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# The use of *in vitro* bioassays to quantify endocrine disrupting chemicals in municipal wastewater treatment plant effluents

Jasen Nelson [a], Farida Bishay [b], Albert van Roodselaar [b], Michael Ikonomou [c], Francis C.P. Law [a,*]

[a] *Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby, BC, Canada V5A 1S6*
[b] *Greater Vancouver Regional District, 4330 Kingsway, Burnaby, BC, Canada V5H 4G8*
[c] *Institute of Ocean Sciences, 9860 West Saanich Road, Sidney, BC, Canada V8L 4B2*

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**Abstract**

*In vitro* bioassays are widely used to detect and quantify endocrine disrupting chemicals (EDCs) in the influents and effluents of municipal wastewater treatment plants (WWTP). These assays have sometimes led to false positive or negative results, partly due to the low EDC concentrations in the samples. The objectives of the present study were: (a) to compare the estrogen screen (E-Screen) and the yeast estrogen screen (YES) bioassays using the 17β-estradiol (E2) or its equivalence and (b) to investigate if a combination of the E-Screen and YES assays can be used to improve the accuracy of EDC detection and quantification. The E-Screen bioassay was conducted with the MCF-7 (BOS) human breast cancer cell line while the YES bioassay employed two different types of recombinant yeast. The influent and effluent samples collected from the five WWTPs operated by the Greater Vancouver Regional District (GVRD) were analyzed by both the E-Screen and the YES bioassays. Since the results of the E-Screen and YES bioassays varied by up to 4-fold on the same split sample of a nominal E2 concentration, the mean value of the E-screen and YES bioassays was used to represent the EDC activity of a given WWTP sample. Results of these studies showed that the E2 equivalent concentration in each WWTP sample was consistently higher than 1 ng/L, a concentration that may potentially cause endocrine disruption in different aquatic species. The composition of selected EDCs in a subset of effluent samples was examined using a gas chromatograph–high resolution mass spectrometer (GC–HRMS). EDC composition in 10 WWTP samples correlated with the mean endocrine disrupting activities of the E-Screen and YES bioassays. Results also indicated that secondary treatment plants are comparable to the primary treatment plants in removing EDCs from the final effluents.


*Keywords:* MCF-7; YES; Endocrine; Wastewater; Treatment

## 1. Introduction

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

* Corresponding author. Tel.: +1 604 291 4285; fax: +1 604 291 3496.
*E-mail address:* flaw@sfu.ca (F.C.P. Law).

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discharges of wastewater treatment plant (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 (*Rutulis rutulis*) and flounder (*Platichthys flesus*) caught near WWTP discharge sites (Jobling et al., 1998; Allen et al., 1999). Among the fish sampled in watersheds receiving WWTP discharges, about 70% of the fish were female (Hansen et al., 1998). 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).

The Greater Vancouver Regional District (GVRD) is committed to a receiving environment monitoring (REM) approach of managing its liquid waste discharges in its Liquid Waste Management Plan (GVRD, 2001). This monitoring approach for the receiving environment of all five of GVRD's Wastewater Treatment Plants includes the characterization of WWTP effluent to define the nature of the effluent and potential effects. Within the GVRD there are two primary WWTPs that discharge into the marine environment, and three secondary WWTPs that discharge into the Fraser River (Fig. 1). Primary

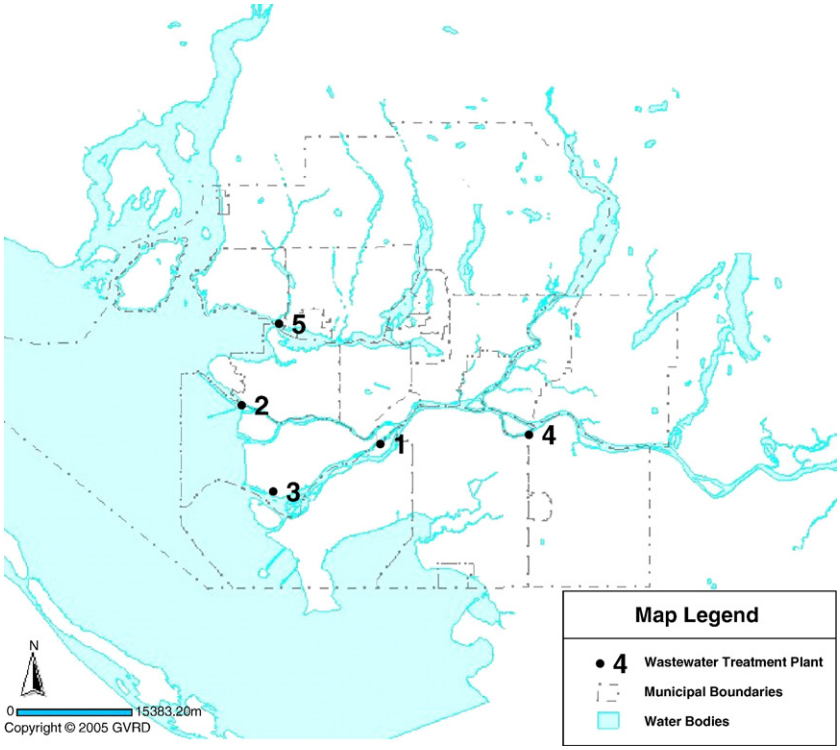


Fig. 1. Wastewater treatment plants of the Greater Vancouver Regional District (modified from GVRD, 2005).

treatment is a mainly mechanical process that removes between 30 and 40% of biological oxygen demand (BOD) and 50% of the total suspended solids (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 of which are at or below the detection limits (Folmar et al., 2002; Desbrow et al., 1998). However, there is a potential for even these low concentrations of EDCs to affect the endocrine system in fish. Therefore, it is important to assess the potential additive or synergistic effects of EDCs at these low concentrations because it may provide more information on the potential for effects in the receiving environment.

Hitherto, there is no consensus among scientists on the best screening methods for determining EDC activities in the aquatic environment. 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). The objectives of the present study were: (a) to compare the estrogenic potencies of 17β-estradiol (E2) using the E-Screen and the YES bioassays, (b) to determine the estrogenic potencies of the influents and effluents in the five municipal WWTPs operated by the GVRD using a combination of E-Screen and YES bioassays, (c) to identify and quantify selected EDCs in a subset of WWTP samples using gas chromatograph–high resolution mass spectrometry (GC–HRMS), and (d) compare calculated endocrine activity from GC–HRMS with measured activity from E-screen and YES bioassays.

## 2. Sample collection and preparation

The larger of the two primary plants (WWTP 2) provides primary treatment to wastewater from approximately 600,000 Vancouver residents 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 liters per day (MLD) with average total suspended solids (TSS) and biological oxygen demand (BOD) of 48 mg/L and 76 mg/L, respectively (GVRD, 2004).

The other primary plant (WWTP 5) provides treatment to wastewater from approximately 160,000 residents and discharges to the ocean, approximately 200 m offshore and 20 m deep. In 2003, the average annual flow was 92 MLD with average TSS and BOD of 54 mg/L and 89 mg/L, respectively (GVRD, 2004).

The largest of the three secondary plants is WWTP 1, which provides treatment to wastewater from approximately 740,000 residents. In 2003, the average annual flow was 485 MLD with average TSS and BOD of 12 mg/L and 13 mg/L, respectively (GVRD, 2004).

The second largest secondary plant is WWTP 3 which provides treatment to wastewater from approximately 120,000 residents. In 2003, the average annual flow was 79 MLD with average TSS and BOD of 8 mg/L and 12 mg/L, respectively (GVRD, 2004).

The third largest secondary plant is WWTP 4 which provides treatment to wastewater from 20,000 residents. In 2003, the average annual flow was 9.2 MLD with average TSS and BOD of <13 mg/L and 11 mg/L, respectively (GVRD, 2004).

### 2.1. Sample collection

WWTP raw influent (untreated) and effluent (treated) samples were collected at each of the five WWTPs (Fig. 1) operated by the GVRD. Grab and 24-h composite samples were collected at each site, throughout the year, the first grab and composite sets (April to May and May to June, respectively) and second grab and composite sets (September to October and October to November, respectively) were collected prior to and after the dry season, respectively. All glassware/funnels were triple rinsed with methanol, then 0.6% HCl in DCM and allowed to dry prior to sample collection. Grab influent and effluent WWTP samples were collected in the morning, using either a bottle sampler with pre-cleaned 1 L glass bottle or stainless steel bucket, and transferred through pre-cleaned stainless steel funnels to 4 L amber glass bottles. Composite samples were collected using flow proportional SIRCO samplers (WWTPs 1, 2, 3 and 5) or a time proportional ISCO sampler (WWTP 4) fitted with glass, Teflon and stainless steel components over a 24-h period either from midnight to midnight (WWTP 1 and 2) or 0700 to 0700 h (WWTPs 3, 4 and 5).

### 2.2. Sample extraction

Samples were extracted on the same day of sample collection. Each 4 L sample was split into 4 aliquots (1 L), and each transferred into its own 2 L separatory funnel containing 60 mL of DCM. The separatory funnel was mixed for 5 min and the layers were allowed to separate for 15 min. The bottom DCM layer was removed, combined and concentrated down to about 4 mL using ROTAVAP and N-EVAP evaporators (Organomation Associates, Berlin, MA, USA) at 40 °C. A 2 mL aliquot was removed and solvent exchanged into ethanol (anhydrous) for bioassay exposures. 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 analysis. Upon arrival at IOS, the samples were reconstituted in DCM. A 0.5 mL aliquot was removed for analysis. A 50 μL 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 high-resolution gas chromatograph–high-resolution mass spectrometry (GC–HRMS) analysis. Details on the methodology used and the criteria used for target analyte identification and quantification are reported elsewhere (Ikonomou et al., submitted for publication).

## 3. Analytical methods

### 3.1. EDC bioassays

E-Screen bioassay was performed according to the procedure of Soto et al. (2004) with modification. YES bioassay was conducted using two different recombinant yeast strains. The procedure of Lorenzen et al. (2004) was used to conduct the YES bioassay of the *S. cerevisiae* strain BJ3505. The procedure of Routledge and Sumpter (1996) was used to conduct the YES bioassay with *S. cerevisiae* culture. Permission to use cell lines for the E-Screen (Soto and Sonnenschein, 1995) and YES (Gaido et al., 1997; Routledge and Sumpter, 1996) bioassays were obtained from the respective researchers.

### 3.2. E-Screen bioassay

The E-Screen bioassay was conducted with the MCF-7 (BOS) cells in 96-well microplates, by modifying the procedure of Soto et al. (2004). Cell counting was performed on a hemocytometer in place of a Coulter Counter. Lyticase (in place of Oxalyticase), sodium dodecyl sulfate (SDS), β-mercaptoethanol and all hormone standards were obtained from Sigma Chemical Company (St. Louis, MO, USA) with the exception of Methyltrienolone (R1881) from New England Nuclear (Perkin-Elmer Life Sciences, Boston, MA, USA).

The 17β-estradiol (E2) standard dilution series ranged from 0.001 nM to 100 nM (i.e., 11 dilutions), plus a solvent (ethanol) blank. The dilutions of the aqueous samples were attained using ethanol. The concentrations used for bioassay exposures were 10-, 1-, 0.1-, 0.01-, 0.001-times the initial concentration. Cytotoxicity was assessed by exposing sample dilutions to 1 nM E2; since this level of E2 produces maximal proliferation, decreased proliferation implies cytotoxicity. Cytotoxicity was confirmed visually using a microscope. Cytotoxicity affected the 10-fold aqueous samples, thus data analysis was limited to the 1-fold sample. An E2 equivalence quotient is obtained for the 1-fold sample based on the dose–response curve of E2. Trichloroacetic acid (TCA) fixation, sulforhodamine B (SRB) staining, and Tris buffer solubilization of dye, were performed according to Soto (1995). The 96-well microplates were scanned at 515 nm and 650 nm with a microplate reader.

### 3.3. Yeast estrogen (YES) bioassays

The E2 standard dilution series, described above, was also used for the YES assays. Standards and environmental extract dilutions were transferred (10 μL), in at least triplicate, into the microplate wells and allowed to dry (approximately 30 min).

The YES bioassays were carried out with two different variations of recombinant yeast. These bioassays are similar in that they have been transformed to include the human estrogen receptor (hER) in their main genome, estrogen response elements (ERE) and lac-Z gene on a plasmid. Essentially, the estrogenic substance binds to an 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 β-galactosidase into the medium. The two YES bioassays employ different chromogenic substances, 2-nitrophenyl-β-D-galactopyranoside (ONPG) is used in the Gaido method, while chlorophenol red-β-D-galactopyranoside (CPRG) is used in the Sumpter method. The intensity of the color is then measured by absorbance; these values are then normalized for the background activity.

A modified version of the Gaido (1997) procedure, allowing for 96-well plates (Sarstedt, Germany), with end-point spectrophotometry measurements (Lorenzen

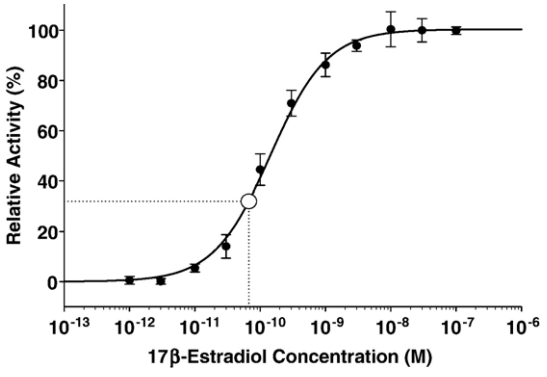


Fig. 2. A typical dose–response curve for 17β-estradiol.

Table 1
Target EDCs measured by GC–HRMS

| 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 |
| 17α-Estradiol | 5.3 | 5.3–5.3 |
| 17β-Estradiol (E2) | 4.9 | 4.9–4.9 |
| 19-Norethindrone (NET) | 11.1 | 9.4–12.6 |
| Mestranol | 6.1 | 6.1–6.1 |
| 17α-Ethynylestradiol (EE2) | 6.9 | 6.8–6.9 |
| Norgestrel | 7.1 | 5.8–9.7 |
| α-Zearalanol | 26.8 | 4.9–52.4 |
| Estriol (E3) | 5.9 | 5.9–6.0 |
| β-Estradiol-3-benzoate | 5.2 | 5.0–5.3 |
| Nonylphenol (NP) | 44.2 | 4.3–70.5 |

LOD values resulted from the analysis of 10 composite samples.

et al., 2004) was followed. Defined fetal bovine serum (FBS) and defined charcoal-dextran stripped FBS were obtained from Hyclone (Logan, Utah, USA). All chemicals and plasticware used in this assay were selected based on the standard operating procedures supplied by the Dr. Ana Soto. Briefly, on day zero, a yeast culture is 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. The culture is diluted 10-fold, by adding 50 mL of growth medium, the next day (day one). The culture is diluted by half, in growth media, the following day (day two). That afternoon, copper sulfate (0.1 M, final) is added to the yeast culture ($OD_{600\ nm}$=0.8–1.0) to induce hER production. Aliquots of 200 μL are transferred to each well of treated 96-well microplates. After 2 min of shaking, the microplates are sealed and incubated overnight at 30 °C, without shaking. After 19–20 h, the yeast is resuspended and 100 μL aliquots are transferred to a 96-well microplate containing 100 μL of assay buffer solution. After 2 min of shaking, the microplates remain in room temperature for 40 min, until the absorbance is read at 415 nm and 595 nm.

The YES bioassay was performed as described in Routledge and Sumpter (1996). The yeast culture was maintained in growth medium containing: 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, and 0.125 mL 20 mM copper (II) sulfate solution to 45 mL single strength minimal medium. On day zero, a yeast culture, 125 μL yeast stock added to 50 mL growth medium, is incubated overnight at 28 °C on a shaker. On day one, 1 mL of the yeast culture is transferred to 50 mL growth medium containing 0.5 mL 10 mg/mL chlorophenol red-β-d-galactopyranoside. This culture is added to treated 96-well microplates, shaken for 2 min and incubated for 5 days at 30 °C. On day six, the microplates were shaken for 2 min, allowed to settle for 1 h prior to reading the absorbance at 540 nm and 620 nm.

### 3.4. Data analysis

Results from the E-screen and YES bioassays were plotted by SigmaPlot (V. 8.02). The E2 dose–response curve was used to quantify the unknown samples. The E2 equivalence (EEQ) was generated based on the E2 dose–response curve (Fig. 2).

### 3.5. Trace organics analysis

Organic analysis for 14 target EDCs (Table 1) was performed on selected samples using a GC–HRMS based trace analytical method (Ikonomou et al., submitted for publication). 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

Table 2
Target EDC concentrations measured by GC–HRMS in influent and effluent WWTP samples

| WWTP | Steroidal EDCs (ng/L) | | | | Industrial EDCs (ng/L) | | |
|---|---|---|---|---|---|---|---|
| | E1 | E2 | E3 | NET* | NPs | BPA | DEHP |
| *Influent* | | | | | | | |
| 1 | 3.3 | 1.2 | 9.1 | 15.2 | 968.3 | 44.6 | 1412.0 |
| 2 | ≤5.8 | 1.9 | 9.2 | 14.8 | 680.2 | 41.9 | 1353.6 |
| 3 | 3.4 | 1.5 | 10.2 | 13.0 | 647.3 | 51.2 | 9032.5 |
| 4 | 5.7 | 0.2 | 12.4 | 13.3 | 1513.2 | 71.8 | 9522.4 |
| 5 | 8.4 | 1.9 | 11.7 | 19.8 | 658.1 | 67.2 | 9191.2 |
| *Effluent* | | | | | | | |
| 1 | 27.2 | 11.2 | ≤4.9 | 14.1 | 1287.3 | 61.1 | 971.2 |
| 2 | 5.9 | 2.0 | 8.9 | 14.2 | 591.7 | 45.4 | 1372.2 |
| 3 | 24.1 | 0.7 | ≤4.9 | 10.4 | 313.7 | 17.3 | 2199.6 |
| 4 | 1.3 | 0.1 | ≤4.9 | 10.9 | 207.5 | 2.9 | 9318.4 |
| 5 | 8.7 | 1.7 | 8.2 | 22.8 | 621.7 | 76.4 | 9232.3 |

Full chemical names provided in Table 1.
(Note: Several chemicals were not included in this table because their concentrations in the samples were below LOD. Mestranol concentrations were below detection in all samples other than WWTP 1 effluent; where it was found to be 6.4 ng/L).

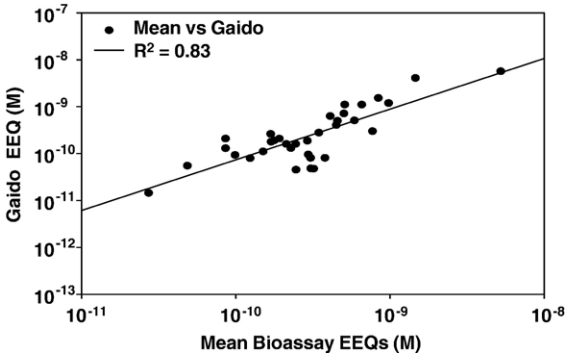


Fig. 3. The relationship between YES (Gaido) EEQ and the mean EEQ of 3 bioassays. Mean EEQ determined by the 3 bioassays for each of the 44 grab and composite samples were plotted against the EEQ of the YES (Gaido) biassay.

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 1.

### 3.6. Conversion of analytical data into 17β-estradiol equivalent units

Several estrogens were selected from the list of GC–HRMS analytes (Table 2) for analysis using the *in vitro* bioassays 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 estrogenic equivalency factor (EEF) was calculated from the ratio of the EC50 for the chemical versus the EC50 of E2. The EEQ of an estrogenic mixture was calculated as the sum of individual chemical concentrations ($C_i$) multiplied by their respective EEF:

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

## 4. Results

The EC50 values of E2 were: 53.2±7.2 pM (14.5±2.0 ng/L) for E-Screen; 242±28 pM (65.9±7.6 ng/L) for Gaido's YES; 203±67 pM (55.3±18.2 ng/L) for Sumpter's YES. The EC50 values were calculated from 44 data points in each of the above bioassays. The EC50 values of E2 for the two YES bioassays were very similar. But the EC50 of E-screen was about 4-times less than those of the YES bioassays.

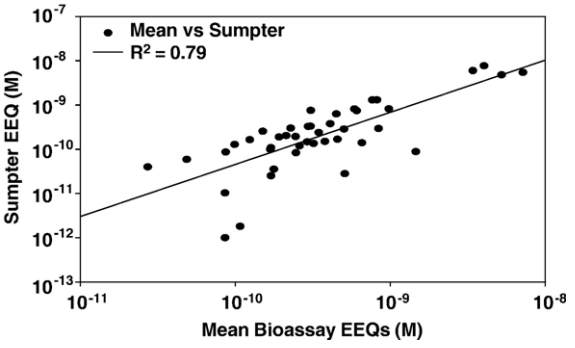


Fig. 4. The relationship between YES (Sumpter) EEQ and the mean EEQ of 3 bioassays. Mean EEQ determined by the 3 bioassays for each of 44 grab and composite samples were plotted against the EEQ of the YES (Sumpter) bioassay.

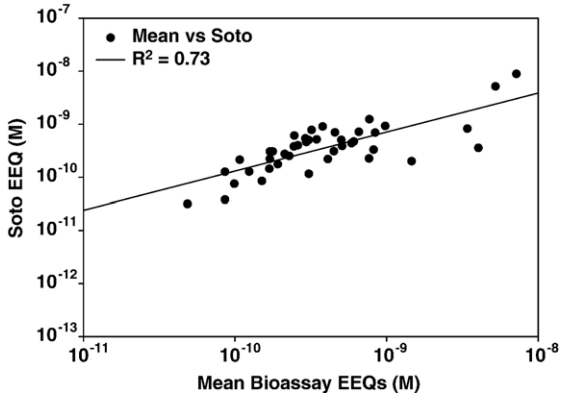


Fig. 5. The relationship between E-Screen EEQ and the mean EEQ of 3 bioassays. Mean EEQ determined by the 3 bioassay for each of the 44 grab and composite samples were plotted against the EEQ determined of the E-Screen bioassay.

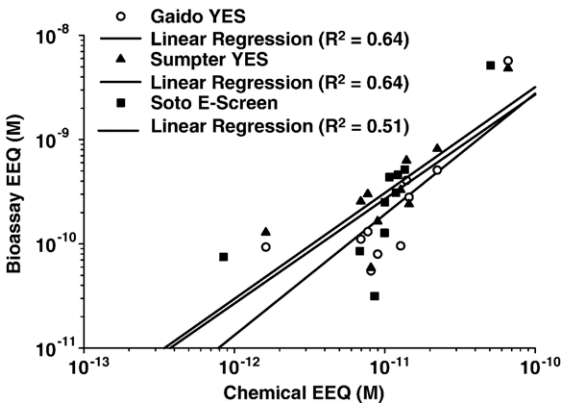


Fig. 6. Correlation between bioassay-derived EEQ and chemical analysis derived EEQ.

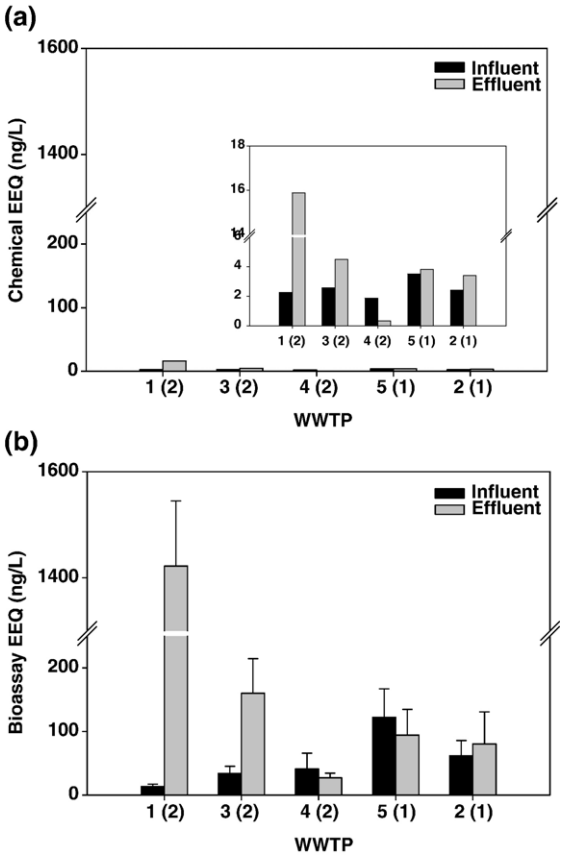


Fig. 7. Chemical analysis-derived EEQ (a) and bioassay-derived EEQ (b) in WWTP influents and effluents. The same *Y*-axis scale is used in (a) and (b); the *Y*-axis scale of the insert in (a) is expanded. The error bars indicate the 95% confidence interval for the bioassay EEQs ($N$=4 per plant influent/effluent).

### 4.1. Bioassay results comparison

The bioassays detect estrogenic activities in most of the 44 individual WWTP samples analyzed with large variation in results. We also have analyzed ten water samples collected at various distances from the outfall of WWTP 5. None of these samples showed estrogenic activity by the bioassays (data not shown).

The bioassays were compared by studying the correlation between the EEQs obtained by the different bioassays. A mild correlation was observed between two of 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. However, a strong correlation was observed between the EEQ of individual assay and the mean EEQ of the 3 bioassays (Figs. 3–4). The correlation and the $R^2$ values are as follows: mean EEQ of 3 bioassays *versus* YES (Gaido) EEQ (Fig. 3), $R^2$=0.83; mean EEQ of 3 bioassays versus YES (Sumpter) EEQ (Fig. 4), $R^2$=0.79; mean EEQ of 3 bioassays versus E-Screen EEQ (Soto), $R^2$=0.73 (Fig. 5).

### 4.2. Chemical analysis

In the present study, only 10 WWTP samples were selected for chemical analysis. The GC–HRMS based analytical method used (Ikonomou et al., submitted for publication), permitted the simultaneous determination of 14 target unconjugated EDCs including natural and synthetic estrogens, see Table 1 for target EDCs. Of the 14 analytes measured, six were consistently detected in all the samples and are presented in Table 2.

### 4.3. Bioassay–chemical analysis comparison

Estrogen concentrations determined by GC–HRMS, were converted to EEQ using estrogen equivalent factors reported in the literature. The bioassay 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. Since chemical analysis was performed for 10 composite WWTP samples, only the bioassay data from the same 10 samples were included in the comparison. The following linear regression correlations were produced: Chemical EEQ versus the YES (Gaido) EEQ, $R^2$=0.64; Chemical EEQ versus the YES (Sumpter) EEQ, $R^2$=0.64; Chemical EEQ versus the YES (Soto) EEQ, $R^2$=0.51. Fig. 6 illustrates these correlations and reveals a clustering of data near the center. Despite a relatively strong correlation, there are approximately one to two-orders of magnitude differences between the chemical and bioassay EEQ.

### 4.4. Estrogenicity of samples

Bioassay-derived EEQ and chemical analysis-derived EEQ also were plotted separately against the WWTP site of sampling (Fig. 7). Several observations can be made regarding the data: (i) chemical analysis-derived EEQ were much lower than the bioassay-derived EEQ in all WWTP samples tested, (ii) influent EEQ are not consistently higher than effluent EEQ in the WWTP. Indeed, WWTPs 1, 2 and 3 show lower influent EEQs than effluent EEQs in both the GC–HRMS analysis (Fig. 7a insert) and the bioassays (Fig. 7b), (iii) the GC–HRMS derived EEQ assumes additivity of the analytes. 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 among 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, we conclude that the WWTPs with secondary treatment are comparable to the WWTPs with primary treatment in removing the EDCs from the influent. Indeed, the EEQ in the influent may actually increase after secondary treatment (Fig. 7).

## 5. Discussion

### 5.1. Complexicity of WWTP

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 treatment WWTPs and three secondary treatment WWTPs (GVRD, 2004) that discharge, respectively into the marine environment and the Fraser River (Fig. 1). 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.

### 5.2. Bioassay-derived EEQ

The dose–response relationships of E2 equivalent concentrations were found to vary by as much as 4-fold among the different bioassays (see Results) implying that the EEQ estimated by the bioassays may vary with the bioassay used in the study. The bioassays can estimate the total endocrine disrupting activity of a WWTP sample because they may account for the additive and synergistic effects of the EDC present. The additive or synergistic effects vary with the biological mechanism(s) based on which the bioassay is developed. Therefore, although the YES bioassays are derived from the same yeast cells, different genetic modifications are made to the cells of the two biassays (Gaido et al., 1997; Routledge and Sumpter, 1996). Moreover, the E-Screen bioassay utilizes the human breast cancer cells, which are very different from the yeast cells (Soto and Sonnenschein, 1995). Murk et al. (2002) determined the estrogenic potency of environmental samples using an estrogen receptor-binding assay and two reporter gene effects assays such as the yeast estrogen screen (YES) and the ER-medicated chemically activated luciferase gene expression (ER-CALUX assay). They found that the estrogen receptor-binding assay consistently yielded an EEQ much higher than the ER-CALUX and the YES assays for the same sample. Results of our studies are consistent with those reported by Murk et al. (2002).

### 5.3. Estrogenic chemicals identified by GC–HRMS

Estrogens are potent chemicals of which the estrogenic effects can be observed in laboratory studies at 1 ng/L (Routeledge et al., 1998). Therefore, although the estrogen chemical concentrations in the WWTP samples are low when compared to the non-estrogen EDCs (Table 2), about 34.5%, 49.2%, and 11.4% of the total EEQ 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 2). The chemical analysis method employed detects only free or unconjugated estrogens, which should account for most of the estrogenicity, as 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-β-D-glucurinide show, respectively 0.21, $1.3\times10^{-3}$, $5.3\times10^{-5}$, and $5.9\times10^{-7}$ of the activity of E2.

In our 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. Plausible explanations may be that there are non-additive effects in the bioassays, resulting in higher EEQ measures, and/or other non-estrogen EDCs in the WWTP sample, which are not included in the GC–HRMS analysis whereas the bioassays measure the total estrogenicity in the sample. Nevertheless, our results from the analysis of the specific 10 samples are in direct contrast to those reported by Korner et al. (2001) where they reported that the EEQ of the effluent samples determined by the E-Screen assay were consistently lower than those obtained by 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.

### 5.4. Absence of EE2 in the WWTP samples

In the present study we are unable to detect any EE2 in 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 generally found in most WWTP effluents (Desbrow et al., 1998; Belfroid et al., 1999) although it 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 be 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).

### 5.5. 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 (Fig. 7b) 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 constrast, the estrogenic activity of the influent samples in WWTPs 1, 2, and 3 was found to be lower than those in the effluent samples (Fig. 7b). Apparently, active estrogens have been generated during water treatment and similar observations have been reported by Servos et al. (in press) 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 WWTPs 1 and 3 (Fig. 7) 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 observation where 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). This conversion is probably conducted by *Escherichia coli* in the feces which are known to produce large amounts of β-glucuronidases which in turn contributes to the enzymatic de-conjugation of conjugated EDCs (Dray et al., 1972).

## 6. Conclusion

The environmental fates of estrogens in WWTP 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 limited in that 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 our 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 help in improving the accuracy of the results. Our results also show that E1 and E2 are the dominant environmental estrogens in the wastewater samples of GVRD WWTPs.

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