FINAL REPORT: ASSESSMENT OF ENVIRONMENTAL ESTROGENS IN THE HUDSON RIVER DRAINAGE SYSTEM

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January 28, 2000

EXECUTIVE SUMMARY

Endocrine modulating chemicals consist of a vast array of 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. These compounds 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 estrogens). Previous experiments in our lab as well as others have shown that exposures to ng/L concentrations significantly alter endocrine and reproductive function in fish.

In view of the above considerations, we proposed to test the hypothesis that **sewage effluent contained endogenous estrogens and/or xenoestrogens at concentrations that disrupt endocrine and/or reproductive function in resident fish of the Hudson River.** Because of the tremendous usage of synthetic estrogens such as ethinyl estradiol in addition to naturally occurring estrogens (i.e. 17β-estradiol), we hypothesized that these compounds will be present in waste water treatment plant (WWTP) effluents at concentrations that lead to estrogenic and subsequent reproductive anomalies in fish populations.

To test the hypothesis that endogenous as well as synthetic compounds were found in sewage effluent in sufficient concentrations to produce estrogenic activity and reproductive toxicity, the following specific aims were carried out:

1. Sewage effluent from a treatment facility on the Hudson River was biologically screened for estrogenic activity using juvenile sunshine bass (*Morone saxalitis* x *chrysops*).

2. To determine the relative concentrations of environmental estrogens, dose-response studies were conducted with 17β estradiol (E2) and estrogen equivalents were determined from vitellogenin responses in sunshine bass.

Results of the investigation indicated concentrations of estrogenic chemicals in the Yonkers WWTP effluent which was sufficient to induce a measureable estrogenic response in juvenile sunshine bass. Sunshine bass previously exposed to 75 percent WWTP effluent for 21 days exhibited induction of plasma VTG 82 percent above reference values, which correlated to 240 ng/L E2 equivalents by the same method. E2 concentrations previously reported in sewage effluent range from 2.7 to 48 ng E2/L (Desbrow et al. 1998) indicating that VTG responses in field fish may result from exposure to a combination of estrogenic compounds in these WWTP effluents. It is likely that other natural (e.g. estrone), synthetic (e.g. ethynylestradiol) and xenoestrogens (e.g. *p*-nonylphenol) increase the E2 equivalent value calculated for sewage effluent. Studies are currently proposed to determine the chemical source(s) of the observed estrogenic activity. It is only through a better and more comprehensive understanding of sources, exposure, dose and response that the Hudson River Foundation can help the public understand and develop policies that are based on sound science. Clearly, underestimating the effect of chemicals may result in serious environmental contamination and adverse ecological health effects, whereas overestimating potential hazards can result in social disruption and undue economic burdens. Therefore, understanding the linkages among sources, exposure, dose and

response will allow communities along the River to make better estimates of hazard and, in turn, better decisions and scientifically sound public policies concerning the Hudson River.

Background

Environmental estrogens refer to a wide range of anthropogenic or naturally occurring compounds that elicit an estrogenic response by mimicking endogenous estradiol. Environmental estrogens mimic the action of endogenous estradiol principally through a receptor mediated mechanism, although indirect estrogenic effects may also occur through the pituitary and/or hypothalamus. Estradiol binds to the estrogen receptor, resulting in a receptor-ligand complex that binds to the Estrogen Response Element (ERE) of DNA, triggering a series of estrogenic events. In mammals, uterine growth is a quantifiable measure of estrogenic activity, whereby in oviparous vertebrates such as fish, the induction of vitellogenin, a precursor of egg yolk protein, is an estrogen receptor mediated response. Typically, compounds with a greater affinity for the estrogen receptor often elicit a more pronounced estrogenic response. However, bioavailability and activity at the target cell also play a significant role in the degree of estrogenicity (Guillette, 1996).

Compounds such as alkylphenol polyethoxylates (APEs) and nonylphenol, a break down product of APEs, have been shown to possess estrogenic activity (Ren et al., 1996; Routledge et al., 1996; White et al., 1994; Jobling et al., 1995,1996), as have several other environmentally-relevant chemicals. Such compounds include polychlorinated biphenyls, as well as the pesticides DDT, methoxychlor and chlordecone, among others. Natural plant and fungal products are also estrogenic. Environmental estrogens may be introduced into the aquatic environment by means of discharge from industrial effluents, municipal sewage treatment facilities and agricultural waste products. Because disruption or alteration of the endocrine system in fish and wildlife species has been associated with adverse reproductive effects, there is concern that exposure to environmental estrogens from these sources may pose a threat to aquatic populations.

While the above environmental estrogens merit investigation due to their occurrence and persistence in the environment, there is increasing evidence that another class of estrogens may be impacting nontarget populations. Research both in the United Kingdom (Harries et al., 1995, 1996; Purdom et al., 1994) and United States (Folmar et al., 1996) have reported estrogenic activity in male fish exposed to sewage effluent. The U.K. Environment Agency reported that the synthetic estrogen, ethinyl estradiol, and endogenous estradiol and estrone' significantly contributed to the estrogenic activity of select sewage effluents (U.K. Environment Agency, 1996). These estrogenic compounds are used extensively in oral contraceptive formulations, hormone replacement therapy and as growth enhancement agents in livestock production. In addition, natural estradiol and estrone are excreted daily by women of reproductive age and female animal livestock.

Concerns regarding potential environmental exposure to pharmaceutical estrogens arise because of their greater potency in relation to the well studied environmental estrogens. For example APEs and some organochlorine compounds are relatively weak estrogens, having 1/50th to 1/10,000th the potency of estradiol (Nimrod and Benson, 1996). There is little information regarding the environmental fate of pharmaceuticals because it is usually assumed that concentrations are lower than those required to elicit biological effects.

In 1990, approximately 10 million American women between the ages of 15 to 44 used oral contraceptives (Peterson, 1995). Oral contraceptives typically contain a combination of estrogen and progestin, with the active ingredients being ethinyl estradiol, typically at dose of 35 ug (Williams and Stancel, 1996). The regimen consists of taking the "pill" for 21 consecutive

days, followed by 7 days without intake, prior to the next 21 day cycle. It has been estimated that 5 to 13 million post-menopausal women are prescribed hormone replacement drugs (Andrews, 1995). The active estrogenic component of hormone replacement drugs are primarily conjugated equine estrogens (estrone, and 17α- and 17β-estradiol) (Bhavnani and Woolever, 1991). The regimen typically involves daily intake of 0.625 mg of conjugated estrogens for 25 consecutive days, followed by five days with no drug treatment.

Expected introduction Concentration

As part of the environmental assessment for all drugs, pharmaceutical companies are required by the US Food and Drug Administration to calculate an expected introduction concentration (EIC) of products to the aquatic environment as a result of patient use (CDER, 1995). The EIC is an initial calculation used to determine if further environmental assessment is warranted. As such, the EIC does not typically consider metabolic processes.

The EIC of ethinyl estradiol was calculated using 1992 US Environmental Protection Agency (EPA) data on the amount of waste water entering publicly owned treatment works in the United States (4.07 x 1013 liters per year) and the estimated kilograms per year of ethinyl estradiol used in oral contraceptive formulations (88 kg/year). Therefore from the present calculations, 2.16 ng/L of ethinyl estradiol has the potential to reach sewage treatment facilities.

Investigations conducted in the United Kingdom quantified ethinyl estradiol in sewage effluent at 0.2 to 7.0 ng/L (U.K. Environment Agency, 1996). As little as 2 ng/L of ethinyl estradiol has been shown to induce vitellogenin and inhibit testicular growth in male rainbow trout (Jobling et al., 1996). Preliminary data in our laboratory indicates similar responses in Japanese medaka (Nimrod and Benson 1998). Therefore, it is quite possible that pharmaceutical products enter the aquatic environment in concentrations sufficient to elicit estrogenic responses. In addition, hormone replacement therapies consisting of conjugated equine estrogens, estradiol and estrone may represent significant loadings to the environment. The U.K. Environment Agency has reported concentrations of estradiol and estrone as high as 40 ng/L in final sewage effluent (U.K. Environment Agency, 1996). In the present discussion, the EIC calculated for estradiol (14.2 ng/L) was based solely upon the use of growth enhancing hormones used in feed livestock. This figure (14.2 ng/L) does not include the endogenous estrogens present in hormone replacement therapy and those that are naturally excreted from reproductive age women. For example, a normal female typically excretes 25 to 100 ug/ day of estradiol at ovulation and 10-80 ug/day during the luteal phase. After menopause, the amount of estradiol excreted drops to 5 to 10 ug, whereas a pregnant women may release as much as 30 ug. Men also excrete approximately 2 to 25 ug of estradiol daily (Williams and Stancel, 1996). Although these endogenous compounds are less potent than the synthetic steroids, they are likely to be present in larger amounts.

The calculation of the EIC assumes that all of the administered drug is excreted from the body unchanged. It also assumes that no further degradation of estrogen occurs. However, both metabolism and biodegradation can decrease the EIC estimates.

Biodegradation in the Environment

Biotransformation studies indicate that estrogens (whether synthetic or naturally derived) are primarily excreted in the urine and/or feces as a sulfate or glucuronide conjugate (Williams and Stancel 1996). However, conjugates are readily hydrolyzed in human gut suggesting that similar metabolic events, involving bacteria, may occur in sewage treatment facilities. For example,

synthetic as well as natural estrogens found in sewage effluent in the United Kingdom were detected as unconjugated, biologically active compounds (U.K. Environment Agency, 1996).

Several studies have examined the biodegradation and concentration of natural and synthetic steroids in activated sewage and waste water effluent. Culture enrichment studies have been conducted in which natural and synthetic estrogens were incubated with microflora from activated sludge and the percent loss of steroids was measured over 28 days (Tabak et al. 1969). Steroids were introduced into the culture enrichment system using two methods and in either case synthetic estrogens (ethinyl estradiol, mestranol) were more resistant to microbial degradation than natural steroids (estrone, estradiol, estriol). Similar findings were reported by Tabak et al. (1981) where concentrations of natural and synthetic steroids were measured in raw and treated sewage from 14 sewage treatment plants in Cincinnati, OH (US). Higher concentrations of synthetic steroids were measured in both the raw and treated sewage when compared to concentrations of the endogenous estrogens. For example, the mean concentration of ethinyl estradiol detected in raw and treated sewage was 1.21 ng/L and 0.81 ng/L, respectively. In comparison, estradiol, estrone and estriol were detected in raw and treated sewage in concentrations ranging from 0.01 to 0.08 ng/L.

Biological Effects of Sewage Effluents

Vitellogenin is a phospholipoprotein synthesized in response to 17β-estradiol receptor stimulation in the livers of oviparous female organisms. Transported to the gonad, it is eventually incorporated into maturing oocytes (Evans 1993). Since expression is relegated predominantly to the female of the species, the appearance of vitellogenin in the male would then be indicative of abnormal hepatic 17β-estradiol receptor activation. Analyses of vitellogenin concentrations in male fish has been extensively employed as an bioindicator of estrogenic activity in feral and caged populations. In a survey of 15 river systems, vitellogenin expression was observed in all fish exposed to effluent from 30 different sewage treatment plants (Purdom et al. 1994). Subsequent studies examining the relationship of vitellogenin expression and distance from sewage treatment plants showed an inverse correlation between distance to the treatment facilities and vitellogenin expression in the caged fish (Harries et al. 1995). Two groups of chemicals were hypothesized to be responsible for the estrogenic activity observed in each study: alkylphenols and/or synthetic estrogens (Purdom et al. 1994; Harries et al. 1995).

To determine the identity of the causative agents, effluent from seven sewage treatment facilities was fractionated chromatographically with the resulting eluents examined for estrogenic activity by a recombinant yeast estrogen assay (U.K. Environment Agency, 1996). Fractions demonstrating estrogenic activity were then subjected to more elaborate chromatographic analyses by HPLC and GC-MS until the structural identities were derived. Primarily three compounds were identified using this methodology: estrone, 17β-estradiol, and ethinyl estradiol. Ethinyl estradiol was found at three of seven sites surveyed at concentrations ranging from 0.2 to 7 ng/L. Concentrations of approximately 2 ng/L have been reported to be estrogenic in male fish (Jobling et al., 1996). The highest concentrations of estradiol and estrone in final effluents were approximately 40 and 80 ng/L, respectively. In the laboratory, rainbow trout exposed to 10 ng/L of estradiol elicited vitellogenin production, whereas, 100 ng/L of estrone was required to generate similar vitellogenin levels. However, exposure of male trout to 25 ng/L of both estradiol and estrone resulted in a significant induction of vitellogenin over that observed with 17β-estradiol alone, suggestive of an additive effect. In the United States, a recent

study examining feral male carp collected down-stream from a municipal sewage treatment facility in Minnesota also demonstrated elevated vitellogenin and reduced serum testosterone (Folmar et al. 1996). However, the causative agents were not identified in this study.

Methods

Objective 1. Biological Screening of Sewage Treatment Effluents

Rationale Since sewage treatment effluents in the United Kingdom have been shown to produce estrogenic responses in caged fish (Jobling and Sumner 1993) and estimated concentrations of endogenous and synthetic estrogens approach levels capable of causing reproductive alterations, we carried out laboratory exposure studies using diluted prechlorinated effluent from the Yonkers WWTP which discharges directly into the Hudson River. The facility was a secondary sewage treatment facility and was chosen based on consultations with Mr. Joseph Marcogliese of the New York Department of Environmental Conservation (Region 3). It is important to note that most of the UK studies found estrogenic activity in effluent resulting from primary treatment facilities (Harries et al., 1995). However, studies in our laboratory indicated significant estrogenic activity with effluent from secondary and tertiary treatment facilities (Tilton et al. 1996). This is significant since every sewage treatment facility on the Hudson River is at least a secondary treatment facility.

The Yonkers facility runs nearly 100 million gallons per day and serves approximately 300,000 people. Effluent was obtained throughout September from the Yonkers facility prior to the chlorination site. Juvenile sunshine bass (*Morone saxatilis* x *chrysops*) and adult male killifish (*Fundulus heteroclitus*) were exposed to untreated water (75% FW, 25% Flax SW) or effluent (75% effluent water, 25% Flax SW), yielding the same salinity in both groups (~ 7.5 ‰). Each group consisited of 5 replicates with 5 animals in each replicate.

After three weeks of daily static-renewal exposure to effluent, plasma samples were isolated and pooled from 5 individuals and analyzed for various biochemcial endpoints indicative of estrogenic activity or altered steroid metabolism.

Vitellogenin and CYP1A, and CYP 3A measurement. Vitellogenin is a phospholipoglycoprotein produced in response to estrogen receptor binding in fish and other egg-bearing animals. CYPlA has been shown to be related to changes in circulating steroid levels in various fish species (Stegeman and Hahn 1994). Plasma VTG was determined by western blot analysis using a universal MAb (HL1364-IC8) provided by Dr. Nancy Denslow (University of Florida).CYP expression was also measured by western blotting utilizing polyclonal antibodies raised against an 18-residue conserved peptide of trout CYPlA1 (Rice et al., 1998) and trout CYP3A27 (Buhler and Wang-Buhler 1998).

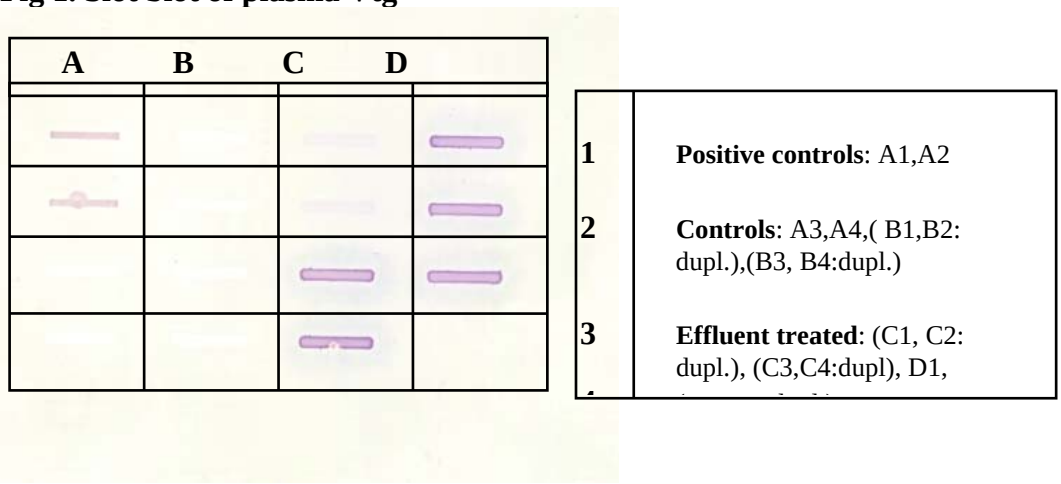
Vitellogenin analysis was performed on blood sera in sunshine bass and in hepatic microsomes in killifish. CYP1A was measured in the hepatic microsomal fraction of both species. Protein concentrations was analyzed using the Pierce colorometric kit.

Serum 17β-Estradiol/testosterone levels, gonadal somatic indices. Steroid levels were measured using a radioimmunoassay kit available through Sigma for the measurement of each hormone. 11-Keto testosterone was combined with testestosterone, since the antibodies recognize both

compounds. Following weighing of the whole fish, gonads and livers were excised from each animal frozen and stored in liquid nitrogen and then weighed. The livers were used for CYP analyses and VTG in killifish.

Vitellogenin levels in both plasma and cytosol from sunshine bass were significantly elevated in effluent exposed animals. Figure 1 is the slot blot of the plasma of sunshine bass probed anti-stripped bass Vtg. Only animals exposed to Yonkers effluent showed any Vtg expression. Hepatic Vtg in sunshine bass was also significantly elevated in effluent exposed animals (Table 1). A linear regression analysis of the data showed a clear positive correlation between plasma and hepatic cytosol Vtg levels, giving a coefficient of correlation of 0.859 (Table 2). In contrast, Vtg was not observed in liver of *Fundulus*.

Fig 1. Slot blot of plasma Vtg



CYP1A expression was significantly elevated (40-45%) in effluent exposed animals of both species (Table 1). In sunshine bass, CYP1A paralleled a significant increase in EROD activity. Although no significant differences could be detected in CYP3A expression in *Fundulus*, values tended to increase in the effluent exposed animals (Table 1). Alterations in CYP isoforms may also significantly alter sex steroid concentrations and feedback loops. However, no apparent difference in circulaing steroids was observed.

Analysis of the effluent for estrogens by ELISA showed the presence of estrogenic compounds (11.1 $\pm$ 2.4 ng/L). These are similar to measurements observed in the UK (Debrow et al., 1998) and capable of eliciting estrogenic reponses in other species (Nimrod and Benson 1998).

Table 1. Effect of 21 d exposure to 75% of Yonkers sewage effluent on plasma and hepatic biochemical indices Values are means +/- SD.

	Morone saxatilis			***Fundulus heteroclitus***		
Endpoint	**Control**	**Effluent**		**Control**	**Effluent**	
Plasma Vtg (Optical density)	2.32 ± 0.40 (n = 4)	38.65 ± 19.14 (n = 4)	p = 0.014	Not measured		
Cytosol Vtg (Optical density)	14.94 ± 1.08 (n = 5)	19.30 ± 2.89 (n = 5)	p = .001	No detectable Vg level		

Plasma protein (mg/ml)	11.01 ± 1.00 (n = 4)	15.49 ± 1.21 (n = 4)	p = 0.001	Not measured		
CYP1A (Optical density)	0.52 ± 0.12 (n = 5)	0.75 ± 0.16 (n = 4)	p = 0.038	0.66 ± 0.09 (n = 4)	0.92 ± 0.19 (n = 4)	p = 0.041
CYP3A4 (Optical density)	Not measured			31.68 ± 20.34 (n = 4)	44.59 ± 3.7 (n = 3)	n.s
EROD (pmol/min/mg)	5.52 ± 3.13 (n = 5)	36.4 ± 7.03 (n = 3)	p < 0.001	Not measured		n.s
Estradiol (pg/ml)	446 ± 239 (n = 5)	420 ± 288 (n = 5)	n.s.	397 ± 153 (n = 5)	429 ± 109 (n = 3)	n.s.
Testosterone (Optical units)	1.29 ± 0.25 (n=5)	1.30 ± 0.22 (n = 5)	n.s.	1.39 ± 0.13 (n=5)	1.40 ± 0 .12 (n = 5)	n.s.

Table 2. Correlation between endpoint parameters

	Morone saxatilis		***Fundulus heteroclitus***	
End points	**R^2**	**P of regr**	**R^2**	**P of regr**
Plasma Vtg vs Cytosol Vtg	0.859 (n= 7)	0.003		
Plasma Vtg vs Plasma protein	0.872 (n= 7)	0.002		
Cytosol Vtg vs Plasma protein	0.922 (n=7)	< 0.001		
Plasma Vtg vs Estradiol	0.081 (n = 8)	n.s.		
Plasma Vtg vs Testosterone	0.050 (n = 8)	n.s.		
Cytosol Vtg vs Estradiol	0.048 (n= 10)	n.s		
Cytosol Vtg vs Testosterone	0.043 (n= 10)	n.s.		
CYP1A vs EROD	0.514 (n= 8)	0.045		
CYP1A vs Estradiol	0.080 (n=9)	n.s.	0.288 (n=8)	n.s.
CYP1A vs Testosterone	0.007 (n=9)	n.s.	0.216 (n=8)	n.s.
CYP3A vs Estradiol			0.004 (n=7)	n.s.
CYP3A vs Testosterone			0.582 (n= 7)	0.046

Objective 2.

The comparative nature of VTG responses among various teleost species may vary tremendously given differences in life histories and reproductive strategies. The present investigation compares plasma VTG induction among three species commonly used in endocrine disruption research. Further comparison of VTG responses obtained from field exposed fish (objective 1) with those observed in laboratory E2 exposed fish provided a means to interpret field responses in terms of relative E2 equivalents.

Adult male Japanese medaka (4 to 6 mos.), adult male channel catfish (12 to 18 mos.) and juvenile hybrid sunshine bass (6 mos.) were exposed to aqueous concentrations of E2 (n=6) ranging logarithmically from 10 to 100,000 ng/L for 21 days. Vehicle control fish were exposed to ethanol. Fish were held in silanized aquaria at a loading capacity of 6 to 9 g/L on a 16:8 hr light/dark cycle. Optimal water quality was maintained for each species with daily 80% static-

renewal.

Exposure water was sampled two times per week to determine actual E2 concentrations. Samples were concentrated utilizing Waters Sep-Pak C-18 columns and E2 concentrations were determined by a competitive EIA with an E2 specific MAb (Munro et al. 1988). Actual E2 concentrations were determined to be 85 to 115% of nominal concentrations 20 minutes post water change.

Blood was obtained from the gill arch of medaka and the caudal vein of catfish and bass, centrifuged at 6,000xg (4^{o}C) for 10 minutes and plasma stored at -80^{o}C until further analysis. For medaka and sunshine bass, plasma VTG was determined by western blot analysis using a universal MAb (HL1364-IC8) provided by Dr. Nancy Denslow (University of Florida). Catfish VTG was detected by similar methods with a catfish MAb provided by Dr. Charlie Rice (Clemson University). VTG was quantified as percent control by densitometry with Scion Image (1998 Scion Corporation). Statistical differences were determined by one-way ANOVA ($p<0.05$). Further analysis was performed using Tukey's test and Dunn's Method for multiple comparisons.

Exposure to E2 for 21 days resulted in a concentration dependent induction of plasma VTG for all species (Figure 2). Juvenile sunshine bass are the least sensitive species to E2 exposure based on threshold and EC_{50} values for VTG expression. Bass demonstrated a significant response above control values at 10,000 ng E2/L, while medaka and catfish had lower threshold values of 1,000 ng E2/L ($p<0.001$). EC_{50} values for bass, medaka and catfish were 1560, 200 and 170 ng E2/L, respectively, indicating that similar responses among these species occur at significantly higher levels of exposure in bass ($p<0.001$). In contrast, bass had the most efficacious response to E2 exposure exhibiting greater maximal VTG expression of 670 percent above control values compared to only 230% for medaka and catfish ($p<0.001$). These data demonstrate that this commonly used indicator of estrogen exposure in laboratory and field studies cannot be directly compared across species due to differences in sensitivity. This supports previous evidence that factors such as species, age and sensitivity need to be critically evaluated prior to selection of an animal model for studies with estrogenic compounds (Routledge et al. 1998).

Comparing VTG expression in field exposed fish with laboratory derived E2-VTG dose response curves provides a means to describe field exposures in terms of relative E2 equivalents. Channel catfish previously exposed *in situ* to WWTP effluent for 21 days expressed plasma VTG 14 to 38 percent over control fish. Using the laboratory-based E2-VTG dose response data for catfish as a standard curve (Fig. 2), the VTG responses of field-exposed catfish were found to correspond to E2 equivalent concentrations between 27 and 115 ng E2/L (Table 3). Sunshine bass previously exposed to 75 percent WWTP effluent for 21 days exhibited induction of plasma VTG 82 percent above reference values, which correlates to 240 ng/L E2 equivalents by the same method. E2 concentrations previously reported in sewage effluent range from 2.7 to 48 ng E2/L (Desbrow et al. 1998) indicating that VTG responses in field fish may result from exposure to a combination of estrogenic compounds in these WWTP effluents. It is likely that other natural (e.g. E2, estrone), synthetic (e.g. ethynylestradiol) and xenoestrogens (e.g. *p*-nonylphenol) increase the E2 equivalent value calculated for sewage effluent. Studies are currently proposed to determine the chemical source(s) of the observed estrogenic activity.

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