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Linkages Between Population Demographics and Municipal Effluent Estrogenicity

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Complex mixtures of chemicals known to modulate fish endocrine function have been identified in municipal effluents (Nimrod and Benson 1997). Perhaps the most potent of effluent estrogen contaminants is ethinylestradiol (EE2), a synthetic estrogen pharmaceutical (Nimrod and Benson 1997). A major formulation in birth control pills, EE2 is prescribed to over ten million women annually in the United States (Arcand-Hoy et al. 1998). Endogenous estrogenic compounds that have similar potencies to EE2 are likely to be present in effluents at higher levels (Tilton et al. 2002; Ternes et al. 1999).

Both endogenous and synthetic estrogens are known to induce vitellogenesis in male fish following binding and activation of nuclear estrogen receptors (Nimrod and Benson 1997). Consequently, *in vivo* male vitellogenin induction is widely used to study contaminant estrogenicity under laboratory and field conditions. *In vitro* assays, including the yeast estrogen screen (YES) assay, are also utilized to screen compounds for estrogen activity (Miller et al. 2001) and monitor estrogenicity of effluents and surface waters (Desbrow et al. 1998). In this assay, human estrogen receptor (ER) and estrogen response elements (ERE) are integrated into the genome of a yeast, *Saccharomyces cerevisae*.

Previous studies used a toxicity identification evaluation (TIE) procedure to explore municipal effluent estrogenicity. Using the YES assay and chemical analyses, Desbrow et al. (1996) identified EE2, estrone and estradiol as causative estrogenic compounds in a United Kingdom municipal effluent. Other studies evaluated the relationship between *in vitro* YES activity and *in vivo* male fish vitellogenin (VTG) induction. Fawell et al. (2001) observed similar sensitivity between YES activity and *in situ* male rainbow trout VTG following exposure to several municipal effluents. In laboratory studies, Metcalfe et al. (2001) also observed comparable sensitivity between *in vitro* YES assay and *in vivo* Japanese medaka VTG assay responses to natural and synthetic estrogens.

Whereas the estrogenicity of municipal effluents has received considerable attention, the relationship between population demographics and effluent estrogenicity has not been examined. For example, VTG induction was observed in male fathead minnows exposed to the City of Denton, TX, municipal effluent

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during March and December 2000 (Allen et al. 2001; Hemming et al. 2001). This effluent was not estrogenic to male fathead minnows in June (Hemming 2000) and August 2000 (Brooks 2002). Analytical measures indicated that steroids were present in City of Denton, TX, effluent during March and December 2000, but not in June studies (Allen et al. 2001; Hemming et al. 2001). However, nonsteroidals including nonylphenols, bisphenol A, and phthalates were detected at low µg/L levels during March and June 2000 (Hemming 2000; Hemming et al. 2001).

Although effluent estrogenicity may be related to City of Denton, TX, population size changes with enrollment in two universities (Brooks 2002), causative estrogenic contaminants have not been identified. The primary objectives of this study were to: 1. Identify causative estrogenic compounds using a modified identification approach from Desbrow et al. (1996); 2. Utilize *in vivo* VTG responses to confirm *in vitro* estrogenicity; and 3. Determine if population size changes corresponded with effluent estrogenicity.

MATERIALS AND METHODS

This study was performed with final treated effluent from the City of Denton, TX, Pecan Creek Water Reclamation Plant (PCWRP). PCWRP is a conventional activated sludge wastewater treatment plant that operated at a capacity of 15 million gallons per day (MGD) with an average outflow of approximately 12.5 MGD during this study. PCWRP received wastewater from approximately 85,000 residential customers and 150 industries in 2001 (*D. Hunter, City of Denton, pers. comm.*). Bar screens, vortex grit removal, primary clarification, extended aeration, and secondary/final clarification comprised the major PCWRP treatment process sequence. Final effluent received chlorination for disinfection and was dechlorinated with sulfur dioxide prior to discharge. Residence time of PCWRP was approximately eight hours on both study dates; therefore, a sampling time of 1500 was chosen to evaluate treatment of early morning influent.

Effluent samples were collected from PCWRP on 02 May 2001, sample date 1, and 16 May 2001, sample date 2. Sample day 1 was selected as a weekday sampling time during the final week of spring university session. Sample day 2 was selected as a weekday sample during the week following spring 2001 university session break. Ratios of PCWRP influent/effluent flow were similar; 0.99 and 1.01 for sample days 1 and 2, respectively. Effluent grab samples were collected with acid washed 20 L cubitainers and transported to the laboratory on ice. Less than 24 hours after collection, 1 L effluent sub-samples were pre-filtered with 0.7 µm Millipore glass fiber filters and extracted with Empore™ SDB-XC solid phase extraction (SPE) disks that were sequentially conditioned with ACS grade acetone, ACS grade methanol and Milli-Q water (Belfroid et al. 1999). Extraction disks were stored at -80°C prior to analysis.

Effluents were initially screened for estrogenic activity using the YES assay (Desbrow et al. 1996). SPE disks were sequentially eluted with three solvents of decreasing polarity: methanol, methylene chloride and hexane. 30 mL solvent

fractions were reduced to dryness by a nitrogen stream and reconstituted in 300 µL ethanol. 5 mL growth media was inoculated with either estrogen response element (ERE, a negative control) or estrogen response element and estrogen receptor (ERE+ER) cells. Cells were incubated overnight at 30°C and then diluted to an optical density (OD) of 0.057, determined at 630 nm with a Tecan UV/Vis microplate reader. Each solvent fraction (100 µL) was incubated with 700 µL of ERE+ER cells, ERE cells or ethanol. Following a 24hr incubation at 30°C, 100µL of each cell suspension and 400µL of chromogenic substrate (ONPG) was added to a 96 well microplate. Following microtiter plate incubation at 30°C during color development, OD was determined at 405nm. 17β-estradiol was used for standard curve generation. OD values were corrected for ethanol controls, turbidity and ERE interactions according to Payne et al. (2000) where: OD = ERE+ER ($Test_{540nm}$ - ($Test_{630nm}$ - Con_{630nm}) - Con_{540nm}) - ERE ($Test_{540nm}$ - ($Test_{630nm}$ - Con_{630nm}) - Con_{540nm}).

Adult male Japanese medaka were exposed to methanol, methylene chloride and hexane eluents and controls for a 7d static renewal study. Medaka were cultured and tested in Balanced Saline Solution (BSS) (Yamamoto 1975). Preparation of methanol, methylene chloride and hexane eluent fractions from solid phase extraction disks followed YES assay procedures. Two adult male Japanese medaka were randomly placed in each of three replicate 800mL exposure chambers per treatment. 40µL aliquots of reconstituted ethanol eluents from sample day 1 were added to exposure chambers prior to daily renewals. On day 7, fish were anaesthetized with MS-222. After the isthmus was incised, blood was collected with a heparinized hematocrit tube. Blood samples from the two fish in each exposure chamber were pooled. Plasma was isolated by centrifugation at 6,000 x g for 10 min. and stored at –80°C prior to VTG analysis.

Plasma VTG levels were determined following previously reported methods (Foran et al. 2002). Protein content of plasma was measured using bovine albumin standards (Sigma Chemical) and a Bio-Rad Protein Assay protein dye with a Tecan UV/Vis microplate reader (Foran et al. 2002). VTG was determined by SDS-Page and Western blotting with a mouse monoclonal antibody developed against striped bass (Cayman Chemical Co.). After the nitrocellulose image of each gel was developed, gels were scanned and subsequently analyzed using Scion Image software. VTG bands (170 kDa and 130 kDa) were quantified as percent integrated optical density (IOD) relative to positive controls. Positive control livers used for this study were collected from Japanese medaka exposed to 1 µg/mL estradiol for 7d (Foran et al. 2002).

Analysis of effluent estrogen steroids was performed for effluent sample days 1 and 2. Analytical procedures followed previously reported methods (Belfroid et al. 1999, Ternes et al. 1999). Compounds were eluted from solid phase extraction disks with two 15 mL methanol additions. Methanol fractions were combined, mixed with 1 g hot sodium sulfate, and reduced to dryness with nitrogen. Samples were derivatized with 50 µL of a 1000:2:2 mixture of MSTFA (n-methyl-N-(trimethylsilyl)-trifluoracetamide):TMSI (trimethylsilylimidazole):DTE

(dithioerytrol), evaporated to dryness, reconstituted in 100 μL hexane and analyzed with a Hewlett Packard 6890 series gas chromatograph (GC) coupled to a Hewlett Packard 5973 mass spectrometer. A 0.9 mL gas flow rate and a J&W DB-5MS capillary column (30 m x 250 um x 0.25 um) were utilized. For each GC sample run, a temperature profile of 160°C for 1 min, increased to 290°C at 10°C/min and held at 290°C for 10 min was followed for a 24 min total run time. Duplicate samples were analyzed by mass spectroscopy in selective ion monitoring (SIM) mode. Ions 425, 416 and 342 *m/z* were utilized for monitoring EE2, E2 and estrone, respectively. Estrogen quantitation was performed with a three-point calibration curve. Matrix spiked recovery of estradiol was 82%, which was used as a model for estrone and ethynylestradiol; detection limits for all analytes were ≤1 ng/L. Estrogens were not detected in BSS medaka controls.

Statistical analyses of treatment effects were performed by ANOVA and Dunnett's multiple comparison test using SPSS (Version 10.05). Statistical significance of fish response variables was determined at $\alpha = 0.05$.

RESULTS AND DISCUSSION

EE2, E2, and estrone were quantitated in effluent sampled on study day 1 and 2. Average E2 and estrone concentrations of duplicate sample injections were 1.2 and 6.02 ng/L, and below detection and 1.2 ng/L on sample days 1 and 2, respectively. EE2 was not detected in effluent samples from either sample date.

Effluent collected on sample days 1 and 2 were evaluated with the YES assay. Sample day 1 exhibited *in vitro* estrogenic activity, but only in the methanol fraction (Figure 1). Snyder et al. (1999) identified that the TIE approach used in this study fractionates most polar compounds including EE2, E2 and estrone in methanol fractions, moderately polar compounds including nonylphenol ethoxylate degradation products in methylene chloride fractions, and most nonpolar compounds including PAHs, PCBs and organochlorine pesticides in hexane fractions. *In vivo* estrogenic responses were observed in Japanese medaka exposed to fractions of effluent collected on sample day 1. Methanol ($p<0.05$) and methylene chloride ($p<0.001$), but not hexane, fractions of sample day 1 effluent significantly induced vitellogenesis in male fish (Figure 2). The observed discrepancy between *in vitro* and *in vivo* responses to methylene chloride TIE fraction potential resulted from complexation of relatively non-polar compounds with dissolved organic matter (Bowman et al. 2002) or from compounds that were metabolized or not absorbed by *Saccharomyces cerevisae* (EDSTAC 1998).

Previous investigators observed that the City of Denton, TX effluent was estrogenic during certain periods (Allen et al. 2001; Hemming et al. 2001) but not others (Hemming 2000; Brooks 2002). Presence and absence of effluent estrogenicity in these studies appeared to correspond with enrollment of two university student populations (Hemming 2000; Brooks 2002); however, the source(s) of effluent estrogenicity were unknown. Results from this study indicate that the City of Denton, TX effluent was estrogenic to *in vitro* and *in vivo*

bioassays on a sample date concurrent with spring university semesters (Figures 1 and 2). Approximately 39% of 80,000 residential City of Denton water utilities customers were students enrolled at University of North Texas (UNT) or Texas Woman's University (TWU) during the 2001 spring semester (UNT: 25,652 (UNT 2002); TWU: 5,717 (TWU 2002)). Interestingly, YES activity was not observed in effluent samples from sample day 2 (Figure 2). This corresponds to an 86% reduction of enrolled students reported between sample day 1 and sample day 2 (UNT: 3,719 (*R. Anzaldua, UNT, pers. comm.);* TWU: 571 (TWU 2002)). A reduction of E2 and estrone concentrations was also observed on sample day 2.

Whereas previous studies indicated that the YES assay responds to a large number of endogenous and exogenous estrogens, it exhibits greatest sensitivity to natural and synthetic steroids. Desbrow et al. (1996) reported the YES assay as 1000, 5000 and 50000 times less responsive to bisphenol A, ocylphenol and nonylphenol, respectively. Others observed greater sensitivity of YES activity to steroids relative to other xenoestrogens (Arnold et al. 1996). Whereas Routledge and Sumpter (1996) observed EE2 to have higher potency to the YES assay than E2, Metcalfe et al. (2001) reported that EE2 and E2 were approximately equipotent. YES activity to xenoestrogen mixtures at equimolar concentrations was previously reported as additive responses (Payne et al. 2000). In this study, only the sample day 1 methanol fraction was estrogenic to the YES assay.

Metcalfe et al. (2001) successfully utilized *in vitro* YES activity to confirm estrogenic impacts to Japanese medaka. Fawell et al. (2001) indicated that *in vitro* YES responses to effluents predicted *in situ* male VTG induction at most sites; however, effluent at one site induced male vitellogenesis without a corresponding increase in YES activity. In this study, *in vitro* YES activity was employed as an initial screening assay and *in vivo* VTG induction in male fish was used as a confirmatory assay. Whereas YES activity was only observed in methanol fractions from sample day 1 in this study, methanol and methylene chloride fractions significantly increased plasma VTG content in Japanese medaka (Figure 2). An estradiol toxicity equivalent approach was implemented to evaluate relative effluent estrogenicity. A toxic equivalent approach was previously used to compare the response of biologically active TIE fractions in the YES assay to a standard curve of 17β-estradiol, a compound which elicits a similar biological response (Kannan et al 2000). This equivalent approach was used to calculate that the methanol fraction from sample day 1 contained 14 ng/L estradiol equivalents. Although not as responsive to the YES assay as steroid estrogens, degradation products of the nonylphenol ethoxylate surfactants were consistently observed by Hemming (2000) at low µg/L levels in the City of Denton, TX effluent in March and June 2000.

This study confirmed previous reports that estrogenicity of the City of Denton, TX, effluent, as measured by YES activity and VTG induction in adult male fish, coincides with enrollment numbers at two Denton universities. Based on biological and chemical data, natural, and potentially synthetic, steroids and other compounds in the City of Denton's municipal wastewater elicited the observed

estrogenicity in this study. *In vivo* bioassays and other *in vitro* assays should be used concurrent with *in vitro* YES activity when effluents are screened or fractionated for estrogenicity.

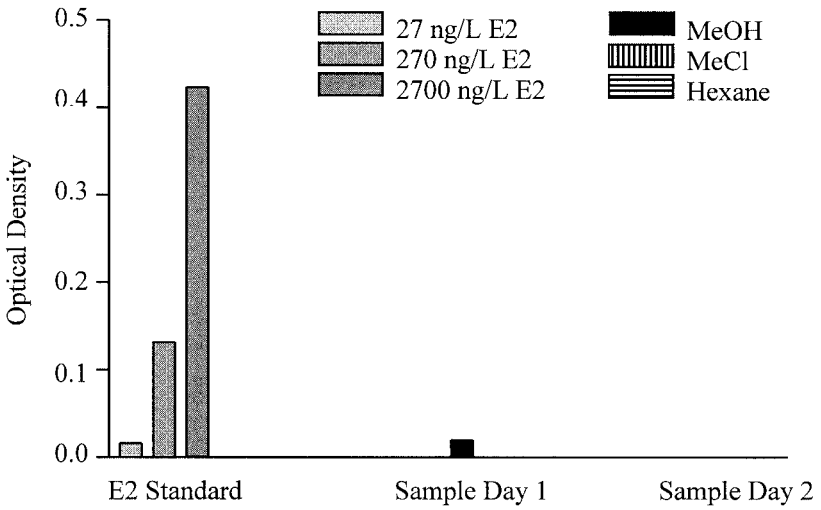

Figure 1. YES assay activity for estradiol standards and solvent fractions eluted from solid phase extraction disks. Sample Day 1 = 02 May 2001, Sample Day 2 = 16 May 2001. MeOH = Methanol, MeCl = Methylene Chloride.

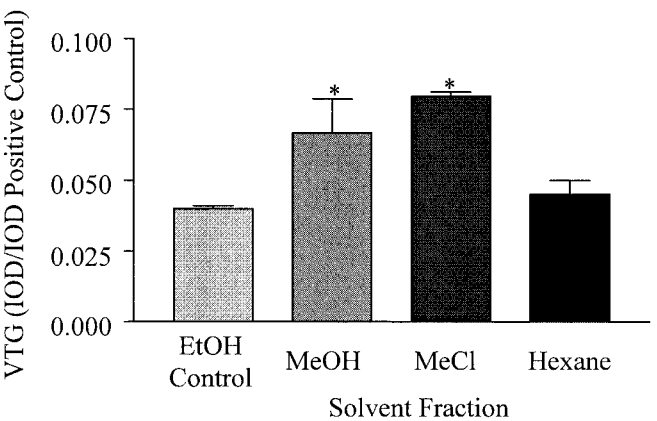

Figure 2. Plasma VTG induction (±SD) in adult male Japanese medaka exposed for 7d to solvent fractions from City of Denton, TX, effluent collected on 02 May 2001. MeOH fraction, $p < 0.05$, N=3; MeCl fraction, $p < 0.001$, N=3.

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