distribution of estrogenic compounds in the storage tank rather than the different temperature in the winter and summer.

4.3 Conclusion

The YES bioassay is an accurate and precise method of quantifying E_2 in distilled water samples; it can also be used to determine the total estrogenic activities of wastewater and biosolid samples. However, the duration by which the extracts can be stored varies with the type of environmental samples. In general, the extract of wastewater can be stored for a longer time than that of the biosolids. To ensure data integrity, WWTP samples should be extracted and analyzed as soon as they are received.

Although the WWTPs in Canada are not designed for the removal of estrogenic chemicals, a majority of the WWTPs is able to reduce a significant amount of EEQs from the raw influents. The treatment methods involving extensive microbial nutrient digestion appear to be quite efficient in removing estrogenic chemicals from the raw influents. Thus future studies should focus on the development of a microorganism-based treatment method for the removal of estrogenic chemicals from the raw influents.

APPENDICES

A. YES BIOASSAY RESULT SUMMARY

Bioassay Results for Winter and Summer Raw Influent:

Site	Winter Raw Influent EEQ (ng/ml)	Standard Deviation	90% CI
J	3.96E-02	1.69E-02	1.61E-02
X	1.89E-02	3.72E-03	3.53E-03
M	5.02E-02	5.50E-03	5.22E-03
N	1.25E-02	4.28E-03	4.07E-03
C	4.50E-02	8.76E-03	8.32E-03
F	4.79E-02	2.88E-03	2.73E-03
P	1.43E-02	6.23E-03	5.91E-03
L	7.82E-02	2.72E-02	2.58E-02
W	8.18E-04	9.67E-05	9.18E-05
B	1.53E-01	6.11E-02	5.81E-02
D	3.84E-01	9.11E-02	8.65E-02
A	7.39E-03	1.55E-03	1.47E-03
R	5.26E-02	1.34E-02	1.27E-02
Site	**Summer Raw Influent EEQ (ng/ml)**	**Standard Deviation**	**90% CI**
B	9.58E-02	4.94E-02	4.69E-02
D	5.28E-01	3.77E-02	3.58E-02
X	1.04E+00	2.78E-01	2.64E-01
N	3.47E-02	1.03E-02	9.77E-03
A	1.67E-03	7.65E-04	7.26E-04
R	3.34E-02	1.12E-02	1.07E-02

Bioassay Results for Winter and Summer Primary Effluent:

Site	Winter Primary Effluent EEQ (ng/ml)	Standard Deviation	90% CI
C	2.88E-02	2.57E-03	2.44E-03
F	3.89E-02	7.84E-03	7.44E-03
P	8.01E-03	1.61E-03	1.53E-03
L	6.64E-02	9.21E-03	8.74E-03
W	6.41E-03	8.82E-04	8.37E-04
A	1.77E-02	1.79E-03	1.70E-03
R	2.46E-02	2.34E-03	2.22E-03
Site	**Summer Primary Effluent EEQ (ng/ml)**	**Standard Devation**	**90% CI**
A	6.64E-03	1.69E-04	1.60E-04
R	8.57E-02	1.79E-02	1.70E-02

Bioassay Results for Winter and Summer Final Effluent:

Site	Winter Final Effluent EEQ (ng/ml)	Standard Deviation	90% CI
J	3.90E-01	4.59E-02	4.36E-02
X	4.98E-02	1.46E-03	1.39E-03
M	1.10E-02	3.79E-03	3.59E-03
N	1.92E-03	2.00E-04	1.90E-04
C	5.41E-02	9.44E-03	8.96E-03
F	3.62E-02	1.12E-02	1.07E-02
P	5.25E-03	2.50E-03	2.38E-03
L	3.09E-02	6.09E-03	5.79E-03
W	5.24E-03	1.05E-03	9.96E-04
B	5.73E-04	1.00E-04	9.51E-05
D	1.89E-02	3.32E-03	3.15E-03
A	1.55E-03	1.06E-04	1.01E-04
R	9.46E-03	3.16E-03	3.00E-03
Site	**Summer Final Effluent EEQ (ng/ml)**	**Standard Deviation**	**90% CI**
B	5.60E-04	3.54E-05	3.37E-05
D	3.09E-02	5.91E-03	5.61E-03
X	1.06E-01	1.95E-02	1.85E-02
N	8.21E-03	1.81E-03	1.72E-03
A	4.74E-03	4.54E-04	4.31E-04
R	1.67E-02	2.68E-03	2.54E-03

Bioassay Results for Winter and Summer Biosolid:

Site	Winter Biosolid EEQ (ng/g)	Standard Deviation	95% CI
C	4.42E+00	2.73E-01	2.59E-01
F	4.52E+00	2.78E-01	2.64E-01
A	1.72E+01	2.08E+00	1.98E+00
P	1.87E+01	2.60E+00	2.47E+00
L	2.46E+01	3.94E+00	3.75E+00
N	7.78E+00	6.15E-01	5.84E-01
R	3.60E+00	5.41E-01	5.13E-01
W	1.57E+00	3.27E-01	3.10E-01
B	1.67E+00	3.77E-01	3.58E-01
Site	**Summer Biosolid EEQ (ng/g)**	**Standard Deviation**	**95% CI**
B	1.18E+00	7.02E-02	6.67E-02
N	1.11E+01	2.79E+00	2.65E+00
A	2.97E+01	2.57E+00	2.45E+00
R	1.22E+00	3.47E-02	3.29E-02

B. REAGENT PREPARATION

1. 10 mM Copper Sulphate

- Weigh out 0.25 g copper sulfate pentahydrate.
- Add 100 ml with distilled water.
- Filter sterilize with 100 ml-0.2 µm filter unit.

2. 10% SDS

- Weigh out 10 g sodium lauryl sulfate
- Add 100 ml distilled water
- Transfer to 100 ml sterile glass bottle.

3. 1 M Sodium Chloride

- Weigh out 58.44 g sodium chloride
- Add 1000 ml distilled water.
- Filter sterilize with 1000 ml-0.2 µm filter unit.

4. Oxalyticase - Lot no. L187F, 50,000 units/mg, 5 mg = 250000 U total

- Make a 200U/µl solution by mixing 1.25 ml of 0.1 M NaCl and 50% Glycerol solution
- Store at 4°C.

5. Z Buffer

- Weigh out: 16.1 g Na_2HPO_4
  5.5 g NaH_2PO_4
  0.75 g KCl
  0.25 g $MgSO_4$
- Mix all the reagents with 800 ml of distilled water.
- Adjust pH to 7.0 while stirring with stir bar on stir plate.
- Bring up the solution to 1000 ml with distilled water.
- Filter sterilize with 1000 ml-0.2 µm filter unit.

6. Amino Acids

- Dissolve LYS-1.8g L-lysine-HCl in 500 ml of distilled water. Sterilize with 500 ml-0.2 µm filter unit.
- Dissolve HIS-1.2 g L-histidine-HCl in 500 ml of distilled water. Sterilize with 500ml-0.2µm-filterunit.

7. Preparation of Yeast growth medium for:

- NaH_2PO_4 (Sodium Phosphate, monobasic) 1.73 g
- Na_2HPO_4 (Sodium Phosphate, dibasic) 1.77 g
- (NH4)2SO4 (Ammonium Sulphate) 2.5 g
- Y.N Base (Yeast Nitrogen Base) 0.85 g
- Dextrose 10.0 g
- Lysine 5.0 ml
- Histidine 5.0 ml
- Distilled Water 490 ml

Mix well and autoclave for 1 hour

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