PROJECT TITLE: Use of bioassays to assess the water quality of wastewater treatment plants for the occurrence of estrogens and androgens.

PRINCIPAL INVESTIGATOR: Daniel Schlenk, Ph.D.
Department of Environmental Sciences
University of California, Riverside
Riverside, CA 92521
Daniel.schlenk@ucr.edu

UC Water Resources Center Technical Completion Report Project No. W-971

Submitted: April 2005

EXECUTIVE SUMMARY

Endocrine disrupting agents encompass a vast array of compounds that have multiple biological targets and degrade water quality, especially if this water is to be re-used for groundwater recharge or agricultural practices. Antiestrogenic and estrogenic activities and chemicals have been observed in effluents from full secondary treatment. Assuming that estrogens and antiestrogens are present in wastewater effluent, the overall aim of this study is to assess the efficiency of treatment processes for the removal of these compounds in one of the major water reclamation producers in the western United States: the Orange County Water District. Utilizing an ***in vivo*** bioassay developed in the PI's laboratory effluents were evaluated after various water treatment processes for the occurrence of estrogenic and antiestrogenic compounds. Treatment processes included reverse osmosis, filtration/chlorination of secondary effluent, ground water filtration, and wetland treatment. *In vivo* estrogenic activity was observed in fish exposed to effluent treated with filtration/chlorination (which is subsequently used for non-potable purposes), ground water and constructed wetlands. No activity was observed in reconstituted water that had been treated with reverse osmosis. Our results also suggest that *in vitro* assays based solely on estrogen receptor ligand activity (YES) may underestimate estrogenic activity of sampled water. Although not as robust a measurement as estrogenic activity, *in vivo* antiestrogenic activity was observed in fish exposed to wastewater samples treated with filtration/chlorination and the wetland. Moreover, wastewater after the wetland treatment seemed to have more antiestrogenic activity than before the treatment. These data indicated the occurrence of antiestrogenic and estrogenic compounds in water following various treatment processes. It is recommended that source identification be considered in future studies utilizing chromatographic fractionation methods to better understand the potential risk associated with these compounds in reclaimed water.

KEY WORDS: Wastewater re-use; endocrine disrupters, exposure assessment, environmental estrogens, environmental androgens, vitellogenin, fish bioassay.

Introduction and Problem Statement

Endocrine modulating chemicals consist of a vast array of natural and xenobiotic chemicals used throughout the world in manufacturing, agriculture, and daily domestic life. Many of these chemicals are classified according to the biological responses they mimic or inhibit following exposure. For example, chemicals which mimic biological responses similar to estrogen or its metabolites, may be classified as environmental estrogens (or anti-androgens), and compounds that mimic androgens would be considered environmental androgens (or anti-estrogens). Compounds altering these important physiological processes may be either derived from industrial or agrichemical sources (i.e. detergents, chlorinated hydrocarbons and pesticides), or from other "naturally occurring" activities (i.e. endogenous steroids).

Most of the literature in recent years has focused exclusively on the estrogens (or anti-androgens). Feminization of fish populations has been observed in the UK near sewage treatment plants (Jobling et al. 1998) and when fish have been exposed to effluent (Rodgers-Gray et al. 2001). However, other studies have indicated the occurrence of androgens (i.e Testosterone) in surface waters and sediments (Tilton et al. 2002; Thomas et al. 2004). In addition, it is likely that other pharmaceutical agents may also have androgenic or anti-estrogenic activities. The anti-cancer therapeutic agent, tamoxifen has been shown to have significant androgenic or anti-estrogenic activities in several fish species (Nimrod and Benson 1996; Mori et al. 1998; Smeets et al. 1999). However, the mechanism(s) for these effects have not been clearly identified.

Concurrent with the development of *in vitro* cell-lines having the estrogen receptor, androgen receptor bioassays have also been developed (Tamura et al. 2001). Although there have not been published reports of the use of such assays for environmental monitoring of androgens, estrogen receptor systems have been used routinely in determining estrogenic activities (Gillesby and Zacharewski 1998; Smeets et al. 1999; Sumpter and Jobling 1995). Recent studies in our laboratory investigating the estrogenic activity of municipal effluents of Mississippi, and New York have indicated that estrogenic activity determined from *in vitro* assays significantly underestimated estrogenic activity observed from *in vivo* assays (Tilton et. al, 2002; Todorov et al., 2002; Huggett et al. 2003; Sapozhnikova et al. 2005). This is likely due to the plethora of mechanisms for endocrine alteration which are not directly mediated through an interaction of the xenobiotic at the steroid receptor. Consequently, it has been recommended that a combination of *in vitro* and ***in vivo*** assays be utilized to assess the endocrine disrupting potential of compounds (Ankley et al. 1998).

Objectives

The objectives of this study were to utilize and develop *in vivo* bioassays for the assessment of environmental estrogens and androgens in sewage effluent. Using a calibrated *in vivo* bioassay to measure equivalent estrogenic (or anti-androgenic) and antiestrogenic (or androgenic) activity at various stages of wastewater treatment, greater information will be gained about the overall balance of estrogenic and androgenic responses following each respective treatment regime, and which treatment method is the most effective at removing both androgenic and estrogenic compounds. The proposed development of in vivo methods

for determining exposure to androgens is designed to complement already existing methods with the same species for evaluation of estrogenic responses to aqueous exposures to wastewaters.

1. Determine estrogenic (anti-androgenic) and androgenic (anti-estrogenic) responses in Japanese medaka and Rainbow Trout fish models using respective hormone equivalent endpoints based upon the ***in vivo*** biological responses in each species.

2. Monitor androgenic and estrogenic activities in wastewater, and monitor the efficiency of wastewater treatment processes for removing androgenic and estrogenic contaminants utilizing estrogen/androgen-based *in vivo* bioassays.

Procedures

Objective 1. Determine estrogenic (anti-androgenic) and androgenic (anti-estrogenic) responses in Japanese medaka and Rainbow Trout fish models using hormone equivalent endpoints based upon the *in vivo* biological responses in each species.

Rationale. The major purpose of this study was to assess water treatment procedures for the removal of endocrine disrupting compounds from sewage effluent. Previous studies in our laboratory successfully characterized estrogenic activity in terms of estradiol equivalents (Huggett et al. 2003; Tilton et al. 2002). Two fish models were utilized: the Rainbow trout model is an extremely sensitive model used predominantly in the United Kingdom to assess estrogenic activity (Jobling et al. 1996); the second model (Japanese medaka) was used extensively in the PI's laboratory in the past to evaluate estrogenic activities (Thompson et al. 2000; Huggett et al. 2003; Sapozhnikova et al. 2005). Since the estrogenic responses have been well characterized, animals can be pre-exposed to 17β-estradiol in concentrations and for a duration known to induce vitellogenesis (100 ng/L). Subsequent, to these calibration studies, animals can be exposed to effluent fractions to identify causative agents which can be identified and quantitated through analytical chemistry.

In contrast to estrogenic evaluations, androgenic activities have not been examined in this manner. In order to quantitatively express androgenic activity, all measurements needed to be expressed relative to the primary androgen, dihydrotestosterone (DHT). Thus, a dose-response experiment using estradiol-treated fish was necessary to derive DHT equivalents.

Androgenic Dose Response Curves

In vivo medaka Dose Response Curves. Adult male medaka were held in aerated water within 2 liter glass aquaria (10 per tank) statically. Animals were exposed to 100 ng/L 17β-estradiol (E2) prior to extract or DHT exposure. This concentration of estradiol was shown to have significant vitellogenin induction in male medaka (Nimrod and Benson 1998; Thompson et al. 2000). Following exposure for 7 days to E2, animals were then treated with

4 separate concentrations of DHT for 7 days. Positive controls consisted of E2-treated animals sacrificed prior to DHT treatment. Negative controls were E2-treated animals receiving no DHT treatment but exposed to control water for 7 additional days.

Livers were collected and analyzed for vitellogenin by ELISA using a commercially available kit from Biosense Laboratories (Bergen, Norway). Five (5) replicates per dose were used to construct the dose-response curve. Attempts to determine DHT equivalents were to be based upon linear extrapolation from the curve.

In vivo trout screening bioassay. Since attempts to calculate DHT equivalents were unsuccessful (see below). Trout were used only to verify estrogenic activities. The trout has a much more sensitive estrogenic response than medaka (Harries et al. 1997; Harries et al. 1996). Trout were obtained from the California Fish and Game Hatchery in nearby Victorville, Ca. Vitellogenin was measured by ELISA using kits purchased from Biosense Laboratories (Bergen Norway).

17β-estradiol exposure

Juvenile rainbow trout were exposed to nominal concentrations of 17β-estradiol of 0 (control, 1ml ethanol only), 0.5 ng/L, 1 ng/L, 10 ng/L, and 100 ng/L in 10 liter of filtered dechlorinated tap water. Measured concentrations were determined as previously described (Huggett *et al.* 2003) with minor modifications. One liter of water sample was taken from the exposure tank and filtered with combined 1.2 um Whatman and 0.45 um Millipore filters (Fisher Scientific, Los Angeles, CA). Estradiol was extracted with the Empore SDB-XC extraction disk previously conditioned with acetone and methanol at a flow rate of 5 ml/min and the disk was eluted with 30 ml of methanol. The extract was evaporated under nitrogen stream until dryness and reconstituted in 0.5 ml of hexane:acetone (65:35, v:v). The resultant mixture was derivatized with 50 ul of Bis-Trimethylsilyl-Trifluoroacetamide (BTSFA, Sigma, St. Louis, MO) at 60°C for 60 min and then evaporated until dryness under stream of nitrogen and reconstituted with 200 ul of hexane. Detection of estradiol was performed using an Agilent Technologies 6890N Gas Chromatography System equipped with 5973 Mass Selective Detector (MSD). The capillary column was HP-5MS 30m x 0.25mm, with 0.25 μm film thickness. The GC conditions were: detector - 290°C; ion source, EI mode; injector - 250°C. Column temperature program started at 80°C, isothermal for 2 min; ramped to 200°C with 20°C/min, followed by 2°C/min to 260°C, and held for 10 min. The mass selective detector was used in Selected Ion Monitoring (SIM) mode. The 17β-estradiol retention time was 23.771 min and the precursor ion was 416 m/z and product ion was 285 m/z.

Analyte recovery was quantified using water samples spiked with 17β-estradiol. The recovery was 60% with a relative standard deviation of 15%. The method detection limit (MDL) was 1 ng/L. The measured concentration of estradiol in the solution for the estradiol exposure was between 79.9% and 122% of the nominal concentration except for 100 ng/L, for which the measured concentration was 28% of nominal.

Plasma VTG production in juvenile rainbow trout exposed to estradiol showed a concentration-related increase after the 0.50 ng/L nominal concentration. The lowest –observed-effect concentration (LOEC) for VTG induction was 1.21 ng/L.

Determination of VTG levels

After termination of the exposure, fish were euthanized in MS-222 (50 mg/L). Blood samples from rainbow trout were obtained by an incision at the caudal peduncle and collecting the blood exiting the incision. Blood was centrifuged at 3000 rpm for 10 minutes at room temperature. After centrifugation, PMSF (Phenylmethyl sulphonyl fluoride; Stock solution 0.1M) was added to the plasma samples at a final concentration of 1mM. The plasma samples were stored at $-80^{o}C$ until analysis.

Vitellogenin concentrations in the plasma were determined using rainbow trout VTG enzyme-linked immunosorbent assay (ELISA) kit supplied by Biosense Laboratories (Bergen, Norway). All assay procedures were followed according to the manufacturer. Briefly, 96-well polystyrene microtiter plates were coated with the capture antibody using 100 ul sodium carbonate coating buffer (50 mM, pH 9.6) per well and incubated at $37^{o}C$ for 2 hours. After incubation, the plates were washed 3 times with 200 ul per well with phosphate buffered saline (PBS) (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.3) with 0.05% Tween 20. One hundred and fifty ul of blocking buffer (1% of bovine serum albumin in PBS) was added to individual wells of the plates for 1 hour. Next, 100 ul of blocking buffer containing the diluted standard or plasma samples was added to the wells and allowed to incubate at room temperature for 1 hour. After the wells were washed 3 times with 200 ul of washing buffer, 100 ul of the diluted detecting antibody was added to all wells and incubated at $4^{o}C$ overnight. Secondary antibody in 100 ul blocking buffer was added to each well and the plates were incubated on an orbit shaker (400 rpm) at room temperature for 1hour. After washing, 100 ul of the color development solution (Ellman) was added to each well and the plates were incubated at darkness for 1 hour. The reaction was stopped by adding 50 ul of 2N H_2SO_4 to all wells. The absorbance was read at 405 nm in a microtiter plate reader. Vitellogenin levels in the plasma samples were calculated based on the standard curve obtained from the relationship between the concentration of the standard rainbow trout vitellogenin and the absorbance (for all cases, standard curves have a $R^2 \geq 0.99$).

Total protein levels in the plasma sample were determined using Coomasie stain using bovine serum albumin as standards (0.25 – 2 mg/ml). Plasma samples were diluted in phosphate-buffered saline (pH 7.4) and transferred to 96-well microtiter plates in triplicates. The absorbance was read at 595 nm.

Vitellogenin levels in the plasma samples were expressed as ng vitellogenin per mg of total protein. Estrogenicity of the wastewater samples was expressed as estradiol equivalent concentrations as described for YES (see below).

Objective 2. **Test the developed biological assays to monitor androgenic/antiestrogenic and estrogenic activities in wastewater, and monitor the efficiency of wastewater treatment processes for removing androgenic and estrogenic contaminants.**

Rationale. Effluent will be tested with each ***in vivo*** fish model and samples possessing either high levels of 17β estradiol or DHT equivalent activities will be identified. Hypotheses can be derived from these data with regard to causative agent(s) with subsequent fractionation of effluents showing either 17β estradiol or DHT activity.

As the major purpose of this study is to assess water treatment procedures for the removal of

endocrine disrupting compounds from sewage effluent, samples were taken from the Orange County Water District, as Orange County Sanitation District does not implement tertiary treatment,. Tertiary treated effluent was sampled from the Green Acres Treatment Plant at the Orange County Water District to evaluate filtration/chlorination as a removal process. To evaluate constructed wetland removal, water was sampled prior to and after the Prado Wetland. Reverse Osmosis and ground water filtration were evaluated in a project currently funded by the Water Environment Research Foundation.

Water sample collection

The Prado Wetland (33°54" N; 117°40" W) is located in Riverside County, California, USA and consists of 50 shallow ponds that have been utilized to remove nitrogen from water originating from the Santa Ana River, which consists of effluent from tertiary treated wastewater from Riverside County. Water samples were collected at the entrance and at a site that where water had flowed through approximately 2/3 of the wetland. Water samples from the two sites were collected in 80-gallon Polyvinyl Chloride (PVC) containers in September 2002 and 2004 (for medaka exposure), and July 2003 (for rainbow trout exposure).

The Green Acres Plant at the Orange County Water District, California, USA treats about 7.5 million gallons of secondary treated wastewater per day from the Orange County Sanitation District (OCWD) using direct filtration followed by disinfection with chlorine. The resultant disinfected water is used primarily for irrigation in Orange County. The water sample was collected in April 2003 and May 2004 in an 80-gallon container. Since the water sample contained high levels of chlorine (approximately 4 mg/L) and ammonium (approximately 3 mg/L) which were lethal to rainbow trout, sodium thiosulfate was added to dechlorinate the water sample and the water sample was aerated continuously for several days prior to exposure to eliminate chlorine. Attempts were made to evaluate raw influent, but fish could not survive.

Orange County "tap water" was treated with reverse osmosis prior to ionic reconstitution to maintain fish osmolality. Control water was treated with salts to mimic the conductivity and chemistry of Santa Ana River water and then pumped into a 40 liter reservoir. The water was distributed by gravity to 40 individual acrylic aquaria containing 10 x 2-3 week-old juvenile fish.

The shallow groundwater was collected from a well screened 25-65 feet below the ground surface and had a subsurface residence time of approximately three to six months from the time it entered the subsurface. All plumbing was Teflon until reaching the trailer where 0.5 m or less lengths of polyvinyl chloride pipe was used to transfer water into a holding reservoir which was pumped into a distributing reservoir as described above.

Carbon-filtered Riverside tap water was used as ultimate control. Fish were maintained in heated water baths with temperatures varying from 21-24.2^{o}C and on a 16:8 light dark cycle. Fish were sampled after 21 days of exposure.

In vivo assays

After multiple range finding studies, Japanese medaka were utilized for androgenic assessment and rainbow trout were used for estrogenic assessments. For androgenic

assessments, sexually mature male medaka were pre-treated with 100 ng E2/L for 7 days and then to effluent of varied dilutions for 7 days. Negative and positive controls were used as described above.

For estrogenic assessments, juvenile rainbow trout were exposed to 9 L of diluted (33%) wastewater samples (3 liters of waste water/6 liters of filtered tap water) in 20 L tanks with aeration in a static renewal system for 14 days. Control fish were exposed to filtered dechlorinated tap water only. Water temperature was maintained at 14 ± 1°C. Light cycle was14:10 h (light : dark). Each treatment (control and exposed) had three replicates with 2-3 fish for each replicate. The water was renewed every other day. Fish were fed rainbow trout chow at 1% of their body weight during the exposure.

In vitro assay (Yeast estrogen screening)

One liter of the wastewater samples (from all sampling locations) was filtered over 0.45 and 1.2 um Whatman filters (Clifton, NJ, USA). The water was passed over an Empore SDB-XC extraction disk (Mt. Pleasant, SC, USA). After extraction, the Empore filters were eluted with 30 ml of methanol, with the eluate evaporated to dryness and resuspended in 100 ul of ethanol. The sample was stored at 4°C until use for YES.

The YES assay was performed according to methods previously published (Desbrow *et al.* 1998; Huggett *et al.* 2003). Briefly, 100 ul of the extracted samples or standard concentrations of 17β-estradiol (10^{-4} – 10^{-14} ng/L in ethanol) were added to 700 ul of a yeast cell suspension (with OD_{600nm} = 0.057) in microcentrifuge tubes. The tubes were incubated with caps open at 30°C for 5 days. The dried sample was resuspended in 100 ul of buffer (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1mM $MgSO_4.H_2O$, and 50 mM 2-Mercaptoethanol, pH 7.0) and 400 ul of 10 mg/ml chromogenic substrate (O-nitrophenyl β-D-galactopyranoside, Sigma, St. Louis, MO). Incubation was carried out at 37°C for 1 hour for color development. Absorbance was measured at 405 nm using a microtiter plate reader (model: *Vmax*, Molecular devices, Sunnyvale, California, USA). The method detection limit of the YES assay was 1 ng/L. Estrogenic activity of the water sample by YES assay was expressed as estradiol equivalent concentrations (EEQs).

Statistical Analysis

All statistical analyses were performed using the Statistical Analysis System package (SAS, version 8.0, Cary, NC) unless otherwise stated. Before analysis, data were evaluated for normality with the Shapiro-Wilks test, and for equality of variance using Levene's test. Since assumptions of normality and equal variance were violated, data were not transformed and a nonparametric test (Kruskal-Wallis test or Mann-Whitney *U*-test) was used to test the difference in vitellogenin levels between control and treatment groups. The significance level was set at $p \leq 0.05$. For estradiol exposure, dose response curves were generated by SigmaPlot software (SPSS Inc, Chicago, Illinois, USA).

Results

Estrogenic Activity

Green Acres Plant Assessment

Juvenile rainbow trout exposed to treated wastewater effluent from the Green Acres Plant for 14 days had a higher level of plasma VTG than control fish (Figure 1). The increase in plasma VTG levels in the exposed fish relative to the control fish was 4936%. Based on the concentration-response curve ($R^2 = 0.999$), the estradiol equivalent concentration of this tertiary-treated water was 16.92 ± 16.48 ng/L (Figure 2). YES assays indicated that the tertiary-treated wastewater had an estradiol equivalent concentration of <1 ng/L (Figure 3).

Prado wetland assessment

After 7 days of exposure, plasma vitellogenin levels in the male Japanese medaka exposed to 33% of the water entering and exiting the Prado wetland were not significantly different ($P > 0.05$ for all pairwise comparisons. Figure 2). However, juvenile rainbow trout exposed to 33% of the water entering and exiting Prado wetland for 14 days had elevated levels of plasma vitellogenin compared to control fish ($P < 0.05$). The percentage increase in vitellogenin levels in the fish exposed to Prado water relative to the control fish was 2038% for influent water and 1114% for effluent water. There were no significant differences in plasma vitellogenin levels in trout exposed to Prado influent or effluent water ($P > 0.05$. Figure 2). Estradiol equivalent concentrations of the entering and exiting water sample of Prado wetland were 24.34 ± 23.17 ng/L and 29.80 ± 28.11 ng/L. No significant differences in EEQ were observed between water entering and exiting Prado wetland (Figure 2). YES assays showed that the water samples from the entering and exiting sites had estradiol equavalent concentrations of 2.57 and <1 ng/L respectively (Figure 3).

Reverse Osmosis assessment

No significant increase of vitellogenin was observed in medaka (Figsures 4 and 5) or trout (data not shown) following continuous or static renewal exposure to salt-reconstituted water after reverse osmosis treatment.

Ground Water assessment

Significant induction of vitellogenin was observed in medaka (male and juvenile) continuously exposed to shallow groundwater in April or October for 21 days (Figures 4 and 5). It was unclear whether the source for induction originated from leachate derived from the delivery system, as shallow ground water prior to delivery and provided to fish in a static renewal system did not significantly induce Vtg. It should be noted, however, that control water sampled prior to or after delivery failed to induce Vtg. It may be possible that compounds in the ground water caused leaching of estrogenic compounds from the tubing of the delivery system. Alternatively, continuous exposure to ground water as opposed to intermittent (static renewal) exposure may allow accumulation of estrogenic compounds from the ground water.

Androgenic/Anti-estrogenic Assessments

Enhanced Vitellogenin expression resulting from E2 exposure was downregulated by subsequent DHT treatment (Figure 6). However, a dose-dependent relationship was not observed. Consequently, further studies using DHT equivalents were not carried out in trout.

Antiestrogenic compounds were observed in wastewater samples from the Green Acres Treatment Plant (Figure 7). Antiestrogenic activity was not present in Santa Ana River water entering Prado wetland. However, water after the wetland treatment had more anti-estrogenic activity than before the treatment.

Conclusions

- Treatment of water with reverse osmosis was the most effective treatment for removing biologically active estrogens.
- *In vitro* methods of estrogen measurement significantly underestimated overall concentrations in tertiary treated wastewater and wetland treated water.
- Estrogenic activity was observed in surface water (Santa Ana River) and appeared to be partially, but not significantly reduced (~50%) by constructed wetland treatment.
- Estradiol equivalent concentrations exceeded "measured" concentrations of specific estrogens previously measured by analytical chemistry.
- Anti-estrogenic activity was observed in tertiary treated effluent and the Santa Ana River following passage through the Prado Wetland indicating constructed wetlands or filtration/chlorination do not completely remove potential endocrine disrupting compounds.
- Source identification using bioassay guided fractionation is recommended to determine identity and relative risk of these compounds in reclaimed water.

Literature Cited

Ankley, G., Mihaich, E., Stahl, R., Tillitt, D., Colborn, T., McMaster, S., Miller, R., Bantle, J., Campbell, P., Denslow, N., Dickerson, R., Folmar, L., Fry, M., Giesy, J., Gray, L. E., Guiney, P., Hutchinson, T., Kennedy, S., Kramer, V., LeBlanc, G., Mayes, M., Nimrod, A., Patino, R., Peterson, R., Purdy, R., Ringer, R., Thomas, P., Touart, L., VanderKraak, G., and Zacharewski, T. (1998). Overview of a workshop on screening methods for detecting potential (anti-) estrogenic/androgenic chemicals in wildlife. *Environmental Toxicology and Chemistry*, **17**(1), 68-87.

Desbrow, C., Rutledge, E. J., Brighty, G. C., Sumpter, J. P., and Waldock, M. (1998). Identification of estrogenic chemicals in STW effluent: 1. Chemical fractionation and in vitro biological screening. *Environmental Science & Technology*, **32**(11), 1549-1558.

Gillesby, B. E., and Zacharewski, T. R. (1998). Exoestrogens: Mechanisms of action and strategies for identification and assessment. *Environmental Toxicology and Chemistry*, **17**(1), 3-14.

Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, M., Sumpter, J. P., Taylor, T., and Zaman, N. (1997). Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environmental Toxicology and Chemistry*, **16**(3), 534-542.

Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, P., Routledge, E. J., Rycroft, R., Sumpter, J. P., and Tylor, T. (1996b). A survey of estrogenic activity in United Kingdom inland waters. *Environmental Toxicology and Chemistry*, **15**(11), 1993-2002.

Huggett, D.B., C.M. Foran, B.Brooks, J. Weston, B.P. Peterson, E.M. Marsh, D.Schlenk (2003) Comparison of In vitro and in vivo bioassays for estrogenicity in fractionated effluent from Municipal Wastewater Effluents. Toxicological Sciences 72:77-83.

Jobling, S., Nolan, M., Tyler, C. R., Brighty, G., and Sumpter, J. P. (1998). Widespread sexual disruption in wild fish. *Environmental Science & Technology*, **32**(17), 2498-2506.

Jobling, S., Sheahan, D., Osborne, J. A., Matthiessen, P., and Sumpter, J. P. (1996). Inhibition of testicular growth in rainbow trout (Oncorhynchus mykiss) exposed to estrogenic alkylphenolic chemicals. *Environmental Toxicology and Chemistry*, **15**(2), 194-202.

Mori, T., Matsumoto, H., and Yokota, H. (1998). Androgen-induced vitellogenin gene expression in primary cultures of rainbow trout hepatocytes. *Journal of Steroid Biochemistry and Molecular Biology*, **67**(2), 133-141.

Nimrod, A. C., and Benson, W. H. (1996). Estrogenic responses to xenobiotics in channel catfish (*Ictalurus punctatus*). *Mar Environ Res*, ***42***, 155-160.

Nimrod, A. C., and Benson, W. H. (1998). Reproduction and development of Japanese medaka following an early life stage exposure to xenoestrogens. *Aquatic Toxicology*, **44**(1-2), 141-156.

Rodgers-Gray, T. P., Jobling, S., Kelly, C., Morris, S., Brighty, G., Waldock, M. J., Sumpter, J. P., and Tyler, C. R. (2001). Exposure of juvenile roach (Rutilus rutilus) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. *Environmental Science & Technology*, **35**(3), 462-470.

Routledge, E. J., Sheahan, D., Desbrow, C., Brighty, G. C., Waldock, M., and Sumpter, J. P. (1998). Identification of estrogenic chemicals in STW effluent: 2. In vivo responses in trout and roach. *Environmental Science & Technology*, **32**(11), 1559-1565.

Sapozhnikova, Y., A. Mcelroy, S. Snyder and D. Schlenk (2005) Estrogenic activity measurement in wastewater using in vitro and in vivo methods In: *Methods of Aquatic Toxicology* Lewis Publishers; Boca Raton, FL..

Smeets, J. M. W., Rankouhi, T. R., Nichols, K. M., Komen, H., Kaminski, N. E., Giesy, J. P., and van den Berg, M. (1999). In vitro vitellogenin production by carp (Cyprinus carpio) hepatocytes as a screening method for determining (anti)estrogenic activity of xenobiotics. *Toxicology and Applied Pharmacology*, **157**(1), 68-76.

Snyder, S. A., Keith, T. L., Verbrugge, D. A., Snyder, E. M., Gross, T. S., Kannan, K., and Giesy, J. P. (1999). Analytical methods for detection of selected estrogenic compounds in aqueous mixtures. *Environmental Science & Technology*, **33**(16), 2814-2820.

Sumpter, J. P., and Jobling, S. (1995). Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environmental Health Perspectives*, **103**((Sup 7)), 173-178.

Tamura H, Maness SC, Reischmann K, Dorman DC, Gray LE, Gaido KW (2001). Androgen

receptor antagonism by the organophosphate insecticide fenitrothion. TOXICOLOGICAL SCIENCES 60 (1): 56-62.

Thomas KV, Balaam J, Hurst MR, Thain JE (2004). Identification of in vitro estrogen and androgen receptor agonists in North Sea offshore produced water discharges Environmental Toxicology and Chemistry 23: 1156-1163.

Thompson, S., Tilton, F., Schlenk, D., and Benson, W. H. (2000). Comparative vitellogenic responses in three teleost species: extrapolation to in situ field studies. *Marine Environmental Research*, **50**(1-5), 185-189.

Tilton, F. A. (1999). “Variable Release of Estrogenically Active Municipal Wastewater: A Biochemical and Chemical Characterization,” Masters, University of Mississippi, Oxford, MS.

Tilton, F. W.H. Benson, and D.Schlenk (2002) Evaluation of Estrogenic Activity from a Municipal Wastewater Treatment Plant with Predominantly Domestic Input. Aquatic Toxicology 61:211-224.

Todorov, J.R., A. A. Elskus, D. Schlenk, P. L. Ferguson, B. J. Brownawell, and A. E. McElroy (2002) Estrogenic Responses of Larval Sunshine Bass (*Morone saxatilis* X *M. chrysops*) Exposed to New York City Sewage Effluent. Marine Environmental Research 54:691-695.

Figure 1. Plasma vitellogenin levels in juvenile rainbow trout exposed to wastewater from the Green Acres Plant (left panel) and the estimated estradiol equivalent concentrations for the wastewater (right panel). * $P < 0.05$.

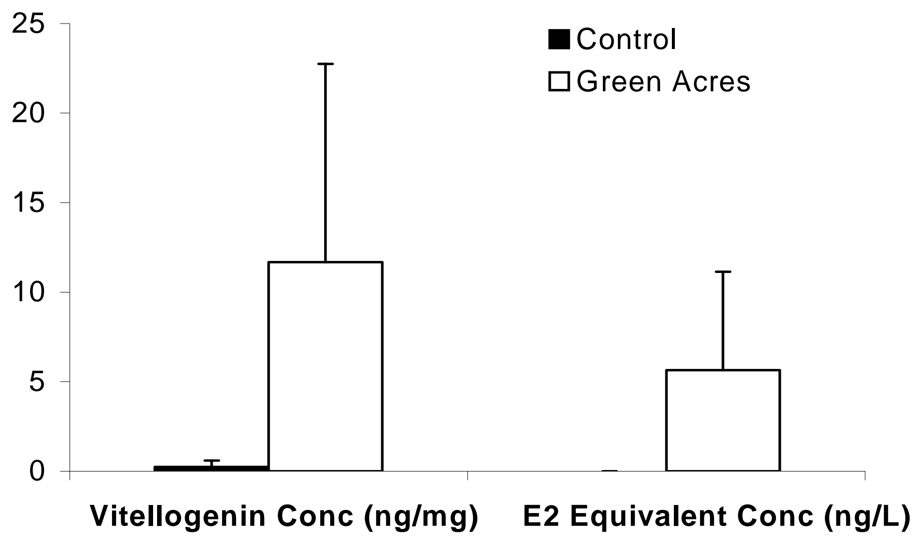


Figure 2. Plasma vitellogenin levels in juvenile rainbow trout exposed to wastewater entering and exiting Prado Wetland and the estimated estradiol equivalent concentrations for the wastewater (right panel). * *P* <0.05.

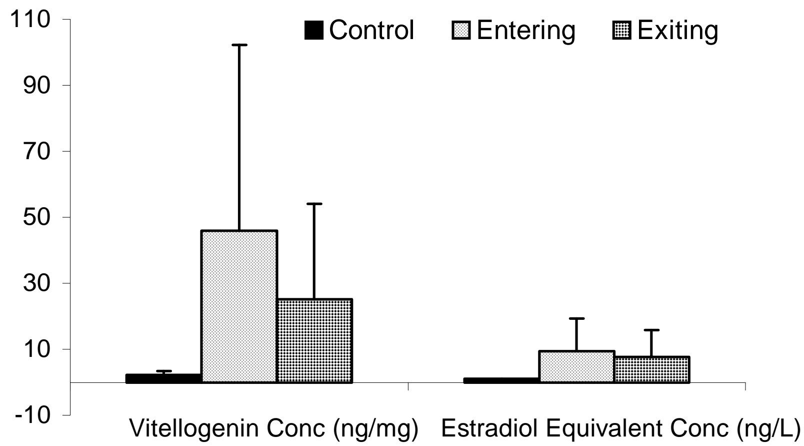


Figure 3. Estradiol equivalent concentrations estimated by yeast estrogen screening assay of wastewater samples from the Green Acres Plant and Prado Wetland.

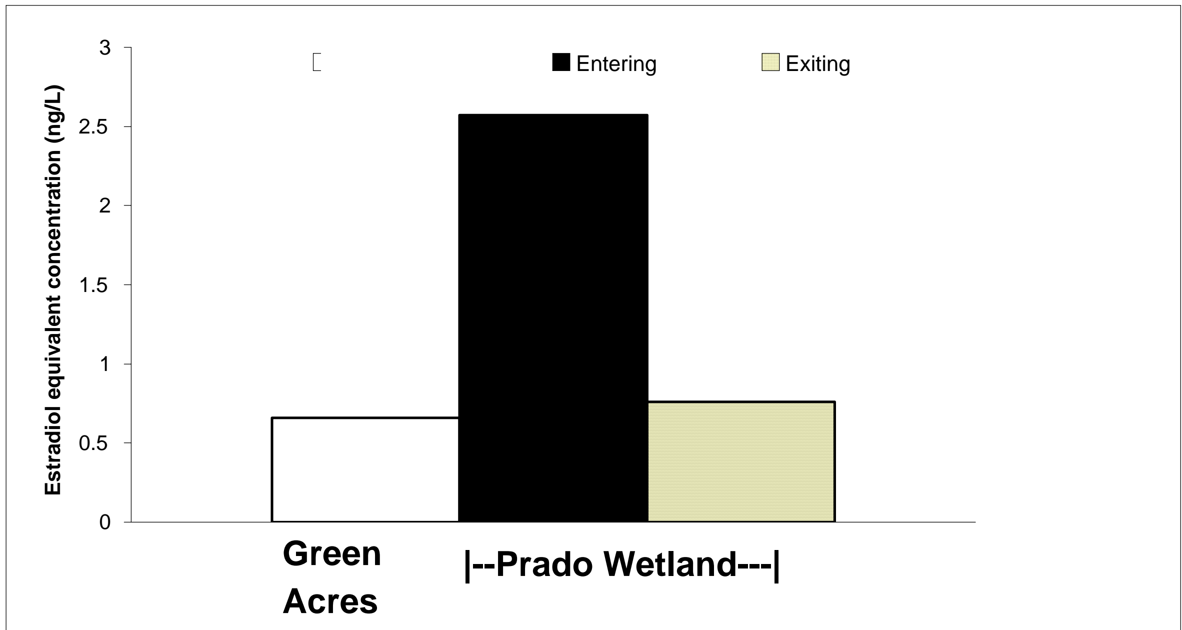


Figure 4. Vitellogenin (Vtg) expression in male medaka continuously exposed to reconstituted reverse osmosis water (Control) and shallow ground water (SAR) for 21 days. (* $p \leq 0.05$)

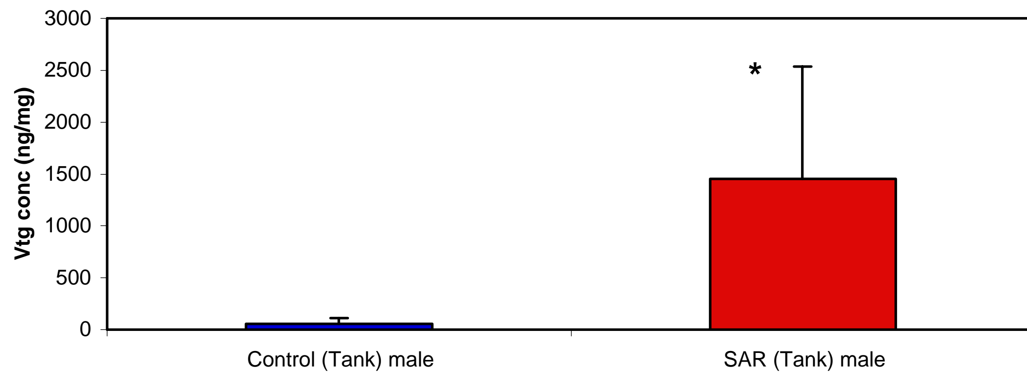


Figure 5. Vtg expression in male medaka continuously exposed to reconstituted reverse osmosis water (Control) and shallow ground water (SAR) for 21 days. (* $p \leq 0.05$)

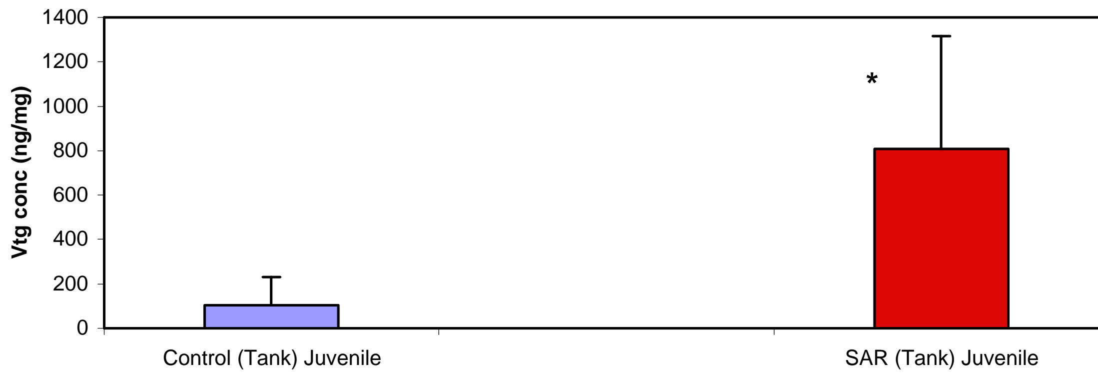


Figure 6. Hepatic vitellogenin (Vtg) concentrations in E2 pre-treated (7 days) Japanese medaka followed by DHT exposure. (*: $p \leq 0.05$)

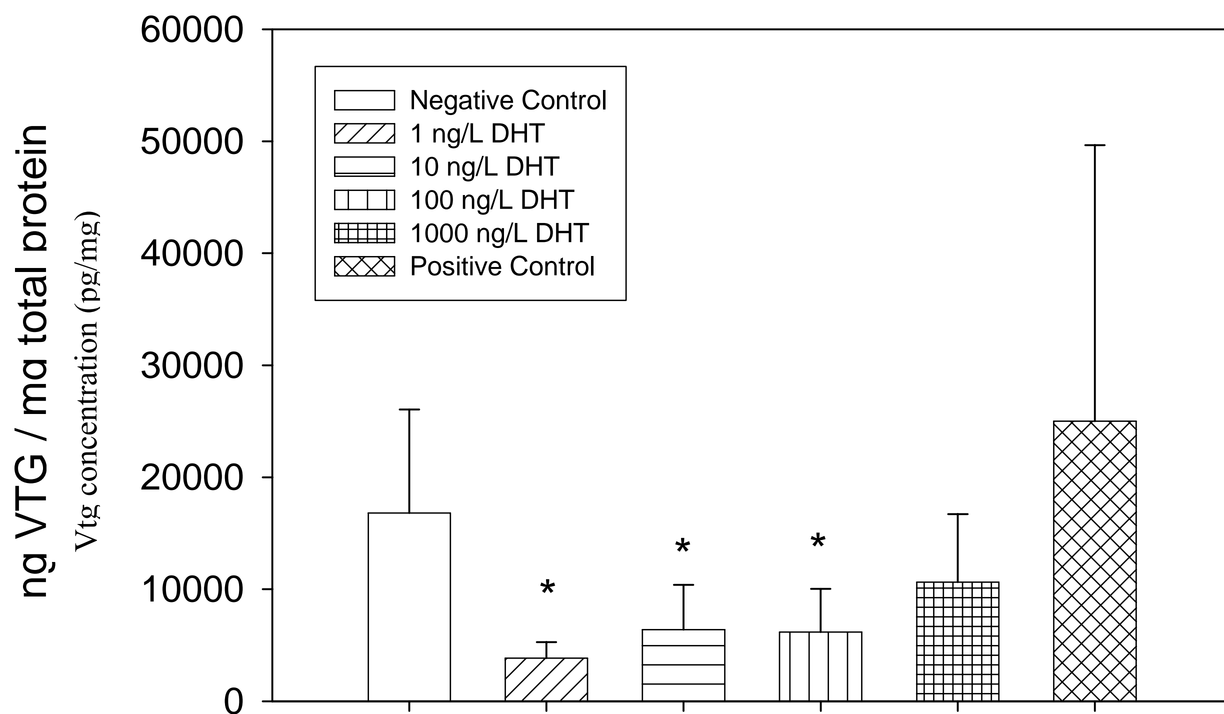
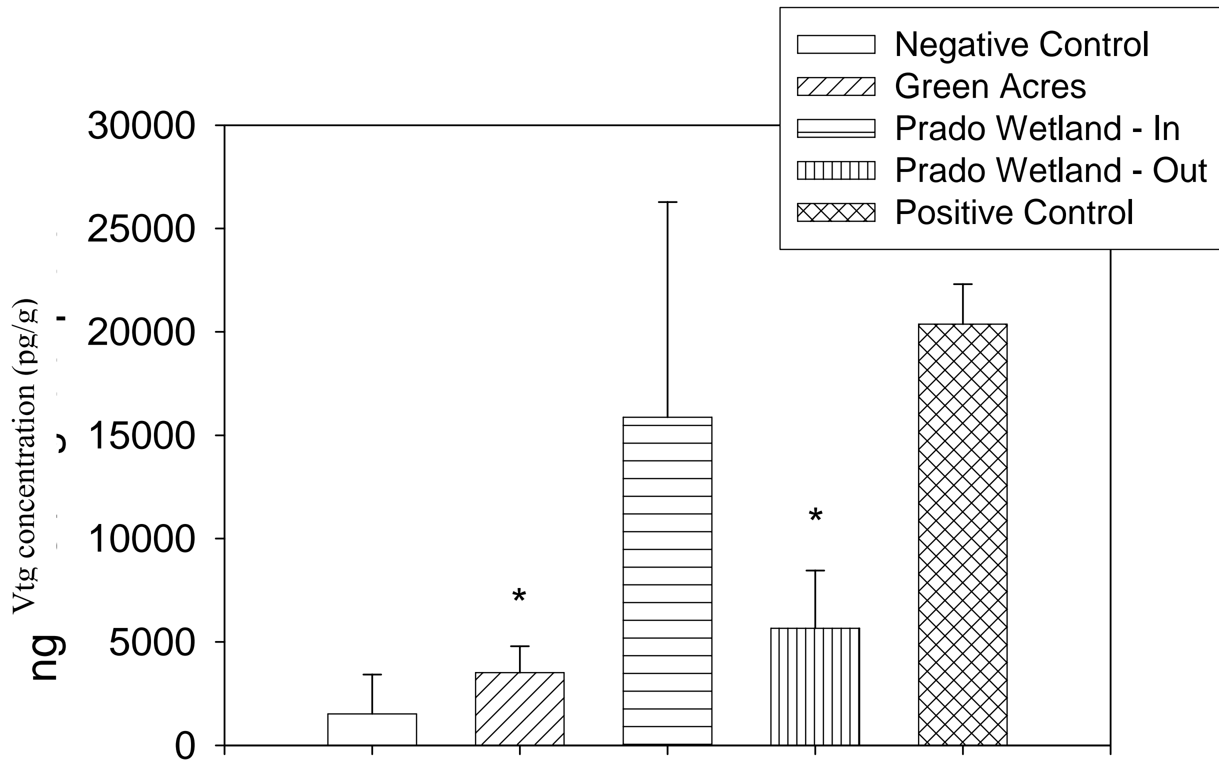


Figure 7. Hepatic vitellogenin (Vtg) concentrations in E2 pre-treated (7 days) Japanese medaka followed by exposure to wastewater samples. (*: $p < 0.05$)



Publications:

Xie, L., Y. Sapozhnikova, O. Bawardi, and D. Schlenk (2005) Evaluation of wetland and tertiary wastewater treatments for estrogenicity using in vivo and in vitro assays. Archives of Environmental Contamination and Toxicology 48:82-87.

Xie, L. Hinton, D. and D. Schlenk (2004) Evaluation of shallow groundwater from California for estrogenic activity using Japanese medaka (*Oryzias latipes*). Abstracts of the 25th Annual Meeting of the Society of Environmental Toxicology and Chemistry. Portland, OR

Shi, D. Xie, L., and D. Schlenk (2004) Inhibition of Vitellogenin Expression in 17 ß-Estradiol Treated Male Japanese Medaka by Subsequent Dihydrotestosterone Exposure. Abstracts of the 25th Annual Meeting of the Society of Environmental Toxicology and Chemistry. Portland, OR

Xie, L., Sapozhnikova, Y., Bawardi, O., Woodside, G. and D. Schlenk (2003) Evaluation of Estrogenicity in Tertiary Treated Wastewater Effluent from Orange County Water District, California, USA. Abstracts of the 24th Annual Meeting of the Society of Environmental Toxicology and Chemistry. Austin, TX