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Science of the Total Environment xx (2007) xxx–xxx

Science of the Total Environment
An International Journal for Scientific Research into the Environment and its Relationship with Humankind

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Screening California surface waters for estrogenic endocrine disrupting chemicals (EEDC) with a juvenile rainbow trout liver vitellogenin mRNA procedure

V. de Vlaming [a,*], A. Biales [b], D. Riordan [c], D. Markiewicz [d], R. Holmes [e], P. Otis [f], R. Zander [e], J. Lazorchak [b]

[a] *School of Veterinary Medicine: APC, 1321 Haring Hall, University of California, Davis, CA 95616, USA*
[b] *US Environmental Protection Agency, National Exposure Research Laboratory, 26 West Martin Luther King Drive, Cincinnati, OH 45268, USA*
[c] *Department of Water Resources, Division of Environmental Services, 3251 S Street, Sacramento, CA 95816, USA*
[d] *Aquatic Toxicology Laboratory, School of Veterinary Medicine, University of California, Davis, CA 95616, USA*
[e] *Central Valley Regional Water Quality Control Board, 11020 Sun Center Drive #200, Rancho Cordova, CA 95670, USA*
[f] *North Coast Regional Water Quality Control Board, 5550 Skylane Boulevard, Suite A, Santa Rosa, CA 95403, USA*

Received 24 January 2007; received in revised form 29 March 2007; accepted 16 June 2007

Abstract

Concern regarding the 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. The objective was to screen surface water samples for estrogenic activity using 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 surface waters in California's Central Valley and northern area 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. 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. Overall, results reveal that a majority of the California surface waters tested were below EEDC detection threshold concentration for the screening procedure utilized.
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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 Chichester, 2005; Propper, 2005; Sumpter,

* Corresponding author: School of Veterinary Medicine, 1321 Haring Hall, University of California, Davis, CA, USA. Tel.: +1 916 718 8290; fax: +1 916 487 8177.
E-mail address: vldevlaming@ucdavis.edu (V. de Vlaming).

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doi:10.1016/j.scitotenv.2007.06.026

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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 occurrence in aquatic ecosystem 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). 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 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. 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).

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 Chichester, 2005; Sumpter, 2005). In response to this 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).

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

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. 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. 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 (e.g., Hiramatsu et al., 2005). 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 presumed 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. Fish liver Vtg or zona radiata protein 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

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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 [a]	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

[a] 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.

US EPA, Cincinnati (Lattier et al., 2002; Biales et al., in press) 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 northern California. Sampling site locations and selection rationales are summarized in Table 1. Locations of the sites are depicted in Fig. 1. A total of 66 different sites were sampled. Several sites were immediately downstream of WWTFs, others 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. Most samples were collected during low flow and low dilution periods during July through mid-September. 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) where they were at 4 °C in darkness until exposure set-up (all within 48 h of sample collection).

2.2. Ambient water screening

There were nine sampling and screening events. The date and sites sampled are summarized below by event.

Event 1—Eleven sites were sampled on March 29, 2005. Four of the sites were in the Russian River watershed (northwestern California) and were sampled to represent spring high flow conditions (Table 1). Six sites were in the California Central Valley (Table 2). Four of these sites were downstream of WWTF outfalls and two sites were on agriculture-dominated waterways in the Sacramento River watershed.

Event 2—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 3—Five sites were sampled on June 1, 2005. Two of the sites were downstream of WWTF outfalls

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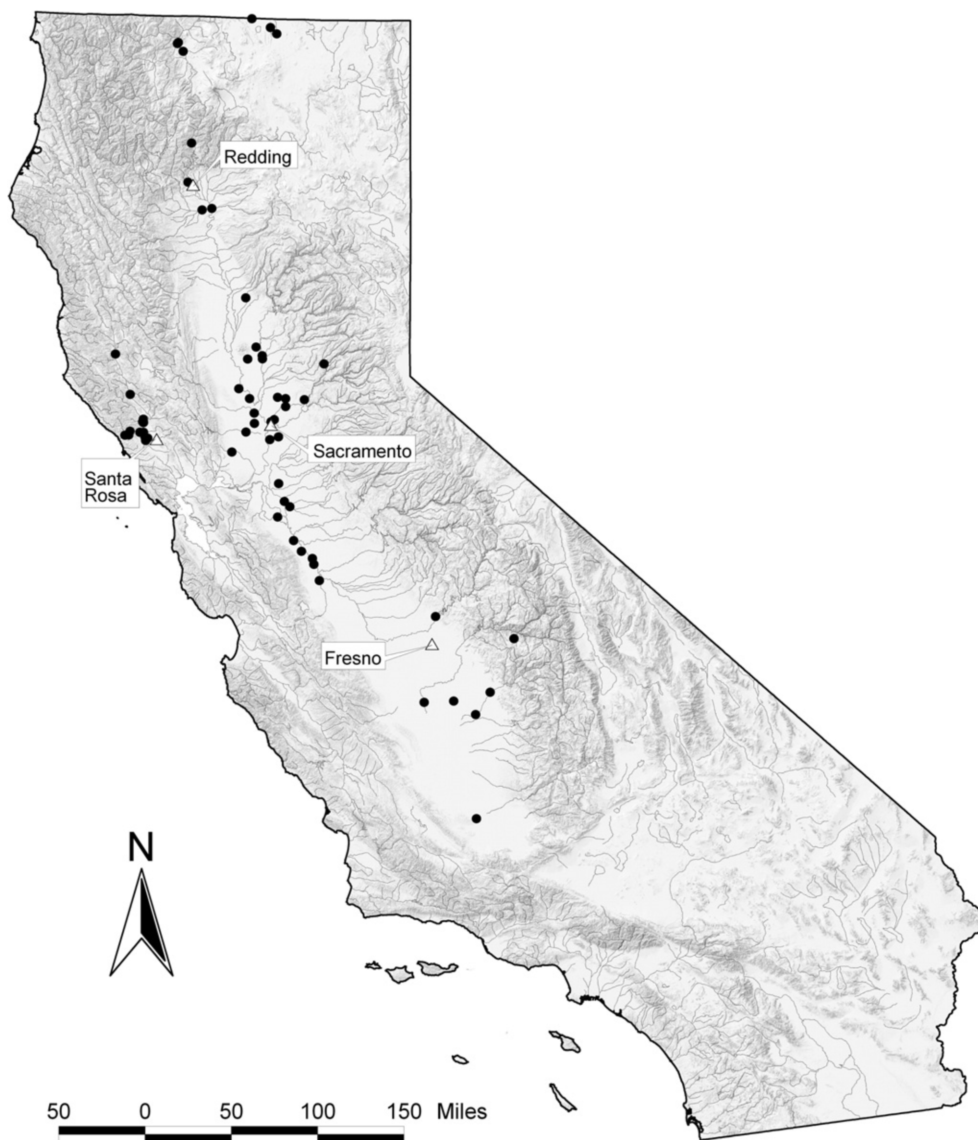

Fig. 1. Location of sites sampled for estrogenic endocrine disrupting activity.

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 4—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 5—On July 12, 2005 nine sites were sampled. Six of these sites were on waterways in the San Joaquin River watershed (California Central Valley) 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 6—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 7—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.

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

Site description	Land use description [a]	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

[a] 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.

Event 8—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 5). 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 9—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

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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-h 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 2 L beakers filled with 1.5 L of water. 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.

All ambient water exposure events included at least three sets of controls: (1) laboratory control water, (2) positive control—17α-ethinylestradiol (EE2), a common component of birth control pills sometimes detected in waste treatment facility discharges), and (3) solvent control—0.001% methanol (to assure that the solvent for the positive control was free of estrogenic effects). One group of positive controls was sacrificed after a 24-h exposure for comparison with positive controls exposed for 8 days.

There were three fish per replicate with five replicates per treatment. 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. Exposure temperature was 15° ± 2 °C; photoperiod was 16L/8D. Exposure duration was 8 days. Approximately 80% of water in each beaker was changed every other day. Fish were fed 2 h prior to water renewal. At renewal particulate matter (e.g., food, feces, etc.) was siphoned out of the beakers.

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 into 1 mL of Tri Reagent® in 1.5 mL snap-top micro-centrifuge tubes. 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. Tubes were stored at −18 °C (for no more than 7 days) until a space in a −80 °C freezer was available. The micro-centrifuge tubes were shipped to US EPA on dry ice via overnight delivery.

2.7. Vitellogenin mRNA analysis

2.7.1. RNA isolation for SybrGreen PCR analysis and reverse RNA transcription

RNA isolation for SybrGreen PCR analysis and reverse RNA transcription was as described in Biales et al. (in press).

2.7.2. Q-RT SybrGreen PCR system design and validation

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 of the correct size was confirmed using the Agilent 2100 Bioanalyzer. Additionally, the

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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 et al., 2003). All Vtg expression data were normalized to 18S expression.

2.7.3. Q-RT SybrGreen PCR analysis

250 nM of each primer (18 S 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 20 s at 95 °C, 20 s at 60 °C and 20 s 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. The $C(t)$ was selected to be in the linear phase of amplification. 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).

2.8. Calculations and statistical analyses

2.8.1. Relative quantification of gene transcription

RNA quantification was performed using a modified form of the equation proposed by Pfaffl et al. (2002) 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%. Quantities were determined for each experiment independently and are reported as relative Vtg mRNA 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; Schmid et al., 2003).

2.8.2. Comparisons between treatments

Statistical analyses were performed on $\log_2$ transformed normalized quantities of Vtg mRNA. The appropriateness of performing parametric statistics on the log-transformed data were assessed using Bartlett's test and Shapiro–Wilks test ($\alpha=0.01$). Means of treatments were compared 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) up-regulation, not down-regulation, was expected.

3. Results

Prior to initiating screening of surface water samples an experiment was conducted to determine whether juvenile rainbow trout mixed-sex and all female groups respond differently to EE2. The level of Vtg mRNA expression did not differ between the two groups, allowing flexibility in subsequent screening of ambient waters. Moreover, obtaining all male juvenile trout for experiments is essentially impossible without genetic phenotyping. Mixed sex trout are not available year-round at most hatcheries. All female juvenile trout are available year-round at many hatcheries.

Juvenile rainbow trout exhibited an EE2 concentration-liver Vtg mRNA response in laboratory control water (Fig. 2) and in EE2-spiked surface water samples (Fig. 3). Neither surface water EE2-spiked sample resulted in liver Vtg mRNA levels different than fish in laboratory control water positive controls (Fig. 2, one-way ANOVA with Dunnett's multiple comparison, $P<0.05$; Fig. 3, two-way ANOVA with Tukey's multiple comparison, $P<0.05$).

To assess the effects of exposure duration one group of positive controls was sacrificed after 24 h for comparison with positive controls exposed for 8 days. Data from screening Events 2 through 9 document that levels of liver Vtg mRNA in positive controls exposed to EE2 for 8 days were significantly higher than in fish exposed for 24 h and are consistent with measured EE2 concentrations (two-way ANOVA with Tukey's multiple comparison, $P<0.05$). Typically, the 24-h exposure mRNA response was 2 to 8% of the 8-day response.

The juvenile rainbow trout liver Vtg mRNA procedure was applied to screen 113 ambient water samples (82 in this study and 31 in a previous study—de Vlaming et al., 2006). There were 13 sampling events (nine in this study and four in the previous project) 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. Background mRNA levels in control water and solvent controls as well as responses of positive controls were equivalent in the screening events. A total of six samples (5% of total samples) resulted in marginal, but statistically significant, induction of liver Vtg mRNA (one-way ANOVA with Dunnett's multiple comparison,

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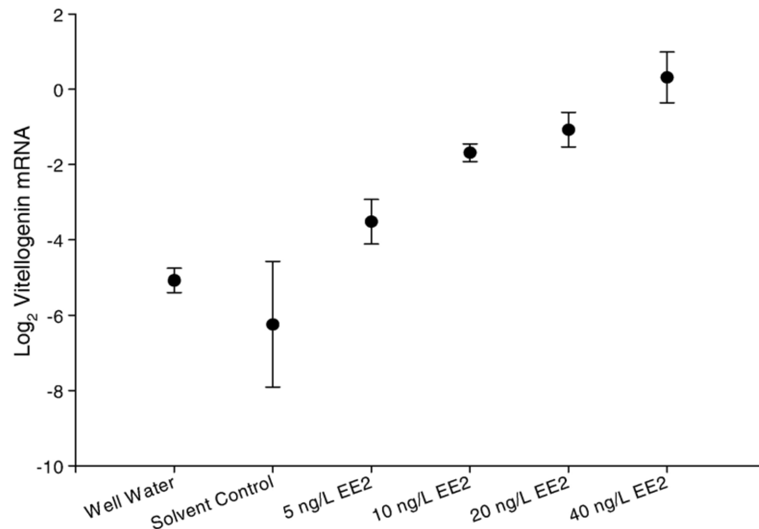

Fig. 2. Relationship between EE2 concentration and Vtg mRNA (mean ± SE) in juvenile rainbow trout during a 24-h exposure.

$P<0.05$). No ambient water sample evoked Vtg mRNA responses equivalent to those in positive controls.

Two samples that produced statistically significant Vtg mRNA responses were collected during screening Event 2 (May 10, 2005) and Event 4 (June 13, 2005) from Colusa Basin Drain (Tables 3 and 4). 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 marginal, but statistically significant, liver Vtg mRNA responses were collected during sampling Event 6 (July 26, 2005) from Elk Bayou, during Event 8 (August 30, 2005) as an effluent sample from the West Roseville WWTF, Pleasant Grove and from Santa Rosa Creek at Willowside Road, and during Event 9 (September 13, 2005) from Butte Creek, an agriculture-dominated waterway that discharges into Sacramento Slough (Tables 5, 6 and 7, respectively). The strongest mRNA signal in all exposure events came from the Elk Bayou sample. Colusa Basin Drain, Elk Bayou, and Butte Creek are agriculture-dominated waterways.

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 2) and 10 ng EE2/L

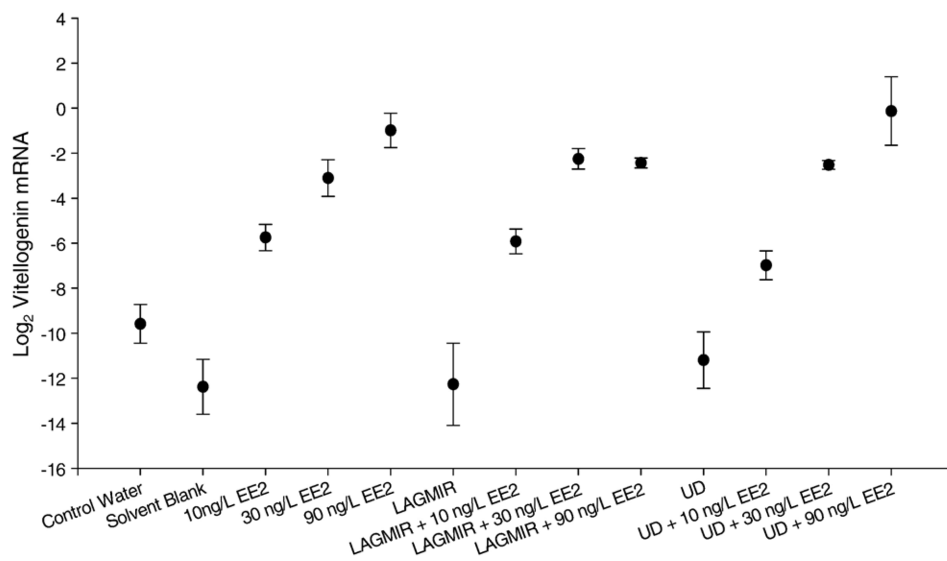

Fig. 3. Liver Vtg mRNA (mean ± SE) in juvenile rainbow trout exposed to EE2 in ambient water samples. LAGMIR = Laguna de Santa Rosa at Mirabel. UD = Putah Creek immediately below UC Davis WWTF outfall. EE2 concentrations are nominal.

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Table 3
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 [a]
Positive control (10 ng/L EE2) @ 24 h	0.0657 ± 0.0103 [a]
Positive control (10 ng/L EE2) @ 8 days	1.0160 ± 0.2800 [a]
Colusa Basin Drain @ Knights Landing	0.0192 ± 0.0099 [b]
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

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

(Event 4) 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 (at or near the threshold for the procedure) or the results may have included false positives. With α set at 0.05 and concentration of EEDCs near procedure detection limit, false positive can be expected in 5% of all samples.

Table 4
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 h)	0.3374 ± 0.0254 [a]
Positive control (10 ng/L EE2) (8 days)	0.5068 ± 0.2212 [a]
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 [b]
Sycamore Slough @ Hwy 45	0.0028 ± 0.0003
Old Alamo Creek @ Vacaville WWTF	0.0046 ± 0.0011

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

Table 5
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 h)	0.1429 ± 0.0585 [a]
Positive control (10 ng/L EE2) (8 days)	1.3298 ± 0.5607 [a]
Elk Bayou @ Laspina	0.0208 ± 0.0115 [b]
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

[a] Statistically different compared to solvent blank (ANOVA with Dunnett's test, one-tailed $P<0.05$).
[b] 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 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 h)	0.0390 ± 0.0027 [a]
Positive control (10 ng/L EE2) (8 days)	0.9867 ± 0.0695 [a]
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 [b]
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 [b]

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

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Table 7
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 h)	0.0255 ± 0.0015 [a]
Positive control (10 ng/L EE2) (8 days)	1.0561 ± 0.0956 [a]
Yuba River @ Marysville	0.0012 ± 0.0002
Butte Creek @ Durham Rd	0.0020 ± 0.0004 [b]
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

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

When two treatment groups showed similar levels of Vtg mRNA, log transformation of the data prior to analysis sometimes reversed the relative magnitude of the means of Vtg mRNA. In two cases, this transformation caused a treatment to differ significantly from the control while a sample with higher untransformed Vtg mRNA was not significantly different compared to the control. Among the samples collected on August 30, 2005 (Table 6) a significant increase in mRNA was not detected in fish exposed to the sample collected from Sacramento Slough (Vtg mRNA = 0.0018), while the Vtg mRNA elicited by the sample collected at West Roseville WWTF was significant (Vtg mRNA = 0.0014). The results of this Dunnett's test stem from a difference in the relative magnitudes of means in the two treatments consequent to log transformation of the data. This same phenomenon occurred with the Butte Creek and the Russian River samples collected on September 13, 2005 (Table 7). In both cases, the marginally significant Vtg mRNA levels were barely detectable by our methods and may represent false positives.

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 at Mirabel, Yreka Creek, Russian River at Johnson Beach, Laguna de Santa Rosa at Occidental Road, and Laguna de Santa Rosa at Delta Pond).

4. Discussion

Quantitative RT PCR Vtg mRNA analysis of juvenile rainbow trout livers from fish exposed to 113 ambient water samples collected from California surface waters indicated that only six samples (5% of total) may have contained EEDCs. These six samples induced Vtg mRNA responses less than 2% of the positive control suggesting low EEDC concentrations (at or near the threshold for the procedure) and may have included false positives. 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-h exposures. Thus, the detection limit for EE2 in 24-h exposures was at least 5 ng/L. Data collected in this study revealed that response in 8-day exposures is considerably greater than in the 24-h exposures, implying a lower detection level in the longer term exposure.

In relation to method detection limit, our literature review indicates 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 (EEDC review article in preparation by de Vlaming). Therefore, we believe that the detection limit of the procedure used was sufficient (with the possible caveat of exposure duration) to meet the objectives of this study. Furthermore, a literature review (in preparation by de Vlaming) 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 commonly below 1 and 5 ng/L, respectively. Moreover, the low frequency of detecting estrogenic activity in the 113 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. No common pattern could be deciphered in the six samples yielding an

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estrogenic response. Four of the six were from agriculture-dominated waterways, one was collected from a small urban creek, and one was a WWTF effluent.

To establish a more definitive assessment of EEDC occurrence in California surface waters 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.

The ambient water samples yielding the marginal Vtg mRNA responses were in 8-day, rather than 24-h exposures (de Vlaming et al., 2006). The liver Vtg mRNA procedure with 24-h, or even 8-day, exposure may not effectively detect chemicals that are weakly estrogenic 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 4 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 5.4 µg/L nonylphenol (a concentration not commonly observed in surface waters) in a continuous flow system, significant induction of liver Vtg mRNA and plasma Vtg was detected after 5 and 13 days, respectively (Hemmer et al., 2001). However, in a follow-up study Hemmer et al. (2002) reported that statistically significant hepatic Vtg mRNA and plasma Vtg were not detected until day 15–16 of exposure of male sheepshaed minnows to 5.6 µg/L nonylphenol; significant response to E2 was noted on day eight of exposure. These and other studies imply that estrogenic responses to alkylphenols (and possibly other EEDCs) require longer exposures compared to synthetic and natural estrogens. Thus, further development of the juvenile rainbow trout liver Vtg mRNA procedure should include investigation of exposure duration, weak EEDCs, and environmentally relevant EEDC mixtures.

The various EEDC screening methods have strengths and limitations, and users should be thoroughly aware of the confines of whatever 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. 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 h after cessation of exposure to EEDCs. Induction of Vtg, including Vtg mRNA procedures, in male and juvenile fish has proven useful for screening for EEDC exposure, but several investigations (e.g., Jobling et al., 1996; Gimeno et al., 1998; Panter 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 always reflect important reproductive responses. Another potential limitation was expressed by Shi et al. (2006), suggesting that Vtg may not be a female-specific protein in fish, but involved in immune responses, including males, to bacterial infections. Therefore, these endpoints seem to be useful for detection of EEDC exposure, but may not be reliable predictor of impairment.

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 developmental or reproductive effects, a large number of articles summarizing laboratory experiments report effect concentrations 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 northern California for estrogenic activity indicated that none contained high concentrations of EEDCs. Five percent

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of the samples induced a marginal, but statistically significant, estrogenic response. EEDC concentrations in these samples were at or near the response threshold for the biological screening procedure used in the study and may have included false positives. While the occurrence of EEDCs that must bioaccumulate (e.g., alkylphenols) to illicit endocrine disruption may be a possibility, natural and synthetic estrogens do not appear to be significant, continuous-occurring contaminants in the California waterways sampled.

Acknowledgments

Funding for this project was provided by the California State Water Resources Control Board Surface Water Ambient Monitoring Program. Linda Deanovic, Stephanie Fong, Joy Khamphanh, and Kevin Reece contributed technical assistance. We are indebted to Bobbye Smith, US EPA Region 9, for her support and advocacy.

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Please cite this article as: de Vlaming V et al. Screening California surface waters for estrogenic endocrine disrupting chemicals (EEDC) with a juvenile rainbow trout liver vitellogenin mRNA procedure. Sci Total Environ (2007), doi:10.1016/j.scitotenv.2007.06.026

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