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Screening California Surface Waters for Estrogenic Endocrine Disrupting Chemicals (EEDC) with a Juvenile Rainbow Trout Liver Vitellogenin mRNA Procedure

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Abstract

Concern regarding possible occurrence of chemicals that disrupt endocrine system functions in aquatic species has heightened over the last 15 years. However, little attention has been given to monitoring for estrogenic endocrine disrupting chemicals (EEDCs) in California's freshwater ecosystems. Our objective was to screen surface water samples collected in the Central Valley and northeastern area of California for estrogenic activity. The screening procedure utilized vitellogenin (Vtg) mRNA quantification in livers of juvenile rainbow trout by real-time reverse transcriptase polymerase chain reaction (Q-RT PCR). Vtg mRNA analysis of livers from fish exposed to 113 ambient water samples collected from California surface waters indicated that six samples (5% of total) may have contained EEDCs. The six samples induced marginal, but statistically significant, increases of Vtg mRNA. No ambient water sample evoked Vtg mRNA responses equivalent to those in positive controls (all responses were less than 2% of the positive control response). Thus, EEDC concentrations in these samples were low (at or near the threshold for the procedure) or results may have included false positives. Overall, results imply that a majority of the surface water samples tested were below EEDC detection threshold concentration for the screening procedure utilized. To establish a more definitive assessment of EEDC occurrence follow up screening at sites where statistically significant, but weak, estrogenic activity was observed is recommended.

Keywords: Estrogenic endocrine disruption; vitellogenin mRNA; rainbow trout; California surface waters.

1. Introduction

Concern regarding chemicals that potentially disrupt endocrine system functions in wildlife and aquatic organisms has heightened markedly over the last 15 years (e.g., Mills and Chickester, 2005; Propper, 2005; Sumpter, 2005; Sumpter and Johnson, 2005; Norris and Carr, 2006). Several types of chemicals have been shown to affect the function of various endocrine glands. While aquatic ecosystem occurrence of all these endocrine-active chemicals is of concern, the most intensely investigated chemicals are those that mimic or inhibit effects of the vertebrate female reproductive hormones, estrogens. The most common estrogens occurring in the bloodstream of female vertebrates are estrone (E1), 17β-estradiol (E2), and estriol (E3). E2 is the most potent natural estrogen. Chemicals other than natural and synthetic (e.g., 17α-ethynlestradiol, EE2) estrogens that have been suggested to possess estrogenic properties include alkylphenols, bisphenols, polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), dioxins, organochlorine and other pesticides, and phthalates (e.g., Birkett, 2003a; Norris and Carr, 2006). Considerable attention has been devoted to alkylphenols. Alkylphenol polyethoxylates such as nonylphenol ethoxylates and octylphenol ethoxylates are non-ionic components in many detergents, emulsifiers, surfactants, pesticide formulations, and solubilizers. Alkylphenol ethoxylates frequently occur in wastewater treatment facility (WWTF) effluents as well as in industrial and agricultural discharges; their degradation products, alkylphenols, have weak estrogenic properties in some fish species. Bisphenol A, a widely used monomer for epoxy resins and polycarbons, also possesses weak estrogenic properties. Natural, synthetic, and estrogen mimics (xenoestrogens) have been identified, by biological and/or chemical procedures, in aquatic ecosystems of several countries around the globe (e.g., Gomes and Lester, 2003).

Various sources of estrogenic endocrine disrupting chemicals (EEDCs) have been identified or proposed. WWTFs have received the most scrutiny. Biological and or chemical screening of effluents have provided a majority of the data, but several studies, most relying on dispersal of caged male or juvenile fish in the vicinity of outfalls

(generally with a reference site upstream of the discharge point), or the collection of resident phenotypic male fish in an area downstream of outfalls, have linked estrogenic responses to constituents in WWTF discharges (e.g., Birkett, 2003b).

While critical to successful reproduction in vertebrate females, estrogens are not typically produced or are produced in very low quantities by males. Exposure of male fish to natural and/or synthetic estrogens as well as to estrogen mimics, in the laboratory has resulted in feminization/sex-reversal, intersex/ovo-testes, impaired sex differentiation, inhibition of testicular growth, spermatogenesis inhibition, decreased capacity to fertilize eggs, reduced male sex hormone (testosterone and/or 11-ketotestosterone) production, and altered reproductive behavior (e.g., Mills and Chickester, 2005; Sumpter, 2005). In response to the vast literature on fish sexual development and/or reproduction, ambient water quality benchmarks/criteria for EEDCs are being developed in the United Kingdom (e.g., Grist et al., 2003). Given the accumulating concern and the paucity of data on California surface waters, screening ambient waters and effluents for estrogenic activity is in order.

In female fish, amphibians, reptiles, and birds one essential function of ovarian-produced estrogen is to evoke the expression of the vitellogenin (Vtg) gene(s) in the liver (a tissue specific gene). Vtg is the phosphoprotein precursor of egg yolk proteins. Liver synthesis of Vtg is estrogen dependent and it is transported in the bloodstream to the ovary during oogenesis/oocyte maturation. The Vtg gene(s) exists in the liver of male fish, but Vtg normally cannot be detected (or occurs at very low concentrations) in the bloodstream of males because they produce no or very little estrogen. The male fish liver Vtg gene(s) can, however, be activated by estrogen (e.g., de Vlaming et al., 1980). The appearance of Vtg in the plasma of adult male or juvenile fish is widely accepted as evidence of exposure to estrogenic chemicals. In fact, this response is the most common diagnostic tool for detecting estrogenic substances in aquatic ecosystems and effluents as well as in laboratory experiments. An important characteristic of Vtg induction in male and juvenile fish is the specificity of the response to estrogens or estrogen mimics.

Induction of the liver cell Vtg gene(s), as well as the zona radiata gene(s), evokes transcription to gene-specific mRNA that is subsequently translated into Vtg and zona radiate proteins (ZRP). Real-time reverse transcriptase polymerase chain reaction (Q-RT PCR) of Vtg and ZRP mRNAs has been applied to screening for EEDCs. Fish liver Vtg or ZRP mRNA has been quantified by several as a biomarker of exposure to EEDCs (e.g., Mills et al., 2003; Robinson et al., 2003; Aerni et al., 2004; Garcia-Reyero et al., 2004; Zhang et al., 2005).

Thorpe et al. (2000) reported that exposure of juvenile rainbow trout (*Oncorhynchus mykiss*) to natural estrogen (17β-estradiol, E2) or to 4-tert-nonylphenol for 14 days yielded a concentration-dependent induction of plasma Vtg. The response relates to the fact that natural estrogen production is low in juveniles. Others have utilized juvenile rainbow trout (e.g., McClain et al., 2003; Thorpe et al., 2000, 2003; Van den Belt et al., 2003; Allard et al., 2004; Nakari, 2004; Xie et al., 2005) or juveniles of other fish species (Beresford et al., 2004; Garcia-Reyero et al., 2004; Hahlbeck et al., 2004; Versonnen et al., 2004; Vermeirssen et al., 2005; Zhang et al., 2005) to screen for exposure to estrogenic compounds. Use of juvenile fish has notable advantages. They are less expensive and require much less space (tank size and water volume) and effort to maintain compared to adult male fish.

The objective of this investigation was to apply an EEDC screening procedure with juvenile rainbow trout to surface freshwater samples collected in California. The screening procedure was to be relatively straight-forward, able to be completed in a reasonable time-frame, and economically feasible. The concept was to adapt the adult male fathead minnow Vtg mRNA procedure developed at US EPA, Cincinnati (Lattier et al., 2002; Biales et al., 2007) for use with juvenile rainbow trout.

2. Materials and Methods

2.1 Sites and sampling

Sampling sites selected for this study are on waterways in the California Central Valley and northwestern California. Sampling site locations and selection rationales are summarized in Table 1. Locations of the sites are depicted in Figure 1. Several sites were immediately downstream of WWTFs, other were selected to represent agriculture or urban land use. Many sites were sampled only once (to expand spatial coverage) whereas some 'core' sites were sampled up to seven occasions (for temporal coverage). Samples were collected between late March and mid-September. A preponderance of samples were collected July through mid-September because this is a period of low flows and low dilution. Ambient water samples were collected mid-channel when possible, as subsurface grabs in one gallon (3.8 L) amber bottles. These samples were packed immediately in wet ice for transport to the University of California, Davis (UC Davis) Aquatic Toxicology Laboratory (ATL). In the laboratory the samples were refrigerated at 4° C in darkness until test set-up (all within 48 hours of sample collection).

2.2 Ambient water screening

There were a total of 13 sampling and screening events. The date and sites sampled are summarized below by event.

Event 1--Eight sites on waterways in the Sacramento River or San Joaquin River watersheds (California's Central Valley) were sampled on September 9 or 10, 2003. All sites were immediately downstream of WWTF outfalls.

Event 2--Seven sites on waterways in northwestern California were sampled on September 17, 2003. Four sites on the Russian River were sampled to represent low flow conditions without WWTF input. Two Shasta River sites were sampled to represent irrigated agricultural land use. A Yreka Creek site was downstream of a wastewater land disposal area.

Event 3--Eight sites were sampled on July 28, 2004. Four sites were on waterways in the Sacramento River or San Joaquin River watersheds. All four sites were downstream of WWTF outfalls. Four other sites were on waterways in the Tule Lake (north central California) area and were selected to represent irrigated agriculture land use. A Yreka Creek (northwest California) site was downstream of a wastewater land disposal area.

Event 4--On September 15, 2004 eight sites were sampled. Four of these sites were on California Central Valley waterways downstream of WWTF outfalls. Four other sites were in the Russian River watershed; all were selected to represent agricultural and light urban land use.

Event 5--Eleven sites were sampled on March 29, 2005. Four of the sites were in the Russian River watershed and were sampled to represent spring high flow conditions. Six sites were in the California Central Valley. Four of these sites were downstream of WWTF outfalls and two sites were on agriculture-dominated waterways in the Sacramento River watershed.

Event 6--On May 10, 2005 nine sites were sampled. Five of the sites were downstream of WWTF outfalls in the California Central Valley and two were on agriculture-dominated waterways in the Sacramento River watershed. One site was on the Laguna de Santa Rosa in the urban City of Santa Rosa (northwest California).

Event 7--Five sites were sampled on June 1, 2005. Juvenile rainbow trout were exposed to these ambient samples for 24 hours and eight days before harvesting livers. Two of the sites were downstream of WWTF outfalls and three were on agriculture-dominated waterways. Two of the agriculture-dominated sites and one of the sites downstream of a WWTF were in the California Central Valley. One of the agriculture-dominated sites and one of the sites downstream of a WWTF were in northwestern California.

Event 8--Again on June 13, 2005 five sites in the California Central Valley were sampled. Two of the sites were downstream of WWTF outfalls and three were on agriculture-dominated waterways.

Event 9--On July 12, 2005 nine sites were sampled. Six of these sites were on waterways in the San Joaquin River watershed with agriculture being the major land use. Three other sites were in City of Santa Rosa area (northwestern California). These sites were in urban areas with some land use devoted to agriculture and dairies.

Event 10--Ten sites were sampled on July 26, 2005. Seven of the sites were in the San Joaquin River watershed; five of these were on agriculture-dominated waterways, one site in an urban area, and one site downstream of a WWTF outfall. Three sites were in the urban City of Santa Rosa area that included some agriculture and dairy land use.

Event 11--Samples were collected at seven sites in the California Central Valley on August 9, 2005. Five sites were downstream of WWTF outfalls and two sites were on agriculture-dominated waterways.

Event 12--On August 29 and 30, 2005 14 sites were sampled. Four sites were in the urban City of Santa Rosa area that included some agriculture and dairy land use (same sites as in Event 9). Six sites in the California Central Valley were downstream of WWTF outfalls. Two sites were in the urban City of Sacramento area and two sites were on agriculture-dominated waterways in the Sacramento River watershed.

Event 13--Eleven samples were collected on September 13, 2005. Three of these sites were on the Sacramento River and four were on tributaries to this river. The remaining four sites were situated so as to assess potential effects of the Healdsburg WWTF's waste pond on the Russian River.

2.3 Fish

Juvenile rainbow trout (size range: 3.1 to 5.4 cm) were obtained from Thomas Fish Company, Anderson, CA. Fish were acclimated for 4 to 7 days prior to initiating experiments in laboratory control water (see below) at the temperature maintained during testing. Each day of acclimation included changing approximately 50% of the water in 37.85 L aquaria. Two AS-1 air stones aerated each tank and the fish were fed Silver Cup Trout Chow, #1 Crumble after the water change.

2.4 Water and water quality

Control water in all experiments consisted of one part deionized water to 1.7 parts well-water (very hard) to achieve a hardness of approximately 200 mg/L. Positive controls and the solvent control were constituted in this water.

In 24-hour ambient water exposures hardness, alkalinity, ammonia, temperature, pH, dissolved oxygen, and specific conductivity were measured prior to test initiation and at termination (with the exception of hardness and alkalinity). In 8-day exposures the same procedure was followed except hardness and alkalinity also were measured at test termination. In the 8-day exposures ammonia was measured at initiation and on day 2 prior to water change out. At each water change out pH, DO, temperature, and specific conductivity were recorded.

2.5 Exposures

Juvenile rainbow trout exposures were in either 9.5 L rectangular aquaria filled with 7.6 L of water (screening Events 1 & 2) or 2 L beakers filled with 1.5 L of water (all other ambient water screening events). Beakers were covered with plastic Petri dishes to prevent splashing and loss of fish. Each exposure chamber was provided with an air bar adjusted so as to maintain dissolved oxygen at 9.8 mg/L O_2. Upon completion of an experiment exposure chambers were washed with laboratory glassware soap, thoroughly

rinsed, soaked in an acid bath, acetone rinsed, triple-rinsed with deionized water, and dried.

Exposure chambers were placed into a recirculation, constant temperature bath. Chambers/replicates belonging to the same treatment groups were randomly placed in the bath to avoid potential location effects. For screening Events 1 and 2 temperature was maintained at 12°±2°C. In all other experiments temperature was set at 15°±2°C because an experiment (conducted prior to ambient water screenings) revealed that Vtg mRNA production was higher than at 12° C. Photoperiod in all experiments was 16L:8D.

Juvenile trout exposure was for 24 hours in screening Events 1 though 4. Fish were not fed during the exposures. Exposure duration in screening Events 5 through 13 was eight days. Exposure duration was extended to eight days with the thought that weak estrogenic chemicals and/or chemicals that have to bioaccumulate to a threshold level would be more likely detected. Approximately 80% of water in each beaker was changed every other day. Fish were fed 2 hours prior to water change out. At change out particulate (e.g., food, feces, etc.) matter was siphoned out of the beakers

All experiments involved at least three sets of controls: (1) Laboratory control water, (2) Positive control (to assure that fish were responding to an estrogenic substance), and (3) Solvent control (to assure that the solvent for the positive control was free of estrogenic effects). The positive control was 17α-ethinylestradiol (EE2, a common component of birth control pills and frequently detected in waste treatment facility discharges). The solvent control in all experiments was 0.001% methanol.

Screening Events 1 and 2 consisted of three fish per replicate with three replicates per treatment. For all other screening events there were three fish per replicate with five replicates per treatment.

2.6 Liver harvesting

In this project, assessing exposure to estrogenic compounds was gauged by quantifying Vtg gene expression (i.e., Vtg mRNA) in liver samples from juvenile rainbow trout. Upon termination of exposure to a known estrogenic substance or to ambient water samples fish were placed in an ice bath to immobilize them. When immobilized the celomic cavity was opened by an incision from the anal vent to the mouth. A segment of liver (approximately 10 mg.) was collected, avoiding rupture of the gall bladder, and placed in fixative. Liver Vtg mRNA analyses for screening Events 1 through 4 were performed at the UC Davis Lucy Whittier Molecular & Diagnostic Core Facility and for Events 5 through 13 at the US EPA laboratory in Cincinnati, Ohio. For Q-RT TaqMan® PCR analyses at Lucy Whittier Molecular & Diagnostic Core Facility liver pieces were placed into 500 µL of lysis buffer (Nucleic Acid Purification Lysis Solution, Applied Biosystems, Foster City, CA) in one well of a 96 deep-well plate. These plates were stored at -18°C until analyzed. For mRNA analyses performed by US EPA, liver pieces were placed into 1 ml of Tri Reagent® in 1.5 ml snap-top micro-centrifuge tubes. Tubes were stored at -18°C (for no more than 7 days) until a space in an -80°C freezer was available. The micro-centrifuge tubes were shipped to US EPA on dry ice via overnight delivery.

Each fish liver piece was fixed and analyzed individually in screening Events 1 and 2. In all other screening events liver pieces from the three fish in each replicate chamber were placed into the same fixative vial. So, each replicate consisted of a composite of three liver pieces that were subsequently homogenized together and entered into the Vtg analysis process.

2.7 Vitellogenin mRNA analyses

2.7.1 RNA isolation for the Q-RT TaqMan® PCR analysis (UC Davis)

To extract total RNA from the liver tissues, proteinase K and two grinding beads 4 mm diameter, SpexCertiprep, Metuchen, NJ were added and the tissues homogenized in a

GenoGrinder 2000 (SpexCertiprep) for 2 min at 1000 strokes per minutes. Protein digest was done at 56°C for 30 min followed by a 30 min period at -20°C to reduce foam and precipitate RNA. Total RNA was extracted from the tissue lysates using a 6700 automated nucleic acid (ANA) workstation (Applied Biosystems) according to the manufacturer's instructions. Contaminating gDNA was digested using RNase free DNase I (Invitrogen, Carlsbad, CA) at 37°C for 10 minutes. The absence of gDNA contamination was confirmed by analyzing DNAase digested tRNA without a RT-step.

2.7.2 RNA isolation for SybrGreen PCR analysis (US EPA)

Liver tissue was thawed at room temperature and homogenized for 4 min at 30 cycles s^{-1} in a Retsch MM300 with 3.2 mm grinding beads (BioSpec). Homogenized samples were incubated for 10 minutes at room temperature. 100 µL BCP (Molecular Research) was added and samples were vortexed. Following a 10-minute incubation at room temperature, samples were centrifuged for 15 minutes at 12000 g at 4˚C. 400 µL of the aqueous phase was removed and transferred to a nuclease-free tube containing 500 µL isopropanol. Samples were vortexed and incubated for 10 minutes at room temperature. Samples were then centrifuged at 12000 g for 10 minutes and the supernatant was removed. The RNA pellet was washed with 1 ml 75% ethanol and either stored at -20˚C until precipitations could continue or incubated at room temperature for 2 – 10 minutes. Tubes were then spun for 10 minutes at 12000 g and the supernatant was aspirated. RNA was resuspended in 20 µL RNAse-free water (Ambion) and quantity and purity were determined spectrophotometrically. RNA with 260:280 ratios greater than 1.7 was considered to be of satisfactory quality. An aliquot of RNA was diluted to 250 µg/µL and stored at -20˚C.

2.7.3 Reverse transcription (RT) reaction of total RNA (UC Davis)

Complementary DNA (cDNA) was synthesized using 100 units of SuperScript III (Invitrogen), 600 ng random hexadeoxyribonucleotide ($pd(N)_6$) primers (random hexamer primer) 10 U RNAaseOut (RNase inhibitor), and 1 mM dNTPs (all Invitrogen,

Carlsbad, CA) and 20 µL of DNase digested tRNA in a final volume of 40 µL (Table 3). The reverse transcription reaction proceeded for 120 min at 50°C. After addition of 60 µL of water, the reaction was terminated by heating for 5 min to 85°C and cooling on ice. The quality of the cDNA was assessed using a TaqMan® PCR system specific for trout ITS-2 (Internal Transcribed Spacer 2—the spacer sequence between 5.8S and 28S ribosomal RNA genes).

2.7.4 Reverse transcription (RT) reaction of total RNA (US EPA)

0.5 µg total RNA was reverse transcribed in a 20 µL reaction containing 25 units MuLV reverse transcriptase (Applied Biosystems), 2.5 µM random hexamers, 0.4 µM oligo-dt (New England Biolabs), 5 mM $MgCl_2$, 25 units RNase inhibitor, 1X PCR buffer II (Applied Biosystems) and 1 mM dNTPs. Reactions were incubated at 25˚C for 10 minutes, followed by 42˚C for 45 minutes and at 5 minutes, 95˚C inactivation. All reactions were done using a PE 9700 thermocycler (Perkin Elmer). Resulting cDNA was diluted 1:5 with water.

2.7.5 Q-RT TaqMan® PCR system design and validation (UC Davis)

For each target gene, two primers (Table 3) and an internal, fluorescent labeled TaqMan® probe (5´ end, reporter dye FAM (6-carboxyflourescein), 3´ end, quencher dye TAMRA (6-carboxytetramethylrhodamine) were designed using Primer Express software (Applied Biosystems, Foster City, CA). The forward primer of the vitellogenin TaqMan® system was placed over exon junction 4-5 to eliminate background signals on co-extracted genomic DNA. To normalize the Vitellogenin raw data, a TaqMan® PCR assay targeting the ITS-2 region of rainbow trout was used (Kelley et al., 2004). The TaqMan® PCR assays were validated as described before (Leutenegger et al., 1999). Briefly, a standard curve was generated on a positive control using 2-fold dilutions analyzed in triplicate. From the slope of the standard curve the amplification efficiency was calculated as a measure of analytical sensitivity using the formula $E = 10^{1/-s}-1$.

Analytical specificity was determined by sequencing PCR product using standard sequencing protocols.

2.7.6 Q-RT SybrGreen PCR system design and validation (US EPA)

Primers for Vtg were designed from the published Vtg sequence (NCBI accession number AF169287) using Primer 3 software (Rozen and Skaletsky, 2000). To minimize unwanted primer interactions, maximum self- and 3'- complimentarity were limited to a maximum score of 3 and 1, respectively. Primers were designed to span an intron to distinguish competing signal from genomic DNA contamination from target amplification. Reaction conditions were optimized with respect to primer concentration and the amplification of a single amplicon was confirmed using the Agilent 2100 Bioanalyzer. Additionally, the amplification of a single product was confirmed in every reaction included in the data set using melting curve analysis. Reaction efficiency was determined for both the Vtg and 18S primer sets using the LinReg applet (Ramakers, 2003). Quantification was carried out using a modified version of the $2^{\Delta\Delta Ct}$ method, incorporating an efficiency correction (Pfaffl, 2002). All Vtg expression data were normalized to 18S expression.

2.7.7 Q-RT TaqMan® PCR analysis (UC Davis)

Each PCR reaction contained 400 nM primer and 80 nM probe concentration for the respective TaqMan system and commercially available PCR mastermix (TaqMan® Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 µL of the diluted cDNA sample in a final volume of 12 µL. The samples were placed in 96 or 384 well plates and amplified in an automated fluorometer (ABI PRISM 7900 HT FAST Sequence Detection System, Applied Biosystems). AB's standard amplification conditions were used: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C. Fluorescent signals were collected during the annealing temperature and CT values extracted with a threshold of 0.04 and baseline values of 3-15.

2.7.8 Q-RT Sybrgreen PCR analysis (US EPA)

250 nM of each primer (18S or Vtg) was added to 20 µl PCR reactions containing 1X Dynamo real time PCR hot start master mix (New England Biolabs). Thermocycling parameters were as follows: 15 min at 95°C, then 40 cycles of 20S at 95°C, 20S at 60°C and 20S at 72°C. Fluorescence data were collected at the end of each cycle. Following the amplification reaction a melting curve analysis was carried out between 60°C and 95°C, fluorescence data were collected each 0.1 °C. Ct was set at a fluorescence value of 0.5 for both 18S and Vtg. All real-time reactions were done in an Opticon 2 DNA Engine (Bio-Rad) and data analysis was done using Opticon Monitor v3.00 (Bio-Rad).

2.7.9 Relative quantification of gene transcription

Quantification was performed using a modified form of the equation proposed by Pfaffl et al. (2003) which incorporates an efficiency correction. QPCR reaction efficiencies used in quantification were determined using the LinReg software (Ramakers et al., 2003) based on raw fluorescence data for each reaction and were averaged per 96-well plate for a given primer pair. For the initial primer quality determination, reaction efficiencies were determined using methods outlined in Livak and Prism, (2001) and were considered acceptable if efficiencies were greater than 90%. Threshold values, Ct, were set at a constant of 0.025 fluorescence units, which was within the linear phase of the amplification reaction. Quantities were determined for each experiment independently and are reported as relative quantity values normalized to the relative quantity value of the 18S ribosomal gene, which has been shown to be highly invariant under most experimental conditions including estrogenic exposure (Goiden et al., 2001; Lattier et al., 2002; Schmidt et al., 2003).

2.8 Replication

For screening Events 1 and 2 there were three replicate tanks per treatment, containing three fish each. In analyzing these data the mRNA expression of every fish as a separate data point was considered in order to achieve greater sensitivity to differences between

treatments. This gain in sensitivity brought a degree of pseudoreplication into the analysis, as fish held in the same tank during the experiment were considered to be independent data points.

This situation was rectified in later experiments, where each treatment consisted of five replicate tanks of three fish each. In these experiments, the livers of all three fish in a tank were homogenized into one sample for RT-PCR analysis, giving five fully independent data points per treatment.

2.9 Comparisons between treatments

Before statistical analysis of experiment results normalized quantities of Vtg mRNA were $\log_2$ transformed in order to increase the normality of the data and to make the variances of treatment groups more equivalent. The appropriateness of performing parametric statistics on the log-transformed data were assessed by examining homogeneity of variance between the treatments and the fit of the data in each treatment to normal distributions using Bartlett's test and Shapiro-Wilks test ($\alpha = 0.01$). We compared the means of treatments using 1-way ANOVA or 2-way ANOVA (one-tailed $\alpha = 0.05$) with a multiple comparisons procedure appropriate to the hypotheses being tested by the experiment (Dunnett's or Tukey's test). All tests comparing means between treatments were performed as one-tailed tests because Vtg gene(s) upregulation, not downregulation, was expected.

2. 10 Statistical power

For each experiment, the sensitivity of the test was determined by calculating the power $(1 - \beta)$ to detect 20% of the difference in Vtg mRNA expression between the negative and positive controls.

3. Results

Prior to initiating screening of surface water samples an experiment was conducted to determine whether different juvenile rainbow trout size groups (approximately 5, 10, or 15 cm standard length) or mixed-sex and all female groups respond differently to 17α-ethinylestradiol (EE2—a common component of female contraceptives). There was not a statistical difference in liver Vtg mRNA response to EE2 (10 ng/L-nominal concentration) among the groups after a 24-hour exposure. The results of this experiment allowed flexibility in subsequent screening of ambient waters. It is impossible to visually sex juvenile rainbow trout. Therefore, obtaining all male fish for experiments is essentially impossible without genetic phenotyping. Mixed sex trout are not available year-round at hatcheries in California. The fact that all female fish (available year-round) responded to EE2 as did mixed-sex fish suggested to us that use of all females is acceptable for estrogenic chemical screening purposes. Furthermore, there were no significant differences in responses among the size groups allowing latitude in fish size.

Another experiment was conducted to determine whether an EE2 concentration-Vtg mRNA response relationship in juvenile rainbow trout livers could be observed. Figure 2 illustrates that an EE2 concentration-liver Vtg mRNA response relationship was observed. In an associated experiment we assessed whether the juvenile rainbow trout liver Vtg mRNA response to EE2 seen in control water is equivalent in ambient water samples. To this end a Putah Creek sample from below the UC Davis WWTF outfall and a Laguna de Santa Rosa sample were spiked with a range of EE2 concentrations. Neither ambient sample resulted in liver Vtg mRNA levels different than those fish in laboratory control water. Concentration-liver Vtg mRNA response trends were noted in EE2 spiked laboratory control water and ambient water samples (Fig. 3).

All ambient water samples were screened with juvenile rainbow trout. In screening Events 1 though 5, exposure duration was 24 hours. Exposure duration in ambient water screening Events 6 through 13 was eight days. Liver Vtg mRNA analyses for ambient water screening Events 1 through 4 were performed at UCD and at the US EPA lab for Events 5 through 13. One experiment was conducted to compare the Vtg mRNA

measurement procedures at the two laboratories. While measurements were not equal, results from the two laboratories were equivalent.

The juvenile rainbow trout liver Vtg mRNA procedure was applied to screen 113 ambient water samples. There were 13 sampling events between September 2003 and September 2005. In all 13 screening events the EE2 positive control(s) resulted in a statistically significant induction of liver Vtg mRNA. A total of six samples (5% of total samples) induced marginal, but statistically significant, increases of Vtg mRNA. No ambient water sample evoked Vtg mRNA responses equivalent to those in positive controls.

Two samples that produced significant Vtg mRNA responses were collected during screening Event 6 (May 10, 2005) and Event 8 (June 13, 2005) from Colusa Basin Drain (in Sutter County near confluence with Sacramento River--Tables 4 and 5). Colusa Basin Drain samples were screened on two other occasions (March 29, 2004 and August 9, 2005) without inducing liver Vtg mRNA. The other four samples that resulted in small liver Vtg mRNA responses were collected during sampling Event 10 (July 26, 2005) from Elk Bayou (in Tulare County near confluence with Tule Creek), during Event 12 (August 30, 2005) as an effluent sample from the West Roseville WWTF, Pleasant Grove, during Event 12 from Santa Rosa Creek @ Willowside Road, and during Event 13 (September 13, 2005) from Butte Creek, an agriculture-dominated waterway (but with urban upstream input from the city of Chico) that discharges into Sacramento Slough (Tables 6 through 8, respectively). Colusa Basin Drain, Elk Bayou, and Butte Creek are agriculture-dominated waterways. Detections of estrogenicity were noted only in 8-day exposures.

The mRNA responses in fish exposed to the two Colusa Basin Drain samples were 3 and 1% of the response to the 5 ng EE2/L (Event 6) and 10 ng EE2/L (Event 8) positive controls, respectively. In fish exposed to the Elk Bayou, West Roseville WWTF, Santa Rosa Creek, and Butte Creek samples the liver Vtg mRNA responses were 1.6, 0.1, 0.2, and 0.2% of the positive control response, respectively. The Santa Rosa Creek sample that yielded a statistically significant response was a duplicate sample at this site. The

primary sample at this site failed to induce Vtg mRNA. Santa Rosa Creek was sampled and screened on two other dates; liver Vtg mRNA was not induced in these two other samples. Moreover, all six samples that were significantly different compared to laboratory control water manifested very weak estrogenic responses. Thus, EEDC concentrations in these samples were low (near the threshold for the procedure) or the results included false positives.

To assess the effects of exposure duration one group of positive controls was sacrificed after 24 hours for comparison with positive controls exposed for eight days. Data from screening Events 5 through 13 document that levels of liver Vtg mRNA in positive controls exposed to EE2 (10 ng/L, nominal concentration) for eight days were significantly higher than in fish exposed for 24 hours and are consistent with measured EE2 concentrations. Typically, the 24-hr. exposure mRNA response was 2 to 8% of the 8-day response.

Several ambient water sites were sampled multiple times without detecting estrogenic activity. In the Central Valley Dry Creek, Sycamore Slough, and Miner's Ravine samples were tested 8, 4, and 4 times, respectively, without detecting estrogenic activity. Eleven Russian River samples, collected at various sites, were tested without detecting estrogenic activity. Estrogenic activity was not observed in nine Laguna de Santa Rosa samples gathered at various sites. Three samples collected on different dates from the following sites did not manifest estrogenic activity (Laguna de Santa Rosa @ Mirabel, Yreka Creek, Russian River @ Johnson Beach, Laguna de Santa Rosa @ Occidental Road, and Laguna de Santa Rosa @ Delta Pond).

The ability (sensitivity) to detect Vtg mRNA improved and remained high in ambient screening Events 5 through 13 (Table 9).

4. Discussion

Applying the Q-RT PCR Vtg mRNA analysis of juvenile rainbow trout livers to fish exposed to 113 ambient water samples collected from California surface waters indicated that six samples (5% of total) may have contained EEDCs. The six samples induced marginal, but statistically significant, increases of Vtg mRNA. No ambient water sample evoked Vtg mRNA responses equivalent to those in positive controls (all responses were less than 2% of the positive control response). Moreover, all six samples that were significantly different compared to laboratory control water manifested very weak estrogenic responses. Thus, EEDC concentrations in these samples were low (at or near the threshold for the procedure) and may have included false positives. Most ambient waters screened in this project were below the detection threshold of the procedure utilized. Most sites were sampled (point-in-time grabs) only once or infrequently so pulses of EEDCs could have gone undetected. The absolute detection limit of the procedure utilized is unknown. In five experiments (procedure development) conducted prior to screening surface water samples statistically significant induction of liver Vtg mRNA was observed at 5 ng/L EE2 with 24-hour exposures. Thus, the detection limit for EE2 in 24-hour exposures was 5 ng/L or less. Data collected in this study indicate that response and, thus, detection limit in 8-day exposures is lower.

To establish a more definitive assessment of EEDC occurrence follow up screening is recommended at sites where statistically significant, but weak, estrogenic activity was observed. Samples to be tested should be collected multiple times (e.g., 4 to 6) on an annual cycle.

A review of the literature reveals that the lowest concentrations of EE2 to have adverse effects on male fish sexual development and/or reproduction are 1 ng/L (fathead minnows—Parrott and Blunt, 2005) and 2 ng/L (zebrafish--Orn et al,, 2003; Segner et al., 2003; Fenske et al., 2005). These adverse effect concentrations were determined in long-term, life-cycle exposures. Adverse effect concentrations of EE2 for all other fish species tested to date are 10 ng/L or greater. Therefore, we believe that the procedure detection limit was sufficient (with the caveat of exposure duration) considering the objectives of

the study. Furthermore, our literature review also revealed that on a world-wide scale, the median and mean concentrations of EE2 and E2, the most potent estrogenic chemicals, in surface waters are below 1 and 5 ng/L, respectively. Moreover, the low frequency of detecting estrogenic activity in the 112 California surface water samples is consistent with EEDC adverse effect concentrations being below median and mean concentrations detected globally in a majority of surface water samples.

The samples yielding the low-level Vtg mRNA response were in 8-day rather than 24-hour exposures. The 24-hour exposure Vtg mRNA procedure appears to be effective for detecting relatively low concentrations of synthetic and natural estrogens. However, the liver Vtg mRNA procedure with 24-hour, or even 8-day, exposure may not effectively detect chemicals with low estrogenicity and/or those that must bioaccumulate to threshold concentrations (e.g., alkylphenols). For example, Panter et al. (2002) reported that significant levels of Vtg could be measured in juvenile fathead minnows exposed to 2 ng/L EE2 after four days, but not until day seven when exposed to 10 µg/L pentylphenol (an environmentally unrealistic concentration of this 'weak' estrogenic chemical). In adult male sheepshead minnows (*Cyprinodon variegates*) exposed to 5.6 µg/L NP concentrations (not commonly observed in surface waters) significant induction of liver Vtg mRNA was seen after sixteen, but not, eight days (Hemmer et al., 2001). Moreover, further development of the juvenile rainbow trout liver Vtg mRNA procedure should include investigation of exposure duration (which is clearly an issue in screening procedures), weak EEDCs, and environmentally relevant EEDC mixtures.

Those interested in utilizing juvenile trout for screening purposes should be aware that all male populations are seldom, if at all, available from hatcheries. Further, mixed-sex juvenile rainbow trout are not widely available from hatcheries, not offered on a year-round basis, and sometimes obtainable only once a year. Therefore, all female juvenile trout are much more feasible for year-round experimental purposes.

The various EEDC screening methods have strengths and limitations, and users should be thoroughly aware of the confines of whatever screening method employed. Plasma Vtg

procedures may be more biologically meaningful than the mRNA methods because they embody 'biological cost' to individuals. Furthermore, proteins are more persistent and can accumulate substantially. Based on a review of the literature, Hiramatsu et al. (2005) concluded that several weeks or months may be required for plasma Vtg to return to baseline in male fish exposed to EEDCs, whereas liver Vtg mRNA generally returns to baseline within 24 to 36 hours after cessation of exposure to EEDCs. Screening for EEDCs with plasma Vtg measurements, however, require longer term exposures thus more labor intensive (feeding, cleaning, water change outs, etc.) and costly.

Induction of Vtg, including Vtg mRNA procedures, in male and juvenile fish has proven useful for screening for EEDCs, but several investigations (e.g., Jobling et al., 1996; Gimeno et al., 1998; Pantner et al., 1998; Giesy et al., 2000; Harries et al., 2000; Cheek et al., 2001; Rodgers-Gray et al., 2001; Sohoni et al., 2001; Van Aerle et al., 2001; Ackermann et al., 2002; Schwaiger et al., 2002; Seki et al., 2002; Kang et al., 2002; Kirby et al., 2003, 2004; MacLatchy et al., 2003; Mills et al., 2003; Pelley, 2003; Robinson et al., 2003; Roy et al., 2003; Allard et al., 2004; Kleinkauf et al., 2004a, b; Pawlowski et al., 2004; Fenske et al., 2005) provide data that indicate these biomarkers are not always associated with significant reproductive effects or do not reflect some important reproductive responses, and therefore, are *not* a reliable predictor of impairment. Thus, detecting exposures to estrogenic chemicals should not be applied to conclusions regarding development or reproductive effects.

An association between plasma Vtg, gonadal morphology, and fertility has been most convincingly demonstrated in wild male roach (Jobling et al., 2002a, b). However, in a review article Hiramatsu et al. (2005) surmise that a relationship between Vtg induction by EEDCs and impairment of reproductive function has not been rigorously demonstrated in other species of wild fishes. Wheeler et al. (2005) also commented that there is a lack of a strong correlative link between Vtg and other significant reproductive endpoints. Likewise, according to Sumpter (2005) effects, such as elevated vitellogenin concentrations and intersexuality have, to date, been studied almost exclusively at the level of the individual, and hence whether endocrine-disrupting chemicals cause

population-level consequences is largely unknown. While many publications imply adverse developmental or reproductive effects of proposed EEDCs on fish, there is no universal accord on these claims. Moreover, several publications lack convincing evidence of adverse effects, a large number of articles report effect concentrations on fish in laboratory experiments which are environmentally irrelevant, and data are almost completely lacking to link EEDCs to fish population declines.

5. Conclusion

Screening 113 samples collected from effluent-, agriculture-, and urban-dominated waterways in the Central Valley and northwestern area of California for estrogenic activity indicated that none contained high concentrations of EEDCs. Five percent of the samples induced a marginal, but statistically significant, estrogenic response. EEDC concentrations in these samples were low (at or near the response threshold for the screening procedure) and may have included false positives. While the occurrence of EEDCs that must bioaccumulate (e.g., alkylphenols) to illicit estrogenic endocrine disruption may be a possibility, natural and synthetic estrogens do not appear to be significant contaminants in the study area waterways.

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Table 1. Sites sampled to screen for estrogenic endocrine disrupting activity in the California North Coast Region.

Site Description	Land Use Description[1]	Latitude	Longitude
Russain River @ Talmadge	light urban, agriculture	39.14493	123.18172
Russian River @ Healdsburg Memorial Beach	light urban, agriculture	38.60316	122.85997
Laguna de Santa Rosa @ Mirabel	urban, agriculture, dairy, WWTF	38.49374	122.89246
Russian River @ Johnson's Beach	light urban, agriculture	38.49953	122.99894
Shasta River @ Highway 263	agriculture	41.78139	122.59678
Yreka Creek @ Anderson Grade Road	light urban, WWTF	41.77258	122.60579
Shasta River @ Montegue	agriculture	41.70895	122.5382
Tule Lake @ Pump D	agriculture	41.92489	121.56654
Klamath Straits Drain @ Stateline Highway 161	agriculture	41.99716	121.77782
Tule Lake @ Pump 10	agriculture, WWTF	41.87158	121.49556
Yreka Creek @ Anderson Grade Road	light urban, WWTF	41.77258	122.60579
Russian River @ Cassini Ranch	light urban, agriculture	38.4646	123.05119
Russian River @ Monte Rio Beacg	light urban, agriculture	38.46664	123.01075
Mark West Creek @ Trenton-Healdsburg Road	light urban, agriculture	38.49397	122.85302
Russian River @ Cloverdale	light urban, agriculture	38.80846	123.00774
Laguna de Santa Rosa @ Mirabel	urban, agriculture, dairy, WWTF	38.49374	122.89246
Laguna de Santa Rosa @ Occidental Road	urban, agriculture, dairy	38.4253	122.82936
Laguna de Santa Rosa @ Delta Pond	urban, agriculture, dairy, aquatic herbicide	38.4510947	122.834617
Santa Rosa Creek @ Willowside Road	urban - reference	38.4452705	122.806976
Russian River US Healdsburg Waste Pond	light urban, agriculture	38.58435	122.85855
Russian River DS Healdsburg Waste Pond	light urban, agriculture	38.57642	122.85674
Healdsburg STP Effluent	WWTF secondary effluent	38.58266	122.86596
Healdsburg Waste Pond	WWTF secondary effluent storage pond	38.57583	122.85833

[1] Land use description includes criteria for selection as a sample site. Many sample sites represent mixed uses. Not all land uses may be listed. WWTF is defined as “waste water treatment facility” and indicates that the sample site may contain such effluent.

Table 2. Sites sampled for estrogenic endocrine disrupting activity in the California Central Valley Region.

Site Description	Land Use Description[1]	Latitude	Longitude
Sacramento River @ Freeport	urban, WWTF, agriculture	38.4565	121.5012
Sacramento River @ Delta	forest	40.9388	122.418
Sacramento River @ Keswick Dam	forest, urban	40.6085	122.4469
San Joaquin River @ City of Stockton outfall	urban, WWTF, agriculture	37.9382	121.3358
Unnamed Trib to City of Lodi WWTP outfall (Dredger Cut)	WWTF	38.0875	121.3983
Live Oak Slough @ Clark Rd	WWTF, agriculture	39.2331	121.6653
Stormdrain outfall to Pleasant Grove Creek @ Opal Rd	urban	38.80276	121.33842
Old Alamo Creek @ Vacaville WWTP	WWTF	38.3472	121.9044
Putah Creek @ UC Davis WWTP	WWTF	38.5174	121.7575
Colusa Basin Drain @ Knights Landing	agriculture	38.7988	121.7255
Sycamore Slough @ Hwy 45	agriculture	38.8809	121.8438
Miner's Ravine @ Dick Cook Rd	WWTF	38.7968	121.1358
Bunch Creek @ Iowa Hills Rd	WWTF	39.0985	120.9286
Dry Creek @ Cook Riolo Rd	WWTF, urban	38.7364	121.3369
Pleasant Grove Creek @ Pettigrew	WWTF, urban	38.8124	121.4245
Orestimba Creek @ River Rd	agriculture	37.413	121.0147
Del Puerto Creek @ Vineyard	agriculture	37.5215	121.1488
Los Banos Creek @ Hwy 140	agriculture	37.27662	120.9553
Turlock Irrigation District Lateral # 5 @ Carpenter Rd	urban, WWTF, agriculture	37.4626	121.0312
Hospital Creek @ River Rd	agriculture	37.6105	121.2309
French Camp Slough @ Airport Rd	agriculture	37.8948	121.2758
Elk Bayou @ Laspina St.	agriculture	36.1512	119.3205
Kaweah Creek @ Rd. 182	agriculture	36.3389	119.1665
Hume Lake @ Long Meadow Creek inlet	WWTF	36.7871	118.91361
Buena Vista Slough @ Tule Elk Park	agriculture	35.2763	119.3196
King's River @ Jackson Ave (near hwy 198)	agriculture	36.256	119.8539
Mill Creek near 5th Ave (City of Visalia)	Urban	36.267	119.5475
San Joaquin River @ Lost Lake Park	agriculture	36.9784	119.732
Steelhead Creek @ Beach Lake Rd	urban, WWTF	38.6079	121.4908
Sacramento Slough @ Hwy 113	agriculture	39.1617	121.5964
Wadsworth Canal @ Franklin/Arcade Rd	agriculture	39.1303	121.7529
Arcade Creek @ Norwood Ave	urban	38.6257	121.4568
Elder Creek @ Howe Ave	urban	38.4801	121.4086
Old River @ City of Tracy outfall	WWTF	37.806	121.4047
Tule Canal @ City of Woodland outfall to Yolo Bypass	WWTF	38.6778	121.6719
Unnamed trib to Willow Slough (City of Davis WWTP outfall)	WWTF	38.5907	121.6678
Eldorado Hills Irrigation District - Reclamation effluent	WWTF	n/a	n/a
City of Roseville WWTP - Dry Creek Plant - effluent	WWTF	n/a	n/a
West Roseville WWTP - Pleasant Grove Plant - effluent	WWTF	n/a	n/a
Butte Creek @ Durham/Dayton HWY	agriculture, urban	39.646	121.78568
Battle Creek @ Gover Rd	fish hatchery effluent	40.39195	122.17778
Cottonwood Creek @ Balls Ferry Rd	agriculture, urban	40.37702	122.28386
Yuba River @ Marysville	urban, forest	39.13421	121.59299

[1] Land use description includes criteria for selection as a sample site. Many sample sites represent mixed uses. Not all land uses may be listed. WWTF is defined as “wastewater treatment facility” and indicates that the sample site may contain such effluent.

Table 3. Sequence of PCR primers and TaqMan® probes.

Target	Primer	Sequence (5'->3')[1]	Length	Probe[2]	Probe sequence (5'->3')[1,2]
ITS-2	tTIS2-2f	TCATCAATCGGAACCTCTGG	156	tITS2-75p	CACCGAGCCGTCCTGGTCTGAACTTA
	tITS2-157r	AAGGAAGAGCGCACGGG			
Trout Vitellogenin	tVit-364f	CCCACTGCTGTCTCTGAAACAG	202	tVit-454p	CCTGAGCTCCAGCCTCCTGCAACTC
	tVit-565r	GACAGTTATTGAGATCCTTGCTCTTG			

[1]Exon-exon junctions are underlined

[2]TaqMan probes are labeled 5' with 6FAM and 3' with TAMRA

Table 4. Liver Vtg mRNA in juvenile rainbow trout exposed to ambient samples collected on May 10, 2005.

Treatment	Vtg mRNA expression (Mean ± SE)
Control Water	0.0027 ± 0.0014
Solvent Blank	0.0023 ± 0.0010
Positive Control (5 ng/L EE2) @ 8-days.	0.6444 ± 0.3516[1]
Positive Control (10 ng/L EE2) @ 24-hrs.	0.0657 ± 0.0103[1]
Positive Control (10 ng/L EE2) @ 8-days.	1.0160 ± 0.2800[1]
Colusa Basin Drain @ Knights Landing	0.0192 ± 0.0099[2]
Sycamore Slough @ Hwy 45	0.0003 ± 0.0001
Old Alamo Creek @ Vacaville WWTF	0.0016 ± 0.0007
Putah Creek @ UC Davis WWTF	0.0005 ± 0.0002
Bunch Creek @ Iowa Hills Rd.	0.0004 ± 0.0002
Miners Ravine @ Dick Cook Rd.	0.0035 ± 0.0021
Dry Creek @ Cook Riolo Rd.	0.0025 ± 0.0018
Pleasant Grove Creek @ Pettigrew	0.0012 ± 0.0004
Pleasant Grove Creek @ Pettigrew (replicate)	0.0002 ± 0.0000

[1] Statistically different compared to solvent blank (ANOVA with Dunnett's test, one tailed $p < 0.05$).

[2] Statistically different compared to control water (ANOVA with Dunnett's test, one tailed $p < 0.05$). However, a T-test comparing this sample to the control water showed no significant difference.

Table 5. Liver Vtg mRNA in juvenile rainbow trout exposed to ambient samples collected on June 13, 2005.

Treatment	Vtg mRNA expression (Mean ± SE)
Control Water	0.0023 ± 0.0009
Solvent Blank	0.0042 ± 0.0016
Positive Control (10 ng/L EE2) (24 hr)	0.3374 ± 0.0254[1]
Positive Control (10 ng/L EE2) (8 day)	0.5068 ± 0.2212[1]
French Camp Slough @ Airport Rd	0.0039 ± 0.0007
Dry Creek @ Cook Riolo Rd	0.0037 ± 0.0011
Colusa Basin Drain @ Knights Landing	0.0056 ± 0.0090[2]
Sycamore Slough @ Hwy 45	0.0028 ± 0.0003
Old Alamo Creek @ Vacaville WWTF	0.0046 ± 0.0011

[1] Statistically different compared to solvent blank (ANOVA with Dunnett's test, one-tailed $P < 0.05$).
[2] Statistically different compared to control water (ANOVA with Dunnett's test, one-tailed $P < 0.05$).

Table 6. Liver Vtg mRNA in juvenile rainbow trout exposed to ambient water samples collected on July 26, 2005.

Treatment	Vtg mRNA expression (Mean ± SE)
Control Water	0.0013 ± 0.0009
Solvent Blank	0.0015 ± 0.0004
Positive Control (10 ng/L EE2) (24 hr)	0.1429 ± 0.0585[1]
Positive Control (10 ng/L EE2) (8 day)	1.3298 ± 0.5607[1]
Elk Bayou @ Laspina	0.0208 ± 0.0115[2]
Kaweah River @ Rd 182	0.0017 ± 0.0004
Hume Lake @ Long Meadow Creek Inlet	0.0008 ± 0.0005
Buena Vista Slough @ Tule Elk Park	0.0086 ± 0.0065
Kings River @ Jackson Ave	0.0024 ± 0.0008
Mill Creek @ 5th Ave (City of Visalia)	0.0056 ± 0.0026
San Joaquin River @ Lost Lake Park	0.0026 ± 0.0012
Laguna de Santa Rosa @ Occidental Rd	0.0059 ± 0.0027
Laguna de Santa Rosa @ Delta Pond	0.0016 ± 0.0005
Santa Rosa Creek @ Willowside Rd	0.0048 ± 0.0042

[1] Statistically different compared to solvent blank (ANOVA with Dunnett's test, one-tailed $P < 0.05$).
[2] Statistically different compared to control water (ANOVA with Dunnett's test, one-tailed $P < 0.05$).

Table 7. Liver Vtg mRNA in juvenile rainbow trout exposed to ambient water samples collected on August 30, 2005.

Treatment	Vtg mRNA expression (Mean ± SE)
Control Water	0.0004 ± 0.0002
Solvent Blank	0.0028 ± 0.0024
Positive Control (10 ng/L EE2) (24 hr)	0.0390 ± 0.0027[1]
Positive Control (10 ng/L EE2) (8 day)	0.9867 ± 0.0695[1]
Sacramento Slough @ Hwy 113	0.0018 ± 0.0010
Wadsworth Canal @ Franklin/Arcade Rd	0.0007 ± 0.0003
Arcade Creek @ Norwood Ave	0.0010 ± 0.0005
Elder Creek @ Howe Ave	0.0004 ± 0.0001
City of Roseville WWTF, Dry Creek Effluent	0.0006 ± 0.0001
West Roseville WWTF, Pleasant Grove	0.0014 ± 0.0003[2]
Bunch Creek @ Iowa Hills Rd	0.0005 ± 0.0003
Laguna de Santa Rosa @ Occidental Rd	0.0010 ± 0.0003
Santa Rosa Creek @ Willowside Rd	0.0007 ± 0.0003
Laguna de Santa Rosa @ Delta Pond	0.0007 ± 0.0002
Santa Rosa Creek @ Willowside Rd (Duplicate)	0.0018 ± 0.0006[2]

[1] Statistically different compared to solvent blank (ANOVA with Dunnett's test, one-tailed $P < 0.05$).
[2] Statistically different compared to solvent blank (ANOVA with Dunnett's test, one-tailed $P < 0.05$).

Table 8. Liver mRNA in juvenile rainbow trout exposed to ambient water samples collected on September 13, 2005.

Treatment	Vtg mRNA expression (Mean ± SE)
Control Water	0.0004 ± 0.0001
Solvent Blank	0.0012 ± 0.0004
Positive Control (10 ng/L EE2) (24 hr)	0.0255 ± 0.0015[1]
Positive Control (10 ng/L EE2) (8 day)	1.0561 ± 0.0956[1]
Yuba River @ Marysville	0.0012 ± 0.0002
Butte Creek @ Durham Rd	0.0020 ± 0.0004[2]
Battle Creek @ Gover Rd	0.0005 ± 0.0002
Cottonwood Creek @ Balls Ferry Rd	0.0003 ± 0.0002
Sacramento River @ Freeport	0.0008 ± 0.0006
Sacramento River @ Delta	0.0007 ± 0.0003
Sacramento River @ Keswick Dam	0.0005 ± 0.0002
Russian River u/s Healdsburg waste pond	0.0003 ± 0.0002
Russian River d/s Healdsburg waste pond	0.0021 ± 0.0011
Healdsburg waste pond	0.0002 ± 0.0001

[1] Statistically different compared to solvent blank (ANOVA with Dunnett's test, one-tailed $P < 0.05$).
[2] Statistically different compared to control water (ANOVA with Dunnett's test, one-tailed $P < 0.05$).

Table 9. Statistical power to detect a 20% induction of Vtg mRNA compared to the response to 10ng/L EE2 (positive control). Positive controls were examined at 24 hours, 8 days, or both time points.

		Power (%)	
Test	**Date**	**24hr**	**8day**
Event 3	7/28/2004	24.4	
Event 4	9/15/2004	78.9	
Event 5	3/29/2005		100
Event 7	6/2/2005	99.7	100
Event 8	6/13/2005		100
Event 9	7/12/2005	93.3	100
Event 10	7/26/2005	99.38	100
Event 11	8/9/2005	92.6	100
Event 12	8/30/2005	99.98	100
Event 13	9/13/2005	95.22	100

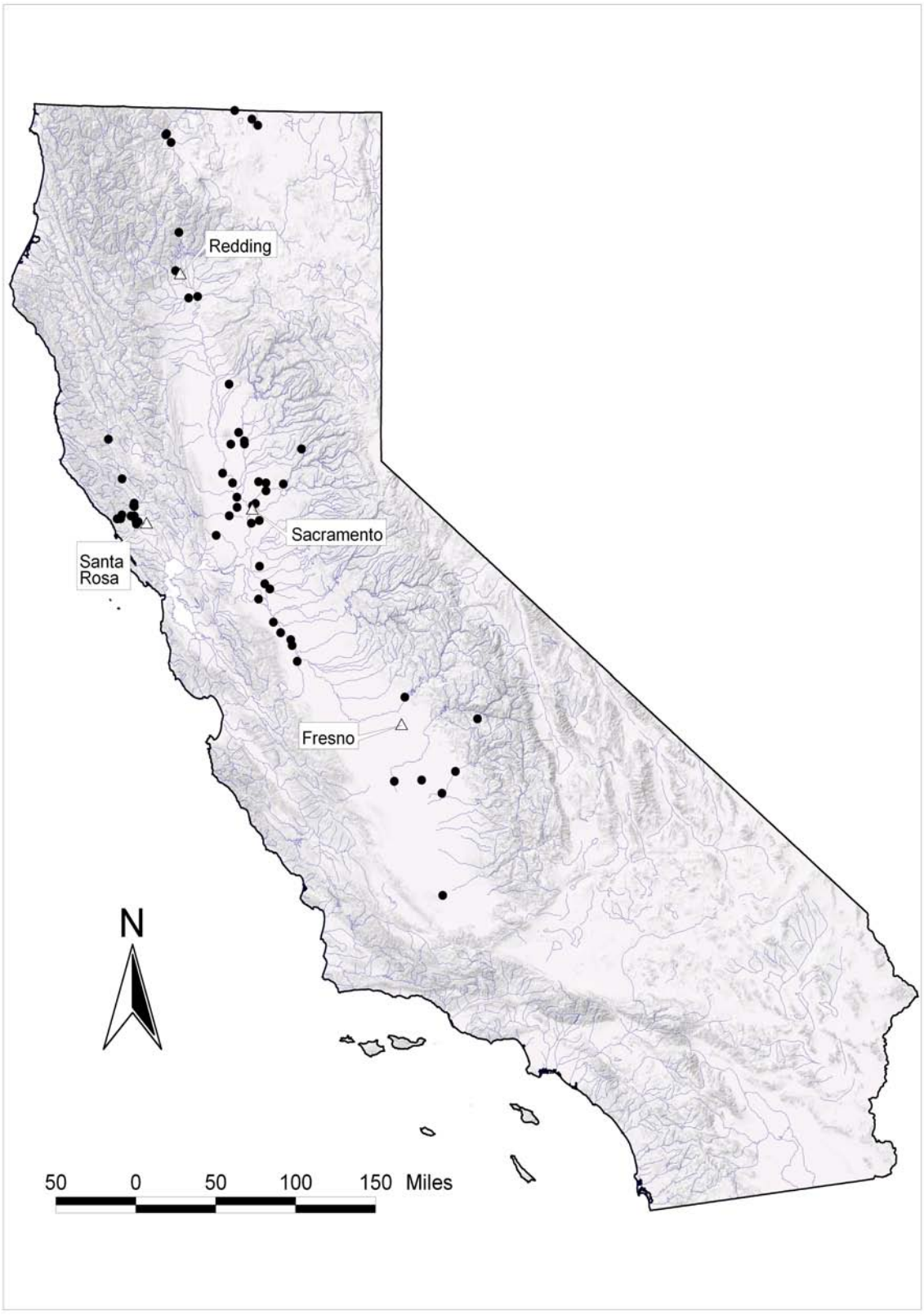

Figure 1. Location of sites sampled for estrogenic endocrine disrupting activity.

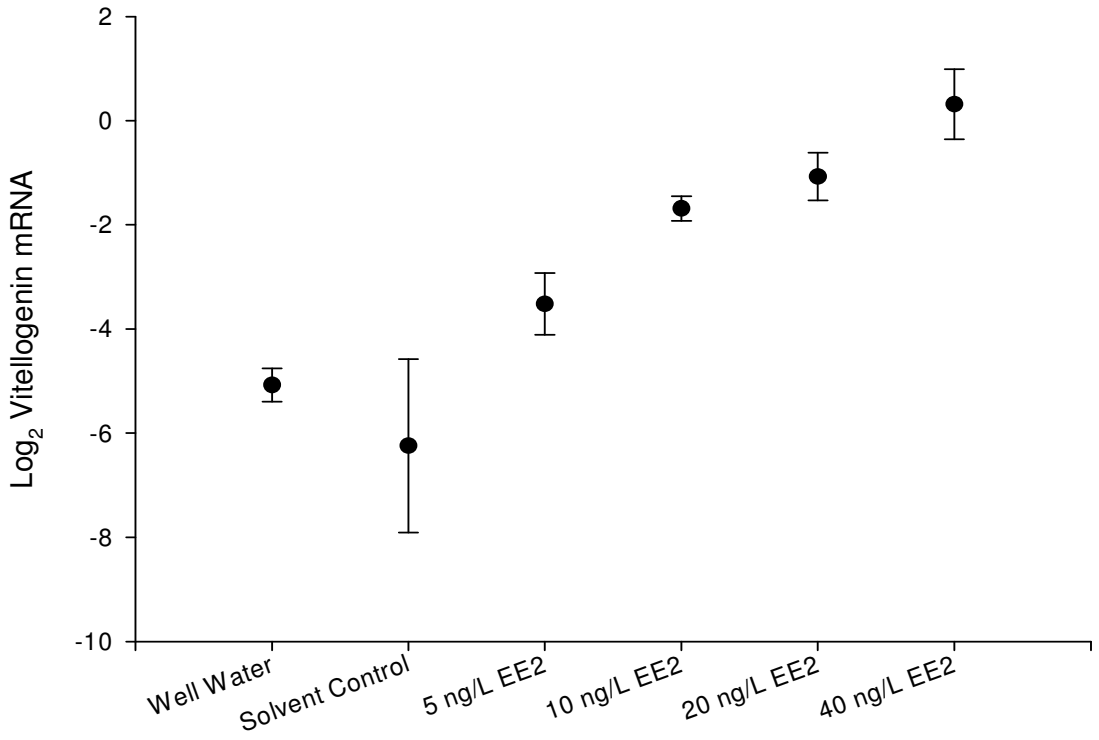

Figure 2. Relationship between EE2 concentration and Vtg mRNA (Mean ± SE) in juvenile rainbow trout during a 24 hour exposure.

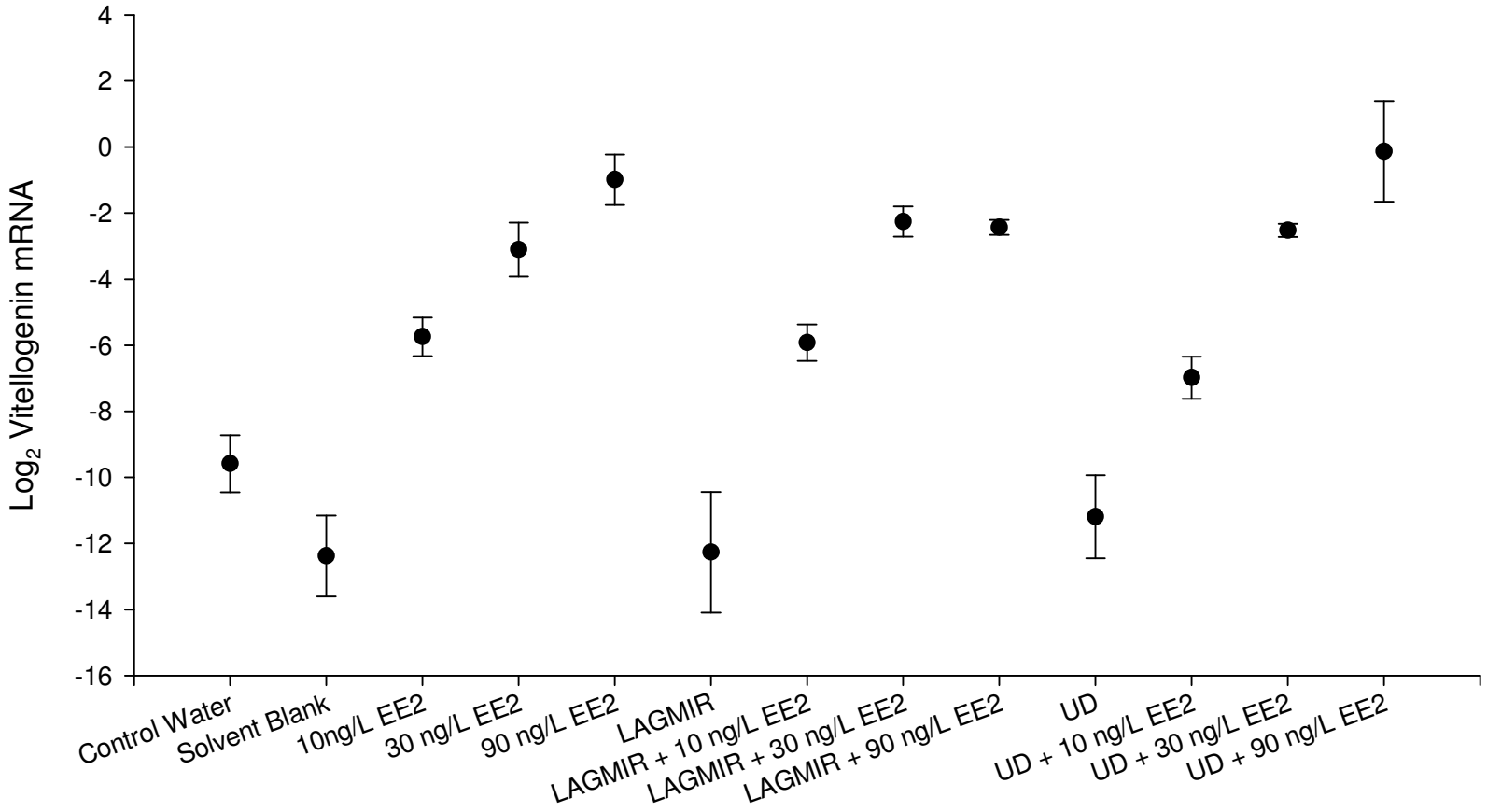

Figure 3. Liver Vtg mRNA (Mean ± SE) in juvenile rainbow trout exposed to EE2 in ambient water samples. LAGMIR = Laguna de Santa Rosa at Mirabel. UD = Putah Creek immediately below UC Davis WWTF outfall. EE2 concentrations are nominal.