PROJECT FINAL REPORT

IDENTIFYING THE CAUSES OF FEMINIZATION OF CHINOOK SALMON IN THE SACRAMENTO AND SAN JOAQUIN RIVER SYSTEM

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CONTRACTOR/GRANT RECIPIENT CONTACT INFORMATION

Program/Principal Administrator
Name: Jane Hartnett, Senior Research Administrator
Institution: Sponsored Projects Office; University of California, Berkeley
Address: 336 Sproul Hall; Berkeley, CA 94720-5940
Phone: 510-643-7365
Email: jhartnett@berkeley.edu

Principal Investigator
Name: Dr. David L. Sedlak, Professor
Institution: University of California, Berkeley
Address: 657 Davis Hall; Department of Civil and Environmental Engineering; Berkeley, CA 94720
Phone: 510-643-0256
Email: sedlak@ce.berkeley.edu

Principal Investigator
Name: Dr. Daniel Schlenk, Professor
Institution: University of California, Riverside
Address: 2207 Geology; Department of Environmental Sciences; Riverside, CA 92521
Phone: 951-827-2018
Email: daniel.schlenk@ucr.edu

Principal Investigator
Name: Dr. Robert B. Spies, President
Institution: Applied Marine Sciences
Address: 4749 Bennett Dr; Suite L; Livermore, CA 94550
Phone: 925-373-7142
Email: spies@amarine.com

Project Locations: University of California, Berkeley
University of California, Riverside
Applied Marine Sciences; Livermore, CA

GRANTING PROGRAM CONTACT INFORMATION

Technical Contact

Name: Lindsay E. Correa, Staff Environmental Scientist
Agency: Delta Stewardship Council
Address: 650 Capitol Mall; 5th Floor; Sacramento, CA 95814
Phone: 916-445-0092
Email: Lindsay.Correa@deltacouncil.ca.gov

Contract/Administrative Contact

Name: Marla Lynch, Contract Analyst
Agency: Delta Stewardship Council
Address: 650 Capitol Mall, 5th Floor; Sacramento, CA 95814
Phone: 916-445-7640
E-mail: marla.lynch@deltacouncil.ca.gov

BACKGROUND

Starting in the early 1990s, scientists began to report the presence of feminized male fish in rivers around the world. Sex reversal in male fish, which is a form of endocrine disruption, has attracted the attention of scientists, policymakers and the general public. Most research on feminization of fish has focused on rivers in which the discharge of municipal wastewater accounts for a significant fraction of the overall flow. In these systems, a large percentage of the male fish often exhibit elevated plasma levels of the lipoprotein vitellogenin and express egg cells in their testes [1-3]. Related studies have demonstrated that the feminization of male fish in these systems usually is attributable to the presence in wastewater effluent of trace concentrations of steroid hormones, such as ethinyl estradiol, 17β-estradiol and estrone [4, 5].

The Sacramento and San Joaquin watersheds likely contain a variety of chemical contaminants that are known to act as (xeno)estrogens. For example, dietary exposures or injections of steroid hormones and pesticides can cause feminization of salmon [6]. Although waterborne exposures to sewage effluent also can cause feminization of Chinook salmon [7], few *in vivo* studies involving waterborne exposures of Chinook salmon to specific contaminants have been performed. However, waterborne exposure to (xeno)estrogens can cause feminization of the closely related species, rainbow trout (*Oncorhynchus mykiss*). Observations with rainbow trout provide insight into the types of chemical contaminants that might be responsible for feminization of Chinook salmon. For example, induction of vitellogenin production has been observed upon exposure of juvenile rainbow trout to steroid hormone concentrations as low as 1 ng/L (Table 1). Waterborne exposure to higher concentrations of nonylphenol and octylphenol, common metabolites of non-ionic detergents, also can induce vitellogenin production in rainbow trout. Although no data are available on effects on fish, certain pyrethroid pesticides and their metabolites (*e.g.,* 3-phenoxybenzyl alcohol) bind to estrogen receptors used in the yeast estrogen screen (YES) *in vitro* bioassay. Although less information is available on the waterborne exposure route, metabolites of the persistent organic pollutants DDT and PCBs (DDE and hydroxylated PCBs, such as 4-hydroxy-2'4'6'-trichlorobiphenyl) also can induce vitellogenin production when exposure occurs via food or by direct injection into the fish.

Salmon that spawn in the Sacramento River and San Joaquin River watersheds are likely exposed to many of the (xeno)estrogens listed in Table 1. Steroid hormones are present in municipal wastewater effluent [8] and in agricultural wastes [9, 10]. For example, we recently reported the presence of the steroid hormone estrone at concentrations up to 17 ng/L in drainage canals in an area of California's Central Valley with a high density of dairy farms. Concentrations of steroid hormones were higher during or shortly after winter storms suggesting that runoff from agricultural operations was an important source of steroid hormones to surface waters [11]. Steroid hormones also were detected in water discharged by fish hatcheries along the American and Mokelumne Rivers indicating that aquaculture may also play a role in feminization of salmon.

Table 1. Chemical contaminants capable of causing feminization of male fish or binding to estrogen receptors.

Contaminant	Threshold Concentration	Assay	Reference
Ethinyl estradiol	1 ng/L	Rainbow trout	[12]
17β-estradiol	10 ng/L	Rainbow trout	[12]
Estrone	44 ng/L	Rainbow trout	[12]
4-nonylphenol	10 μg/L	Rainbow trout	[13]
4-octylphenol	3 μg/L	Rainbow trout	[13]
3-phenoxybenzyl alcohol	10 μg/L	YES Assay	[14]
4,4'-DDE	NA	Rainbow trout	[15]
4-hydroxy-2'4'6'-trichlorobiphenyl	NA	Rainbow trout	[16]

NA = Not applicable

Runoff from farms that use pesticides also may contain xenoestrogens. Although most pesticides do not cause feminization of fish at the concentrations expected in agricultural runoff, elevated concentrations of estrogen-binding compounds have been detected in agricultural areas and it has been hypothesized that the detergents used to deliver the pesticides are the causative agents [17]. Consistent with this hypothesis, data from the Schlenk laboratory indicated feminization of rainbow trout exposed to two commercial detergents (TPA and R-11) and two aquatic herbicides (2,4 D and trichlopyr) that are commonly used in concert in the Central Valley in California. In these experiments, both 2,4-D and R-11 caused vitellogenin induction in exposures when the concentrations of the surfactant and the pesticide were comparable. The ability of the surfactant R-11 to induce vitellogenin production is most likely attributable to the presence of alkylphenol polyethoxylates, which account for approximately 90% of the mass of the R-11 formulation [18]. In surface waters of the Central Valley and Delta, the concentration of alkylphenol polyethoxylates is likely to be significantly higher than that of the xenoestrogenic pesticides.

Surfactants are not the only xenoestrogens associated with pesticide use that could contribute to feminzation of salmon; the pyrethroid pesticides and their metabolites are heavily utilized in Central Valley agriculture and they have demonstrated estrogenic activity. Over 130,000 kg of permethrin was applied in California in 2002 [19] and it is possible that these compounds could also contribute to feminization of salmonids.

Salmon that spawn in the Sacramento and San Joaquin River watersheds also may be exposed to metabolites of persistent organic pollutants (POPs) listed in Table 1 (*i.e.,* DDE and hydroxylated PCBs) through their diets or through contact with contaminated sediments. As female adult salmon return to their home watersheds to spawn, they concentrate these hydrophobic organic compounds in their fat [20] and relatively high concentrations of these compounds may be transferred to their eggs. Thus, exposure of developing fish to elevated concentrations of xenoestrogens could occur through maternal transfer from fish that are exposed to contaminated food in the ocean or the Bay/Delta.

In addition to causing feminization, many of the chemical contaminants listed in Table 1 also could impact salmon reproductive success and survival through other mechanisms. For example, exogeneous steroid hormones can interfere with chemical communication in salmon, which is important to the timing of reproduction [21]. Also, Arsenault *et al.* [22] have shown that growth and insulin-like growth factor-I levels are depressed in juvenile Atlantic salmon (*Salmo salar*) exposed to environmentally relevant concentrations of 4-nonylphenol or 17β-estradiol. Finally, hydrophobic xenoestrogens, such as DDE, can cause immunosupression in salmon [23]. Although the main focus of this research project is feminization of salmonids, information on the source and behavior of these contaminants also will be useful in the evaluation of other, potentially harmful chemical stressors of potential importance to salmon in the Sacramento River, San Joaquin River and Delta ecosystems.

Research on fish feminization by chemical contaminants raises several questions that are particularly relevant to the CALFED Program:

- Has feminization of male salmon played a role in salmon population declines?
- Will feminization affect efforts to protect and restore Chinook salmon and other key fish species such as the Delta smelt (*Hypomesus transpacificus*), Sacramento splittail and tule perch (*Hysterocarpus traski*)?
- Are chemical contaminants in water responsible for feminization of fish in the Bay/Delta ecosystem, and if so, which chemicals are responsible?
- Do (xeno)estrogens cause other related, subtle effects on sensitive fish species?
- Are there any cost-effective actions that can be used to reduce exposure of fish to (xeno)estrogens?

PROJECT GOALS

The main purpose of this research project was to assess the potential importance of endocrine-disrupting chemical contaminants to salmon and other resident species of waters that discharge to the San Francisco-San Joaquin Delta. To achieve these objectives, endocrine disrupting compounds were quantified through a combination of field sampling, state-of-the-art chemical analyses and laboratory bioassays. An initial survey of sites representative of California's inland waters was followed by focused sampling, toxicity identification evaluations and fish exposures at sites where endocrine disrupting compounds were observed.

In parallel with water sampling and site assessments, resident fish were sampled in an effort to detect evidence of feminization. For these analyses, a biomarker for fish feminization, choriogenin, was measured in fish collected from sites where sources of endocrine-disrupting compounds were present. These analyses were complemented by studies in which choriogenin was evaluated after caging fish in locations proximate to water sampling sites.

MATERIALS AND METHODS

Chemicals

Most analytical standards were acquired from Sigma-Aldrich Inc. (St. Louis, MO, USA): mesterolone (min. 97%) [1424-00-6], testosterone (98%) [58-22-0], estrone (min. 99%) [53-16-7], progesterone [57-83-0], estriol (min. 99%) [50-27-1], medroxyprogesterone [520-85-4], androstenedione (min. 98%) [63-05-8], 17α-estradiol (min. 98%) [57-91-0], 17β-estradiol (minimum 98%) [50-28-2], nonylphenol (technical grade) [84852-15-3], octylphenol (97%) [140-66-9], Igepal CA-210 [68987-90-6], and Igepal CO-210 [68412-54-4]. Igepal CA-210 was used as standard for the octylphenol mono- and di-ethoxylates (OP1EO and OP2EO), and Igepal CO-210 for the respective nonylphenol homologues (NP1EO and NP2EO). Igepal CA-210 composition was determined to be 78% OP1EO and 19% OP2EO, and Igepal CO-210 53% NP1EO and 34% NP2EO, by normal-phase HPLC. Estrone-2,4,16,16-d4 (minimum 95 atom % D) was purchased from Isotec Inc. (Miamisburg, OH, USA); 17β-estradiol-2,4,16,16,17-d5 (> 98 atom % D) [221093-45-4] and progesterone-2,2,4,6,6,17α,-21,21,21-d9 (98.5 atom % D) [15775-74-3] were acquired from CDN Isotopes (Pointe-Claire, Quebec, Canada). d2-NP and d2-OP were synthesized in the laboratory by mixing approximately 15 mg NP or OP with a 9:1 D2O:D2SO4 mixture and baking at 170°C for 24 h in sealed glass tubes followed by liquid-liquid extraction into hexane. The 5 deuterated compounds and mesterolone were used to prepare a surrogate standard mixture in acetonitrile (hormones: 1 ng/μL; d2OP and d2NP: 0.1 ng/μL). Heptafluorobutyric anhydride (HFBA) [336-59-4] (Supelco) was used as the derivatization reagent. Organic solvents, such as methanol and acetonitrile, were HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was obtained using a Milli-Q water purification system (Millipore, Billerica, MA, USA). Glassware was baked at 400°C for 4 h in a muffle furnace (Fisher Scientific) before use.

Sample Collection

For the survey of occurrence of estrogenic activity, grab samples were collected from the center of the flow of the river. Site locations are depicted in Figure 1 and are summarized in Table 2. Samples were collected on 6 different occasions during 2006-2007 in previously baked 4-L amber glass bottles. All samples were stored on ice and returned to the laboratory within 2 days of collection.

Solid-Phase Extraction

Water samples were subjected to solid-phase extraction with a reversed-phase disc. For the bioassay samples, 1 L of unfiltered water was extracted through 47-mm C18 Empore extraction disks (3M, St. Paul, MN, USA) previously conditioned with methanol and deionized water. After drying, disks were stored at -20ºC until analysis. Prior to use in bioassays, extracts were removed from the freezer and thawed for 15 min and 10 mL of methanol was added. Methanol extracts were evaporated to dryness under a gentle stream of N_2. The extracts were resuspended in 750 or 800 μL of ethanol and stored at -20ºC until bioassays were conducted (usually within 2 days).

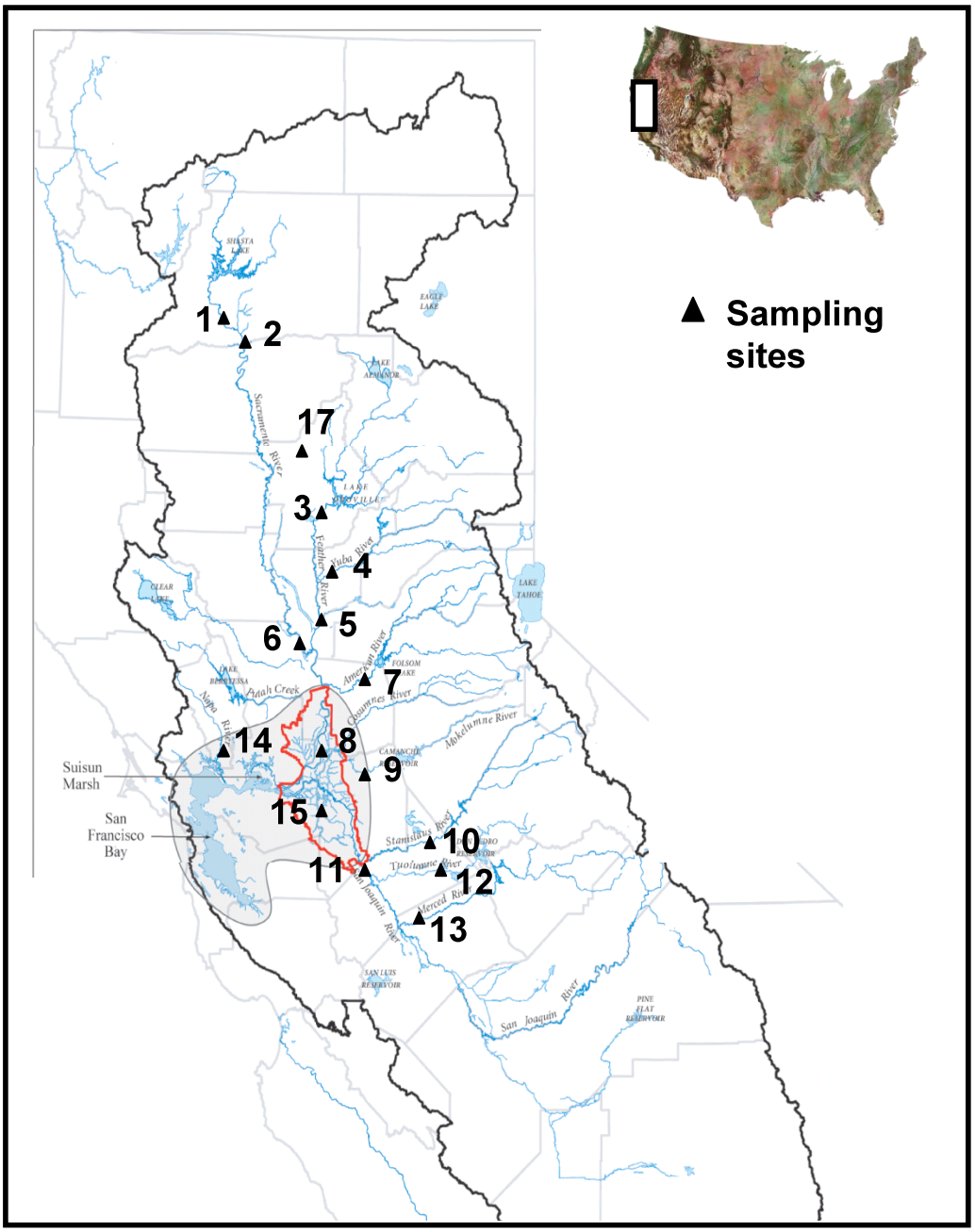


Figure 1. Sampling sites in the San Joaquin and Sacramento River system.

Table 2. Sampling sites and their description in Central Valley of California.

Site ID	Site name	Description	Latitude	Longitude
1	Upper Sacramento River	Site within Redding surrounded by mixed open space and agricultural areas	40º 32.354'	122º 21.390'
2	Battle Creek	Rural area surrounded by agriculture/grazing	40º 23.509'	122º 10.704'
3	Upper Feather River	Near a busy bridge downstream of an urbanized area (Oroville, CA)	39º 29.879'	121º 34.769'
4	Yuba River	Rural area upstream of Marysville, some agriculture and a little grazing	39º 08.519'	121º 34.580'
5	Lower Feather River	Rural area surrounded by crop agriculture	38º 54.031'	121º 35.156'
6	Lower Sacramento River	Border of small town and agricultural area	38º 48.140'	121º 43.200'
7	Lower American River	Dense urban area	38º 34.013'	121º 22.962'
8	Sacramento River in Delta	Agricultural area with low density housing and heavy boat traffic	38º 14.544'	121º 30.912'
9	Mokelumne River	Rural area adjacent to agricultural areas	38º 12.315'	121º 05.581'
10	Stanislaus River	Rural/agricultural area adjacent to small town	37º 49.123'	120º 40.050'
11	San Joaquin River	Heavy agricultural area with heavy road traffic	37º 38.472'	121º 13.737'
12	Tuolumne River	Agricultural area	37º 38.156'	120º 37.098'
13	Merced River	Heavy agricultural area. Area sometimes used for recreational fishing	37º 21.039'	120º 57.737'
14	Napa River	Dense urban area (Napa), intense agricultural areas upstream	38º 18.631'	122º 16.682'
15	Clifton Court Forebay	Located within Skinner Fish Protection Facility	37º 49.503'	121º 35.711'
17*	Butte Creek	Agricultural area adjacent to suburban residential area	39º 40.685'	121º 46.659'

*A location originally referred to as site 16 was abandoned due to safety concerns for the sampling crew. No data from this site are reported.

For chemical analyses, samples were filtered using AP40 glass fiber filters (90 mm diameter, 0.7μm pore size; Millipore) on glass or stainless steel filter holders (Millipore), for 1 or 4 L samples, respectively. The glass filtration unit was previously baked for 4 h at 400°C, and the stainless steel container was rinsed with methanol and deionized water between samples. If necessary, filtered samples were stored overnight at 4°C. Extraction typically occurred less than 24 h after filtration. To provide accurate quantification, 4-L aliquots of filtered water were spiked with 100 µL of the surrogate standard mixture and extracted with 90-mm C18 Empore extraction disks (3M) pre-rinsed twice with 30 mL of methanol and deionized water. After

extracting the samples, the disks were dried and stored at -20°C until elution with 25 mL methanol. After elution, the methanol was evaporated to dryness in a vacuum oven, the extracts were reconstituted in 200 µL acetonitrile, and 50 µL HFBA were added and the mixture heated at 55°C for 90 min. After derivatization, extracts were dried under a gentle nitrogen stream and reconstituted in 100 µL of isooctane with hexachlorobenzene as an internal standard.

Chemical Analyses

Steroid Estrogens and Alkylphenol Ethoxylates: Gas chromatography/tandem mass spectrometry (GC-MS/MS) with isotope dilution or internal standard calibration was performed with a Quattro micro GC (Waters, Milford, MA, USA) with a DB-5 MS column (30 m, 0.25 mm ID, 0.25 μm, J&W Scientific/Agilent Technologies, Santa Clara, CA, USA) using helium at 1.2 mL/min as a carrier gas and the following oven temperature program: 80°C (2 min), 30°C/min to 285°C, 3°C/min to 289°C, and 10°C/min to 310°C (3 min) for a total run time of 15.3 min. 2 µL of sample were injected using a 7683B Series Injector (Agilent) and a split/splitless injector in splitless mode at 270°C. MS analysis was performed using an EI+ source at 190°C and 70 eV. The trap was set at 200 μA, and the GC interface temperature was 290°C. Multiple-reaction monitoring was used for analysis. Limits of detection ranged from 0.1 to 1 ng/L. Peaks with a S/N ratio under 3 were considered non-detects, and a S/N ratio greater than 10 was used as the limit of quantification. An IDMS approach was employed for quantitation, using the deuterated standards and mesterolone to correct for analyte losses during extraction, and matrix interference during derivatization and MS analysis. Absolute recoveries of the surrogate standards ranged from 70 to 120%. Additionally, every sample set included a clean water sample (either deionized or dechlorinated tap water) spiked with 25 ng/L of the analytes. Recoveries of these samples were typically above 100% because of matrix enhancement of the MS response, but concentrations were not corrected for recovery.

Pesticides: For organophosphates and pyrethroids, the methanol/water extracts were analyzed by dual column gas chromatography and/or gas chromatography-mass spectrometry (GC-MS) using conditions which permit the separation and measurement of the target analytes in the extracts. A measured volume of sample was extracted with methylene chloride (DCM) and dried with sodium sulfate, evaporated using Kuderna-Danish (K-D) and solvent exchanged into petroleum ether. The extract was concentrated with micro-snyder (micro K-D) apparatus to approximately 1 mL and adjusted to 2.0 mL with isooctane.

The extracts were analyzed for organophosphate pesticides using gas chromatography (Agilent 6890 plus) equipped with dual 30 meter columns (DB5 and DB17) and dual flame photometric detectors in phosphorous mode. Extracts were also analyzed and confirmed using a Varian 4000 GC-ITD-MS.

The extracts were analyzed for pyrethroid pesticides using gas chromatography (Agilent 6890 plus) equipped with dual 60 meter columns (0.25 mm I.D. and 0.25 µm film thickness) DB5 and DB17 and dual micro-electron capture detectors. Pyrethroid pesticides were confirmed using GC-MS (Varian 4000 GC-ITD-MS) or GC-MS/MS (Varian 1200 triple quadrupole GC-MS).

Carbamate pesticides were analyzed by LC-MS Analysis was performed using an Agilent 1100 series LC-MS quadrupole system coupled to an Agilent 1100 series LC system consisting of a

binary pump, diode array UV-Vis detector (DAD), autosampler, thermostated column compartment and vacuum degasser. The MS was operated with atmospheric pressure electrospray ionization (API-ES) source in positive ion mode.

Chromatographic conditions included an Agilent Zorbax C-18 column, 15cm x 4.6mm i.d. x 5μm (or equivalent) with mobile phase A consisting of water with 5 mM formic acid and mobile phase B consisting of acetonitrile with 5 mM formic acid. A gradient from 5% to 100% acetonitrile was carried out over 25 min. The flow rate was 1.0 mL/min and the column temperature was 38ºC. The MS conditions included API-ES in positive ion mode with the drying gas flow at 12 L/min and temperature at 350ºC.

Triazine herbicides were analyzed by GC/MS/MS. As part of this analysis, a measured volume of sample was extracted with methylene chloride (DCM) and dried with sodium sulfate, evaporated using Kuderna-Danish (K-D) and solvent exchanged into petroleum ether. The extract was concentrated with micro-snyder (micro K-D) apparatus to approximately 1 mL and adjusted to 2.0 mL with isooctane. The extracts were analyzed by gas chromatography using conditions permitting the separation and measurement of the target analytes.

The operating parameters for the Varian 3400/2000 and 3800/4000 GC-MS/ITD included a DB5MS column, (30 m x 0.25 mm I.D. x 0.25 μm film thickness) a Varian 1078 Inlet with an isocratic temp at 240 ºC. The initial temperature was 80ºC and increased 15.0ºC/min to 175ºC, for 0.5 min followed by a 2.5ºC/min increase to 220ºC for 2.0 min and finally a 5.0ºC/min, increase to 260ºC. The MS/MS operating conditions included a trap temperature of 240ºC, a manifold temperature of 80ºC and a transfer line temperature of 280ºC.

Water Quality Parameters

Temperature, pH, dissolved oxygen and conductivity were measured in the field. Total suspended solids, total organic carbon, and nitrate were measured after returning to the laboratory. An aliquot of the river water collected to rinse the sampler and sample jars was poured into a 250 mL stainless steel beaker to measure field parameters. One liter of water was collected in HDPE containers and transported in ice to the laboratory to measure the rest of the parameters. Temperature and pH were measured with a pH52 Microprocessor Waterproof pH and temperature meter (Milwaukee Instruments, Inc., Rocky Mount, NC, USA), dissolved oxygen with a CHEMets Dissolved Oxygen Kit K-7512 (CHEMetrics, Inc., Calverton, VA, USA), and conductivity with a C65 Waterproof electrical conductivity meter (Milwaukee Instruments). Total suspended solids, nitrate and total organic carbon were measured according to standard methods [24]: Method 2540 D for solids, 4110 B for nitrate, and 5310 B for total organic carbon.

Estrogenicity Bioassay

For the *in vivo* bioassays, intraperitoneal injections of extracts were carried out on Rainbow trout (*Oncorhynchus mykiss*) of approximately 5 months (16 ± 3 cm). Fish were obtained from Jess Ranch Fish Hatchery (Apple Valley, CA, USA) and were maintained in a flow-through living-stream system with dechlorinated carbon-filtered municipal water at 13-15ºC and acclimatized for 1 month before experimental use. Organisms were fed daily with a commercial fish feed

(Silver Cup, Murray, UT, USA). Gonadal morphology indicative of gender was not observed in any of the animals indicating the animals were not sexually mature and did not have measurable endogenous expression of vitellogenin [25].

Fish were collected on days 1 and 3, with subsequent bleeding of the fish on day 7. These particular dosage concentrations and exposure durations were determined from previous studies [26]. Following injections, animals were maintained in 20-L aquaria. Water extracts reconstituted in ethanol were administered by injecting 0.1 mL per 100 g fish. Three to four animals in each group were used to evaluate the estrogenic activity of the extracts. On day 7, animals were anesthetized with 1 g/L tricaine methane sulfonate (MS-222) (Sigma) in water and blood samples were collected from the dorsal aorta using a latex-free 1 mL syringe and a 27-gauge needle (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The samples were immediately centrifuged at 5,000-*g* for 5 min and the supernatants immediately frozen and stored at -80ºC. Total protein concentrations of the serum were determined using the Coumassie Blue protein-staining reagent from the Pierce Chemical Company (Rockford, IL, USA).

Plasma levels of VTG were determined by a commercial enzyme-linked immunosorbent assay developed for rainbow trout (ELISA, Biosense Laboratories, Bergen, Norway). To determine estradiol equivalents (EEQs), an E2 dose-response curve was calculated using the two-injection design. Final doses were 0.1, 1, 10 and 100 μg/kg body weight, using no injected animal and animals injected with 0.1 mL ethanol as control and solvent control, respectively. The limit of detection of the method was 0.4 ng VTG/mL plasma and the limit of quantification for EEQs was 0.15 μg/kg body weight.

For the *in vitro* bioassays, hepatocytes were isolated from the Rainbow trout using enzymatic digestion with trypsin followed by mechanical disaggregation and gradient centrifugation using Percoll (Amersham Biosciences, Uppsala, Sweden) as previously described [27]. After cell isolation, the cells were seeded in 48-well plates with a density of $1x10^6$ cells/well. Cells were allowed to settle for 2 hours and then were treated with the water extracts. Three wells were treated for each extract, control and solvent control (0.6 % ethanol) treatment. Cells were incubated for 24 hours at 18ºC and then were resuspended in PBS, centrifuged at 5,200-*g* for 5 min and the pellet was washed twice with PBS. Cells used for PCR were immediately processed for total mRNA extraction. The viability of the cells was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Ethanol was used as a carrier for extract additions to the cells because no cytotoxicity was observed in optimization studies.

Total mRNA was extracted from cells using QIAshredder and RNeasy Mini RNA extraction Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. VTG mRNA was quantified by qPCR by using iScript™ One-step RT-PCR kit with SYBR® Green from Bio-Rad (Hercules, CA, USA) and as a sense primer tVit-364 5'-CCCACTGCTGTCTCTGAAACAG-3' and as antisense primer tVit-565 5'-GACAGTTATTGAGATCCTTGCTCTTG-3' from rainbow trout. β-actin was used as housekeeping gene and as sense primer 5'-GTCCTTCATGATTCTCTGCTGA-3' and antisense primer 5'-ACTCGGGTTCATTTGCATAAACA-3'. 250 nM of each primer (VTG or β-actin) was added to 25 μL PCR reactions containing SYBR® Green RT-PCR Reaction Mix (Bio-Rad,

Hercules, CA, USA), 100 ng mRNA sample and iScript Reverse Transcriptase for One-Step RT-PCR from Bio-Rad. Thermocycling parameters were as follows: 10 min at 50ºC (cDNA synthesis); 5 min at 95ºC (iScript Reverse transcriptase inactivation); 40 cycles of 10 s at 95ºC and 30 s at 56ºC. Fluorescence data were collected at the end of each cycle. Following the amplification reaction, a melting curve analysis was carried out between 60ºC and 95ºC, fluorescence data were collected at 0.1ºC intervals. The C(t) was selected to be in the linear phase of amplification. All real-time reactions were done in an iCycler-MyIQ Single Color Real-Time PCR Detection System (Bio-Rad) and data analysis was done using IQ5 (Bio-Rad). To determine estradiol equivalents (EEQs), an E2 dose-response curve was calculated using different concentrations of E2 ($4x10^{-12}$M, $4x10^{-11}$M, $4x10^{-10}$M, $4x10^{-9}$M, $4x10^{-8}$M, $4x10^{-7}$M, $4x10^{-6}$M and $4x10^{-5}$M).

Toxicity Identification Evaluation (TIE) Analysis

Bioassay-directed fractionation was conducted on selected samples. The SPE extracts from the 4L samples were eluted with 10 mL each of 20, 40, 60, 80 and 100% methanol/water solutions. Each fraction was evaluated for VTG expression by *in vitro* and *in vivo* bioassays. As a positive control, distilled water (4 L) was amended with 17β-estradiol for a nominal concentration of 100 ng/L. River water (4 L) from the Mokelumne River (site 9), a location where elevated estrogenic activity was never detected, served as a negative control. Fractions showing elevated estrogenic activity were evaluated for steroid hormones and detergents and their degradation products.

Replicate fractions with activity were reduced in volume to 400 μL, and 350 μL was injected into a SCL-10AVP Shimadzu HPLC system using a 250 x 4.6 mm Atlantis C18 (5 μM) reverse-phase column (Waters, Milford, MA, USA) with a flow rate of 1 mL/min. The mobile phase was methanol:water with an elution program of 0 to 3 min 40% methanol, followed with a gradient of 3 to 30 min of 40% to 100% methanol. Fractions were collected over 3 min intervals and were evaporated until dryness under a gentle stream of nitrogen. Fractions were resuspended in ethanol and evaluated using the bioassays and chemical analyses described above.

A second round of bioassay-directed fractionation was conducted using samples collected in August 2008 at the Napa River and Sacramento River Delta sites. To enhance the sensitivity of the analysis and retain more hydrophilic compounds, the sample volume was increased to 10 L and the unfiltered water was extracted on 90 mm Empore C18 extraction disks in stainless steel filter holders. As a positive control, dechlorinated tap water (10 L) was amended with 17β-estradiol at 30 ng/L and analyzed. Disks were frozen immediately after extraction and eluted with methanol as described above.

Chriogenin Analysis

Three assays were performed at sites where estrogenic signals were observed with the bioassays. December 14-21, 2009, three scientists surveyed for suitable sites and collected wild fish resident at each site. Fish were brought to Bodega Marine Laboratory for sampling of blood plasma, livers, and gonads from male, juvenile, and female *Menidia*. Blood samples were collected individually in different volumes of buffer depending on fish length, spun in a centrifuge for 5 minutes at 5000 RPM to separate plasma, which was then stored at -80 ºC for

future analysis of choriogenin levels. Liver and gonads were collected and stored at -80 ºC for later analysis. Plasma choriogenin levels were later determined by ELISA.

On February 3, 2010, water was collected from the same sites where fish were previously collected. Site water exposures were performed by exposing 5 replicates of 10 naive fish each (50 fish per site, 150 total fish in 15 aquaria) for 14 days to control, Napa or Sacramento River water. Exposures required daily water changes and water chemistry assessment of dissolved O_2, nitrogen, pH and salinity. Fish mortality was low in controls (1 fish out of 50) and highest in Sacramento River water at ~30%. Choriogenin levels in whole-body homogenates were later determined by ELISA.

Finally, an outplant experiment was performed from July 26 – August 6, 2010. Naïve juvenile fish (~60 days old) were purchased from Aquatic BioSystems in Fort Collins, CO in February 2010, and held in a closed aquarium system for 6 months. Three flow-through buckets (that had previously been soaked in deionized water) containing 5 fish each were outplanted to each site for 14 days. Following the outplant, male and juvenile fish were weighed, and then choriogenin levels were determined in whole-body homogenate by indirect ELISA.

Chorion was isolated via centrifugation from homogenized oocytes and solubilized according to Oppen-Berntsen et al., 1990. Solubilized chorion was then run via polyacrylamide gel electrophoresis (PAGE) on 10% tris-glycine gel, bands were excised and emulsified in 2 mLs of Tris buffered saline (TBS). Emulsified denatured chorion (1 mL volume) was injected into two rabbits (#1667 and #1668) housed at UC Davis Comparative Pathology Lab every two weeks for a 12 week period. The rabbits were exanguinated at close of 12 week period, IgG precipitated from serum with ammonium sulfate. Antibody (isolated IgG) from each rabbit was optimized via Western Blot and ELISA.

Samples of solubilized chorion, whole body homogenate (WBH) from fish exposed to a methanol control or to known aqueous concentrations of ethinylestradiol, or plasma (positive from mature females, negative from depurated males) were loaded onto 10% Tris-Glycine PAGE gels (Lonza) at a volume of 10 μl/lane. PAGE was conducted at 160 volts on a Biorad 1000/500 Power Supply for approximately 1 hour, after which proteins were transferred to a 45 μM nitrocellulose membrane using a semi-dry transfer apparatus (Biorad TransBlot SD). Following transfer, the nitrocellulose membrane was stained with Ponceau Red-S dye to confirm transfer and roughly quantify protein amount per lane via densitometry.

Chorion and plasma samples were blocked for 1-2 hours and WBH samples were blocked for 5-6 hours in 5% non-fat powdered milk and phosphate buffered saline (PBS). After blocking eight 5-minute rinses were performed in PBS + 0.5% Tween-20 (PBST). Chorion and plasma samples were incubated in a 1:5,000 or 1:10,000 dilution of Menidia Ab #1667 in PBS, and WBH samples were incubated in a 1:5,000 or 1:10,000 dilution of #1667 in PBST + 5% milk (to reduce background) overnight at 4 degrees C. Membranes were then rinsed in PBST eight times for 5 minutes each and incubated for 1 hour in Goat-Anti-Rabbbit-Horseradish-Peroxidase (GAR-HRP, Sigma-Aldrich), then rinsed again as before after secondary antibody incubation. Finally, membranes were incubated for three minutes in West Pico Electrochemoluminescence

(Thermo-Scientific) and imaged using a UVP Epi Chemi II darkroom and a Photometrics SenSys digital camera.

The indirect ELISA method used here is adapted from that presented in Palumbo et al, 2009. Samples were diluted 1:10 for whole-body homogenate and 1:5 for plasma in coating buffer (30 mM Na_2CO_3, 70 mM $NaCO_3$, pH 9.6) with protease inhibitors (Complete Mini from Roche). Additionally, known quantities of purified chorion was spiked into diluted sample, and then serially diluted to provide a standard curve for quantification. 50 μL of each sample in coating buffer was added to each well of a Nunc MaxiSorp hi-binding 96-well plate, and incubated for 4-6 hours at 4C. Plates were then washed 2X with 200 μL of BupH Modified Dulbecco's phosphate-buffered saline (PBS; Pierce product no. 28374). Un-bound sites on the plate were then blocked by incubating in 200 μL of 5% non-fat powdered dry milk in PBS for 1.5 hours at 4C. Plates were washed 2X with PBS, and then 50 μL per well of a 1:1000 dilution of antibody in PBS + protease inhibitors for an overnight incubation at 4C. The next morning, plates were washed 4X with 200 μL of PBS. 50 μL per well of a 1:2500 dilution of goat-anti-rabbit secondary conjugated with horse-radish peroxidase (Sigma product no. A 6154) in 5% nonfat powdered dry milk in PBS was added for a 3-4 hour incubation at 4C. Following secondary Ab exposure, plates were washed 4X with 200 μL PBS, and then 50 μl per well of 1-step Ultra TMB for ELISA, from Thermo Scientific, was added. Peroxidase converts TMB into a soluble blue product. This reaction was allowed to progress for 30-60 minutes at room temperature, or until sufficient color had developed. The reaction was stopped by adding 50 μl per well of 2M H_2SO_4, which converts the blue product to a yellow one. Absorbance was then measured at 450 nm on a Tecan GENios plate-reader.

Statistical Procedures

Statistical significance for the bioassays was assessed using a Student's *t* test and one-way ANOVA to evaluate differences between groups, with the use of a SPSS v15.0 software package. A *P*-value of less than 0.05 was considered statistically significant unless otherwise indicated. If an overall significance was detected, Tukey's multiple range tests were performed. mRNA VTG expression data and VTG plasmatic levels were log-transformed prior to statistical analyses to meet the homocedasticity and normality assumptions of parametric tests.

RESULTS AND DISCUSSION

Chemical Characterization of Water Samples (Task 1.1)

Ten sampling events were conducted in the Delta and Central Valley from July 2006 to January 2008: seven during dry-weather conditions and three during storm events. Water samples for the dry-weather events were collected from 15 of the 16 sites depicted in Figure 1; sampling took place on July 10-13, September 12-14, November 27-29, 2006; and January 9-11, March 5-7, April 3-5, August 13-15, 2007.

Data from the dry weather sampling (Table 3; water quality data in appendix Table A1) suggest that concentrations of steroid hormones and alkylphenol ethoxylates are well below the threshold at which feminization of fish has been observed in previous studies (e.g., Table 1).

The steroid hormones analyzed were not detected most of the time during dry weather (Table 3), and when they were detected, concentrations were usually below quantification limits (bql). Site 12 in the Tuolumne River had the highest incidence of quantifiable levels of hormones at 18%, followed by sites 14 and 15 (i.e., Napa River and Clifton Court Forebay) with detection of compounds in 14% of the samples in both cases. For the steroid hormones, estrone (E1) was the most frequently detected compound (quantified in 22% of the samples), followed by 17β-estradiol (7.6%), progesterone (7.6%), and testosterone (4.8%). Median concentrations of these hormones were between 0.2 and 0.7 ng/L.

At least one of the alkylphenol compounds measured was present in every dry-weather sample, but the total concentration of the six compounds was less than 311 ng/L in all cases (median 49.5 ng/L). The most abundant alkylphenolic compounds detected were NP2EO (up to 240 ng/L; median 14 ng/L) and NP (up to 190 ng/L; median 13 ng/L). NP2EO was consistently high in site 8 (Sacramento River in the Delta).

These data were expressed in terms of estradiol equivalents (EEQ) using a previously described approach [11]. Comparison of the frequency distribution of EEQ values for the dry weather with data from rangeland runoff and canals proximate to dairy farms (Figure 2) shows that the known endocrine-disrupting compounds are much lower in the rivers than in these two potential sources. Furthermore, the data from rivers indicate that EEQ values were below the threshold for fish feminization in approximately 99% of the samples.

The first and second storm events (February and December 2007) included samples collected approximately 24 hrs after the start of the storm and 2-3 days later in 5 selected sites (sites 3, 4, 6, 8 and 17). The third event (January 2008) covered 5 sites in the southern part of the sampling area, where storms are less frequent (sites 10, 11, 12, 13 and 15). Steroid hormones and APEs concentrations were also low in these samples, despite elevated concentrations of suspended solids (Tables 4 and 5; water quality data in appendix Tables A2 and A3).

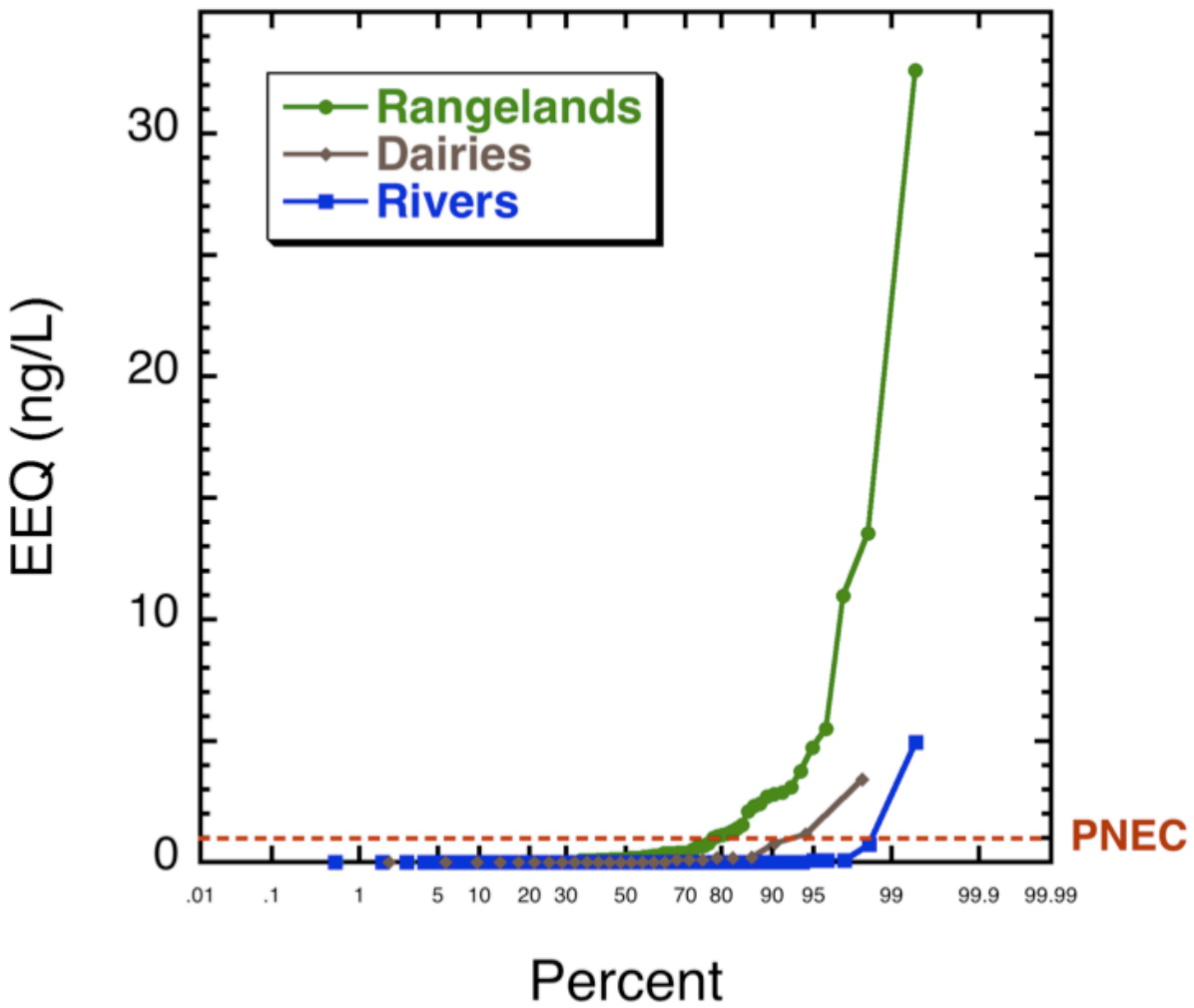


Figure 2. Comparison of estradiol equivalents (EEQ) in the Sacramento and San Joaquin Rivers with data collected in rangeland runoff [11] and canals proximate to dairies [10] within the watershed. PNEC refers to the predicted no-effects concentration for feminization of sensitive fish species.

Table 3. Steroid hormones and APEs in the seasonal screening (dry weather).

Site Month	17α-E2 (ng/L)	17β-E2 (ng/L)	E1 (ng/L)	E3 (ng/L)	P (ng/L)	MP (ng/L)	T (ng/L)	AD (ng/L)	NP (ng/L)	NP1EO (ng/L)	NP2EO (ng/L)	OP (ng/L)	OP1EO (ng/L)	OP2EO (ng/L)
Site 1														
July 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
September 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
November 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	2.3	bdl	0.3	3.0	bdl	bdl
January 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	13.0	15.0	9.5	1.3	1.6	0.9
March 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	11.0	15.0	18.0	0.8	1.1	0.7
April 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	8.5	7.4	6.7	0.7	4.1	6.7
August 2007	bdl	bql	0.57	bdl	bdl	bdl	bdl	bdl	55.0	17.0	80.0	3.4	7.2	11.0
Site 2														
July 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
September 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
November 2006	bdl	bdl	bdl	bdl	0.63	bdl	0.6	bdl	2.4	1.3	2.1	3.8	0.1	0.4
January 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	7.7	8.5	4.1	1.0	0.9	0.5
March 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	33.0	8.0	6.5	0.6	1.0	0.7
April 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	5.8	6.4	18	0.7	6.6	13.0
August 2007	bdl	bql	1.83	bdl	0.14	bdl	bdl	bdl	29.5	9.1	21.0	1.9	6.6	13.0
Site 3														
July 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
September 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
November 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	3.4	0.5	1.0	4.0	0.04	0.02
January 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	5.3	5.1	2.6	1.3	0.7	0.5
March 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	100.0	9.5	8.2	0.7	1.2	0.8
April 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	9.9	7.4	16.0	0.9	6.2	9.7
August 2007	bdl	0.15	0.35	bdl	bdl	bdl	bdl	bdl	16.7	5.7	27.0	1.3	3.7	8.9
Site 4														
July 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
September 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
November 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	17.0	15.0	3.8	3.0	3.8
January 2007	bdl	0.1	23	bdl	bdl	bdl	bdl	bdl	5.2	4.0	2.3	1.0	0.8	0.5
March 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	190.0	8.0	7.1	0.7	1.3	0.9
April 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	18.0	13.0	48.0	1.0	10.0	7.6
August 2007	bdl	bql	0.26	bdl	0.13	bdl	bdl	bdl	37.2	11.0	56.0	2.7	7.2	13.0

bdl: below detection limit. na: not analyzed. **17α-E2**: 17α-estradiol. **17β-E2**: 17β-estradiol. **E1**: estrone. **E3**: estriol. **P**: progesterone. **MP**: medroxyprogesterone. **T**: testosterone. **AD**: androstenedione. **NP**: nonylphenol. **NP1EO**: nonylphenol monoethoxylate. **NP2EO**: nonylphenol di-ethoxylate. **OP**: octylphenol. **OP1EO**: octylphenol monoethoxylate. **OP2EO**: octylphenol di-ethoxylate.

Table 3. Steroid hormones and APEs in the seasonal screening (cont'd.)

Site Month	17α-E2 (ng/L)	17β-E2 (ng/L)	E1 (ng/L)	E3 (ng/L)	P (ng/L)	MP (ng/L)	T (ng/L)	AD (ng/L)	NP (ng/L)	NP1EO (ng/L)	NP2EO (ng/L)	OP (ng/L)	OP1EO (ng/L)	OP2EO (ng/L)
Site 5														
July 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
September 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
November 2006	na	na	na	na	na	na	na	na	na	na	na	na	na	na
January 2007	na	na	na	na	na	na	na	na	na	na	na	na	na	na
March 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	60.0	8.5	12.0	1.0	1.1	0.9
April 2007	na	na	na	na	na	na	na	na	na	na	na	na	na	na
August 2007	na	na	na	na	na	na	na	na	na	na	na	na	na	na
Site 6														
July 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
September 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
November 2006	bdl	bdl	0.4	bdl	0.7	bdl	bdl	bdl	53.0	5.2	6.1	48.0	0.7	1.3
January 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	9.7	4.7	6.5	1.7	0.6	0.4
March 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	27.0	7.4	9.2	0.8	0.9	0.5
April 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	5.7	5.3	27.0	0.6	3.5	7.4
August 2007	bdl	bql	2.25	bdl	bdl	bdl	bdl	bdl	34.0	9.0	24.0	2.0	5.2	11.0
Site 7														
July 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
September 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
November 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	8.5	15.0	0.9	3.6	5.9
January 2007	bdl	bdl	0.4	bdl	bdl	bdl	bdl	bdl	6.0	4.5	3.7	1.6	0.8	0.6
March 2007	na	na	na	na	na	na	na	na	na	na	na	na	na	na
April 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	7.3	3.9	14.0	0.6	3.2	6.1
August 2007	bql	bql	0.29	bdl	bdl	bql	bdl	bdl	28.3	11.0	20.0	2.0	6.0	11.0
Site 8														
July 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
September 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
November 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	17.0	130.0	1.1	0.6	2.9
January 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	23.0	40.0	240.0	1.7	2.4	4.1
March 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	17.0	28.0	140.0	1.4	3.1	4.0
April 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	24.0	19.0	75.0	1.0	5.0	11.0
August 2007	0.35	0.27	0.59	3.74	0.15	0.28	0.27	bdl	36.8	7.5	34	3.2	2.6	8.4

bdl: below detection limit. na: not analyzed. **17α-E2**: 17α-estradiol. **17β-E2**: 17β-estradiol. **E1**: estrone. **E3**: estriol. **P**: progesterone. **MP**: medroxyprogesterone. **T**: testosterone. **AD**: androstenedione. **NP**: nonylphenol. **NP1EO**: nonylphenol monoethoxylate. **NP2EO**: nonylphenol di-ethoxylate. **OP**: octylphenol. **OP1EO**: octylphenol monoethoxylate. **OP2EO**: octylphenol di-ethoxylate.

Table 3. Steroid hormones and APEs in the seasonal screening (cont'd.)

Site Month	17α-E2 (ng/L)	17β-E2 (ng/L)	E1 (ng/L)	E3 (ng/L)	P (ng/L)	MP (ng/L)	T (ng/L)	AD (ng/L)	NP (ng/L)	NP1EO (ng/L)	NP2EO (ng/L)	OP (ng/L)	OP1EO (ng/L)	OP2EO (ng/L)
Site 9														
July 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
September 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
November 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	1.1	6.3	2.7	7.5	13.0	0.1	0.3
January 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	9.1	5.0	4.1	2.7	0.9	1.1
March 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	24.0	8.5	9.2	0.7	1.1	0.8
April 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	5.9	5.8	17.0	0.5	3.4	6.3
August 2007	bdl	bdl	0.69	bdl	bdl	bdl	bdl	bdl	27.9	8.0	26.0	2.0	3.6	7.2
Site 10														
July 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
September 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
November 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	1.1	2.1	3.4	3.2	0.08	0.2
January 2007	bdl	bdl	0.4	bdl	bdl	bdl	bdl	bdl	6.5	3.4	5.1	1.7	1.1	1.1
March 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	46.0	7.4	7.5	0.7	1.2	0.6
April 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	4.2	4.0	9.5	0.4	2.9	5.9
August 2007	bql	bql	bdl	bdl	bdl	bdl	bdl	bdl	47.4	11.0	27.0	4.0	6.7	15.0
Site 11														
July 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
September 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
November 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	4.6	5.2	7.5	1.4	0.2	0.6
January 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	15.0	5.8	11.0	1.8	1.3	0.9
March 2007	bdl	bdl	1.1	bdl	bdl	bdl	bdl	bdl	16.0	11.0	18.0	1.9	1.4	0.7
April 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	12.0	7.4	16	1.4	6.6	11.0
August 2007	bql	bql	0.55	bdl	bdl	bdl	bdl	bdl	39.1	9.2	19.0	1.9	6.8	17.0
Site 12														
July 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
September 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
November 2006	bdl	bdl	bdl	bdl	0.6	bdl	bdl	bdl	1.7	1.7	2.1	5.8	0.1	0.2
January 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	6	3.8	4.4	1.5	1.2	1.5
March 2007	bdl	bdl	3.9	bdl	bdl	bdl	bdl	bdl	25.0	4.6	6.8	0.5	0.7	0.6
April 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	4.0	6.9	61.0	0.4	5.5	12.0
August 2007	0.98	0.78	0.99	6.4	0.23	0.99	0.94	1.46	45.6	15.0	30.0	3.0	6.7	12.0

bdl: below detection limit. na: not analyzed. **17α-E2**: 17α-estradiol. **17β-E2**: 17β-estradiol. **E1**: estrone. **E3**: estriol. **P**: progesterone. **MP**: medroxyprogesterone. **T**: testosterone. **AD**: androstenedione. **NP**: nonylphenol. **NP1EO**: nonylphenol monoethoxylate. **NP2EO**: nonylphenol di-ethoxylate. **OP**: octylphenol. **OP1EO**: octylphenol monoethoxylate. **OP2EO**: octylphenol di-ethoxylate.

Table 3. Steroid hormones and APEs in the seasonal screening (cont'd.)

Site Month	17α-E2 (ng/L)	17β-E2 (ng/L)	E1 (ng/L)	E3 (ng/L)	P (ng/L)	MP (ng/L)	T (ng/L)	AD (ng/L)	NP (ng/L)	NP1EO (ng/L)	NP2EO (ng/L)	OP (ng/L)	OP1EO (ng/L)	OP2EO (ng/L)
Site 13														
July 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
September 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
November 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	0.8	4.2	5.4	3.0	0.3	0.4
January 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	13.0	5.8	19.0	3.3	1.2	1.3
March 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	36.0	6.9	6.8	0.7	0.6	0.5
April 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	6.2	4.7	12.0	0.6	4.4	8.1
August 2007	bql	bql	0.68	bdl	bdl	bdl	bdl	bdl	37.6	11.0	12.0	2.6	8.1	18.0
Site 14														
July 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
September 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
November 2006	0.1	0.21	1.4	bdl	bdl	bdl	bdl	bdl	16.0	23.0	44.0	12.0	2.4	6.7
January 2007	bdl	bdl	bdl	bdl	bdl	bdl	0.1	bdl	10.0	7.4	17.0	1.5	1.0	1.2
March 2007	bdl	bdl	0.6	bdl	bdl	bdl	bdl	bdl	140.0	15.0	17.0	1.5	1.6	0.8
April 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	13.0	10.0	23.0	1.1	8.6	13.0
August 2007	bdl	0.15	3.2	bdl	bdl	bdl	bdl	2.1	30.7	45.0	72.0	1.9	31.0	98.0
Site 15														
July 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
September 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	na	na	na	na	na	na
November 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	17.0	6.4	8.5	5.7	0.2	0.4
January 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	26.0	6.9	23.0	3.0	1.9	2.3
March 2007	bdl	bdl	0.7	bdl	bdl	bdl	bdl	bdl	14.0	6.9	19.0	1.4	1.0	0.7
April 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	11.0	4.9	11.0	1.0	2.4	5.9
August 2007	0.37	0.33	1.0	4.6	bdl	0.54	0.51	1.0	25.0	13.0	13.0	1.9	7.9	16.0
Site 17														
July 2006	na	na	na	na	na	na	na	na	na	na	na	na	na	na
September 2006	na	na	na	na	na	na	na	na	na	na	na	na	na	na
November 2006	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	0.3	0.9	2.5	1.3	0.1	0.4
January 2007	bdl	bdl	bdl	bdl	bql	bdl	bdl	bdl	6.2	4.3	3.4	1.5	1.0	0.9
March 2007	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	5.2	20.0	9.9	0.3	1.2	0.7
April 2007	bdl	0.52	bdl	bdl	bdl	bdl	bdl	bdl	9.1	11.0	85.0	0.7	4.2	7.2
August 2007	bdl	bql	0.4	bdl	0.16	bdl	bdl	bdl	34.0	8.4	27.0	2.1	6.5	13.0

bdl: below detection limit. na: not analyzed. **17α-E2**: 17α-estradiol. **17β-E2**: 17β-estradiol. **E1**: estrone. **E3**: estriol. **P**: progesterone. **MP**: medroxyprogesterone. **T**: testosterone. **AD**: androstenedione. **NP**: nonylphenol. **NP1EO**: nonylphenol monoethoxylate. **NP2EO**: nonylphenol di-ethoxylate. **OP**: octylphenol. **OP1EO**: octylphenol monoethoxylate. **OP2EO**: octylphenol di-ethoxylate.

Table 4. Steroid hormones and APEs before and after storm events (northern sites).

Site Date	17α-E2 (ng/L)	17β-E2 (ng/L)	E1 (ng/L)	E3 (ng/L)	P (ng/L)	MP (ng/L)	T (ng/L)	AD (ng/L)	NP (ng/L)	NP1EO (ng/L)	NP2EO (ng/L)	OP (ng/L)	OP1EO (ng/L)	OP2EO (ng/L)
Site 3														
February 9, 2007 (before)	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	15	11	14	1.0	1.6	1.6
February 12, 2007 (after)	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	6.9	9.5	16	0.31	1.1	1.4
December 19, 2007 (before)	bdl	0.022	0.14	na	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
December 21, 2007 (after)	bql	0.025	0.13	na	bdl	bdl	bdl	bdl	51	bdl	bdl	bdl	bdl	bdl
Site 4														
February 9, 2007 (before)	bdl	bql	bql	bdl	bdl	bdl	bdl	bdl	12	8.0	13	0.63	0.76	1.0
February 12, 2007 (after)	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	7.4	6.9	7.8	0.36	1.0	1.7
December 19, 2007 (before)	0.068	bql	0.23	na	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
December 21, 2007 (after)	0.16	0.12	0.18	na	bdl	0.12	bql	bdl	bdl	bdl	bdl	bdl	bdl	bdl
Site 6														
February 9, 2007 (before)	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	7.8	5.2	6.5	0.41	1.3	1.0
February 12, 2007 (after)	bdl	bdl	bql	bdl	bdl	bdl	bdl	bdl	20	9.5	13	0.69	1.5	1.7
December 19, 2007 (before)	bdl	bql	0.17	na	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
December 21, 2007 (after)	bdl	0.03	0.18	na	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
Site 8														
February 9, 2007 (before)	bdl	bdl	0.48	bdl	bdl	bdl	bdl	bdl	37	22	150	0.92	1.3	2.3
February 12, 2007 (after)	bdl	bdl	0.43	bdl	bdl	bdl	bdl	bdl	43	25	88	1.5	1.5	3.2
December 19, 2007 (before)	bdl	0.035	0.30	na	bdl	bdl	bdl	bdl	150	40	93	1.7	5.1	14
December 21, 2007 (after)	bdl	0.038	0.31	na	bdl	bdl	bdl	bdl	35	100	290	2.5	8.7	25
Site 17														
February 9, 2007 (before)	bdl	bql	bdl	bdl	bdl	bdl	bdl	bdl	15	16	48	0.60	1.9	3.4
February 12, 2007 (after)	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	6.4	5.8	6.8	0.32	0.94	1.2
December 19, 2007 (before)	bdl	bdl	bql	na	bdl	bdl	bdl	bdl	11	bdl	bdl	bdl	bdl	bdl
December 21, 2007 (after)	bdl	bql	bql	na	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl

bdl: below detection limit. na: not analyzed. **17α-E2**: 17α-estradiol. **17β-E2**: 17β-estradiol. **E1**: estrone. **E3**: estriol. **P**: progesterone. **MP**: medroxyprogesterone. **T**: testosterone. **AD**: androstenedione. **NP**: nonylphenol. **NP1EO**: nonylphenol monoethoxylate. **NP2EO**: nonylphenol di-ethoxylate. **OP**: octylphenol. **OP1EO**: octylphenol monoethoxylate. **OP2EO**: octylphenol di-ethoxylate.

Table 5. Steroid hormones and APEs before and after storm events (southern sites).

Site Date	17α-E2 (ng/L)	17β-E2 (ng/L)	E1 (ng/L)	E3 (ng/L)	P (ng/L)	MP (ng/L)	T (ng/L)	AD (ng/L)	NP (ng/L)	NP1EO (ng/L)	NP2EO (ng/L)	OP (ng/L)	OP1EO (ng/L)	OP2EO (ng/L)
Site 10														
January 4, 2008 (before)	bdl	bdl	bql	na	bdl	bdl	bdl	bdl	bdl	bdl	4.8	bdl	bdl	bdl
January 7, 2008 (after)	0.37	0.12	0.22	na	bdl	bdl	bql	1.2	bdl	9.0	11	bdl	bdl	bdl
Site 11														
January 4, 2008 (before)	bdl	bql	0.17	na	bdl	bdl	bdl	bdl	bdl	bdl	4.8	1.7	bdl	bdl
January 7, 2008 (after)	bdl	0.50	1.7	na	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
Site 12														
January 4, 2008 (before)	bdl	1.1	bql	na	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
January 7, 2008 (after)	bdl	0.52	bdl	na	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
Site 13														
January 4, 2008 (before)	bdl	bdl	bdl	na	bdl	bdl	bdl	bdl	bdl	bdl	5.6	bdl	bdl	bdl
January 7, 2008 (after)	bdl	0.7	1.3	na	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
Site 15														
January 4, 2008 (before)	bdl	bdl	0.19	na	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
January 7, 2008 (after)	0.80	3.1	1.2	na	bql	0.61	bql	bql	bdl	bdl	14	bdl	bdl	bdl

bdl: below detection limit. na: not analyzed. **17α-E2**: 17α-estradiol. **17β-E2**: 17β-estradiol. **E1**: estrone. **E3**: estriol. **P**: progesterone. **MP**: medroxyprogesterone. **T**: testosterone. **AD**: androstenedione. **NP**: nonylphenol. **NP1EO**: nonylphenol monoethoxylate. **NP2EO**: nonylphenol di-ethoxylate. **OP**: octylphenol. **OP1EO**: octylphenol monoethoxylate. **OP2EO**: octylphenol di-ethoxylate.

Bioassays of Water Samples (Task 1.2)

Extracts from water samples described in the previous section were analyzed for estrogenicity by an *in vitro* rainbow-trout hepatocyte bioassay and/or *in vivo* whole fish exposure. Vitellogenin (VTG), an egg-yolk protein induced by (xeno)estrogens, was chosen as the biomarker for exposure to these substances. The *in vitro* bioassay consisted of the exposure of isolated trout liver cells to extracts of the water samples and the subsequent measurement and extraction of vitellogenin mRNA from the hepatocytes. The capacity of the samples to induce VTG was compared to E2 in order to express estrogenic activity in E2 equivalents (EEQ). The *in vivo* test was performed by intraperitoneal injection of the water extracts to rainbow trout, and determination of VTG protein levels in plasma after 7-day incubation. Estrogenic potential was also measured against E2.

Unlike the chemical analyses, the bioassays showed more evidence of endocrine-disrupting compounds (Tables 6 and 7). More than half of the sites (2, 3, 4, 6, 7, 9, 10, 11, and 15; i.e. 9 out of 16 sites) presented low or no response in both bioassays; i.e., most samples were under 0.3 µg/kg EEQ *in vivo* and 2 ng/L EEQ *in vitro*. Detection limits were 0.12 ng/L EEQ *in vitro* and 0.1 µg/kg EEQ *in vivo*.

Sites 8 and 14 presented high estrogenicity in both assays: site 8 had the highest *in vitro* activity in July 2006 (160 ng/L EEQ) and the highest *in vivo* activity in March 2007 (5.1 µg/kg EEQ); whereas site 14 showed the highest activity in September 2006, both *in vitro* (68 ng/L EEQ) and *in vivo* (12 µg/kg EEQ). Sites 12 and 13 showed relatively high estrogenicity in the *in vitro* assay and low or none in the *in vivo* test. The highest *in vitro* activity was observed in September 2006, 240 ng/L EEQ in site 12 and 56 ng/L EEQ in site 13. Sites 1 and 5 showed low *in vitro* and high *in vivo* responses. Response was highest in July 2006, 4.8 µg/kg EEQ in site 1 and 7.7 µg/kg EEQ in site 5. Site 17 had detectable *in vitro* activity, ranging from 1.4 to 6.5 ng/L EEQ, but no *in vivo* response. This site is relevant because it is considered prime spawning area for spring-run Chinook salmon.

During the first storm event (February 2007), samples were collected in sites 3, 4, 6, 8, and 17 at approximately 24 hrs after the start of the storm, and resampled 3 days later. Sites 6 and 8 showed an increase in their *in vitro* responses; EEQ increased by approximately 11 and 4 times respectively (Table 8). In contrast, sites 3 and 17 showed activity only at the beginning of the storm. No activity was detected in samples from site 4 (Table 8).

Analysis of storm samples from sites where water discharge information from USGS gauges was available suggested that *in vitro* bioassay response was related to discharge magnitude—*in vivo* analysis showed relatively low estrogenic activity that remained constant before and after the storms. During the first storm event (February 2007), estrogenicity levels in the Sacramento River (sites 6 & 8) were relatively low at the beginning of the storm when discharge was low (Feb. 9, 2007), and they were higher 3 days later (Feb. 12) when discharge was higher (Figure 3). In contrast, estrogenicity levels were high in Butte Creek (site 17) on the first sampling date (Feb. 9) when discharge was high, and below detection limit on the second sampling date (Feb. 12), after the main flushing event had occurred, and discharge was decreasing (Figure 4). Samples were collected in the same sites during the second storm event in December 2007, but

estrogenicity levels were below detection or quantitation limits in all cases (Table 8). Sites 10, 11, 12, 13, and 15 were sampled during a third storm event in January 2008 (Table 9). Bioassay responses also were correlated with water discharge in the San Joaquin River site (site 11) (Figure 5). In the case of all storm events, no correlation was observed between chemical EEQs (i.e., EEQs calculated from the concentrations of estrogenic chemicals measured in the water samples and estrogent equivalent factors cited in the literature) and bio EEQs (i.e., EEQs measured experimentally with the bioassays).

Table 6. EEQs determined in the selected sampling sites by *in vitro* bioassay. Data are presented as ng/L and as mean ± SD (n=4).

		Sampling event					
Site ID	**Site name**	**July 06**	**September 06**	**November 06**	**January 07**	**March 07**	**April 07**
1	Upper Sacramento River	1.8 ± 0.6	3.7 ± 2.9	1.1 ± 0.5	1.7 ± 1.7	bdl	bdl
2	Battle Creek	bdl	0.2 ± 0.1	bdl	bdl	bdl	0.2 ± 0.1
3	Upper Feather River	1.2 ± 1.1	0.9 ± 0.5	4.8 ± 2.8	0.6 ± 0.4	0.5 ± 0.1	0.6 ± 0.3
4	Yuba River	bdl	12.5 ± 11.2	10.4 ± 11.9	0.4 ± 0.3	1.8 ± 0.9	0.9 ± 0.1
5	Lower Feather River	0.3 ± 0.1	15.3 ± 7.0	na	na	bdl	na
6	Lower Sacramento River	bdl	bdl	1.2 ± 0.2	0.9 ± 0.5	bdl	bdl
7	Lower American River	bdl	bdl	bdl	bdl	na	bdl
8	Sacramento River in Delta	164.0 ± 117.7	8.6 ± 6.1	51.2 ± 31.9	107.5 ± 35.6	40.1 ± 11.9	71.3 ± 5.8
9	Mokelumne River	bdl	bdl	bdl	bdl	bdl	bdl
10	Stanislau River	bdl	bdl	bdl	0.5 ± 0.3	bdl	bdl
11	San Joaquin River	bdl	bdl	bdl	bdl	bdl	bdl
12	Tuolumne River	91.6 ± 50.1	241.8 ± 46.3	24.6 ± 4.1	129.5 ± 47.1	68.5 ± 6.3	43.9 ± 14.1
13	Merced River	6.4 ± 3.4	56.1 ± 27.8	0.9 ± 0.4	10.9 ± 7.3	bdl	0.4 ± 0.2
14	Napa River	0.2 ± 0.1	68.3 ± 22.6	13.6 ± 14.1	2.3 ± 0.9	6.8 ± 3.1	10.1 ± 5.4
15	Clifton Court Forebay	bdl	bdl	bdl	bdl	bdl	bdl
17	Butte Creek	na	na	2.8 ± 0.6	6.5 ± 2.0	1.4 ± 0.3	1.6 ± 0.7

bdl: below detection limit (<0.15 ng/L)
na: not analyzed

Table 7. EEQs determined in the selected sampling sites by *in vivo* bioassay. Data are presented as μg/kg w.w. and as mean ± SD (n=3-5).

		Sampling event					
Site ID	**Site name**	**July 06**	**September 06**	**November 06**	**January 07**	**March 07**	**April 07**
1	Upper Sacramento River	4.8 ± 2.8	0.3 ± 0.1	2.5 ± 1.2	1.2 ± 0.3	3.2 ± 0.03	1.1 ± 0.3
2	Battle Creek	bdl	bdl	bdl	bdl	bdl	bdl
3	Upper Feather River	bdl	0.2 ± 0.02	bdl	bdl	bdl	bdl
4	Yuba River	0.2 ± 0.01	0.3 ± 0.01	bdl	bdl	bdl	bdl
5	Lower Feather River	7.7 ± 0.2	3.4 ± 2.4	na	na	bdl	na
6	Lower Sacramento River	bdl	0.3 ± 0.03	bdl	bdl	bdl	bdl
7	Lower American River	bdl	bdl	bdl	bdl	na	bdl
8	Sacramento River in Delta	4.6 ± 5.2	bdl	2.4 ± 0.5	3.1 ± 0.2	5.1 ± 0.7	4.1 ± 1.3
9	Mokelumne River	0.2 ± 0.01	0.2 ± 0.02	bdl	bdl	bdl	bdl
10	Stanislau River	0.3 ± 0.01	0.2 ± 0.01	bdl	bdl	bdl	bdl
11	San Joaquin River	0.2 ± 0.01	0.2 ± 0.1	bdl	bdl	bdl	bdl
12	Tuolumne River	bdl	bdl	bdl	0.8 ± 0.1	0.3 ± 0.1	bdl
13	Merced River	0.2 ± 0.01	0.7 ± 0.03	0.4 ± 0.01	0.7 ± 0.4	bdl	bdl
14	Napa River	0.2 ± 0.01	12.4 ± 0.8	5.2 ± 0.6	0.2 ± 0.01	0.4 ± 0.02	3.1 ± 0.04
15	Clifton Court Forebay	0.3 ± 0.02	0.2 ± 0.02	bdl	bdl	bdl	bdl
17	Butte Creek	na	na	bdl	bdl	bdl	bdl

bdl: below detection limit (<0.15 μg/kg w.w.)
na: not analyzed

Table 8. EEQs during storm events by *in vivo* and *in vitro* bioassays (northern sites).

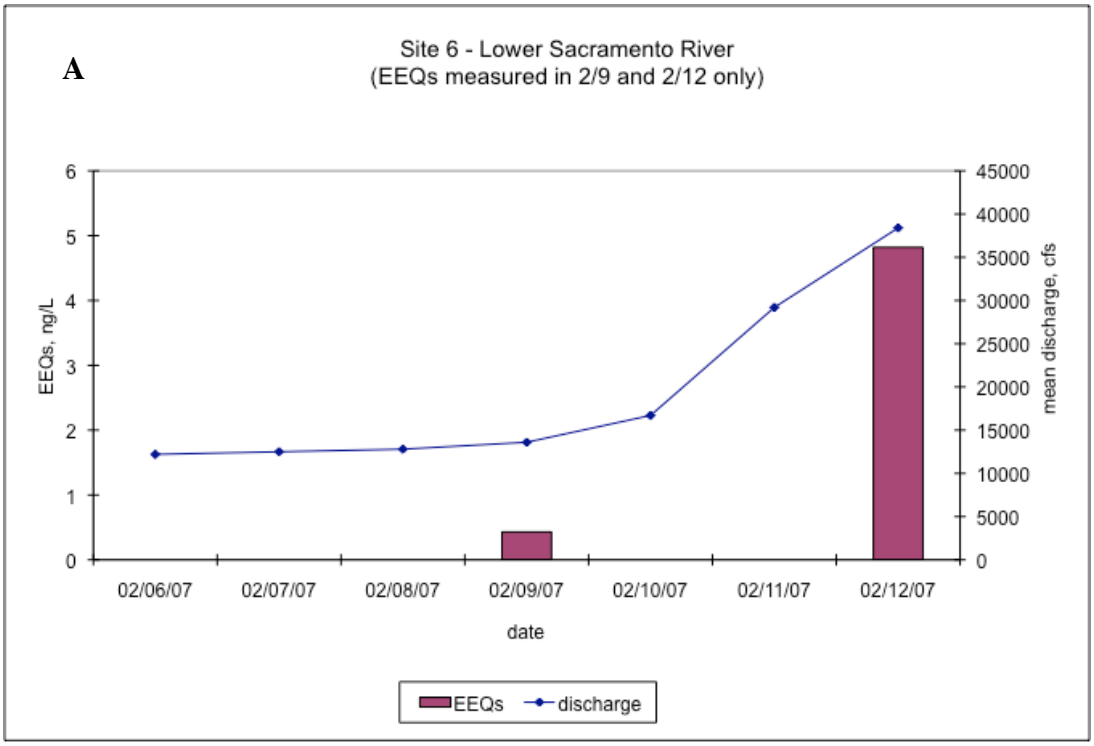
Site **Date**	**EEQ *in vitro* (ng/L)**	**EEQ *in vivo* (µg/kg wet wt.)**
Site 3		
February 9, 2007 (before)	1.22	na
February 12, 2007 (after)	bdl	na
December 19, 2007 (before)	bdl	0.54 ± 0.49
December 21, 2007 (after)	bdl	bdl
Site 4		
February 9, 2007 (before)	bdl	na
February 12, 2007 (after)	bdl	na
December 19, 2007 (before)	bql	0.66 ± 0.61
December 21, 2007 (after)	bdl	0.48 ± 0.43
Site 6		
February 9, 2007 (before)	0.43	na
February 12, 2007 (after)	4.82	na
December 19, 2007 (before)	bdl	bdl
December 21, 2007 (after)	bql	bdl
Site 8		
February 9, 2007 (before)	57.92	na
February 12, 2007 (after)	209.85	na
December 19, 2007 (before)	bdl	bdl
December 21, 2007 (after)	bql	bdl
Site 17		
February 9, 2007 (before)	5.51	na
February 12, 2007 (after)	bdl	na
December 19, 2007 (before)	bdl	bdl
December 21, 2007 (after)	bql	bdl

bdl: below detection limit. na: not analyzed.

Table 9. EEQs during storm events by *in vivo* and *in vitro* bioassays (southern sites).

Site **Date**	**EEQ *in vitro* (ng/L)**	**EEQ *in vivo* (µg/kg wet wt.)**
Site 10		
January 4, 2008 (before)	0.52 ± 0.06	1.72 ± 0.30
January 7, 2008 (after)	0.59 ± 0.13	1.13 ± 0.69
Site 11		
January 4, 2008 (before)	bql	bdl
January 7, 2008 (after)	10 ± 5.8	bdl
Site 12		
January 4, 2008 (before)	bdl	0.66 ± 0.61
January 7, 2008 (after)	290 ± 58	1.05
Site 13		
January 4, 2008 (before)	bql	0.29 ± 0.24
January 7, 2008 (after)	bdl	0.53 ± 0.48
Site 15		
January 4, 2008 (before)	bql	0.60 ± 0.55
January 7, 2008 (after)	1.7 ± 1.3	0.39 ± 0.34

bdl: below detection limit. na: not analyzed.



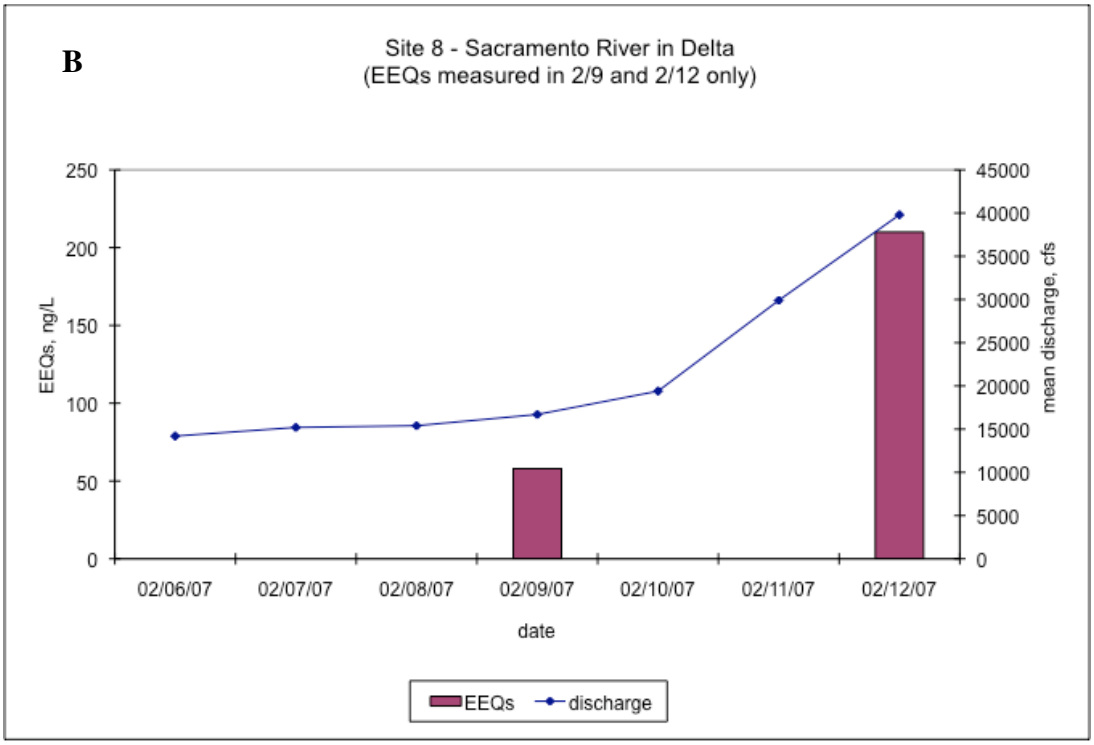


Figure 3. *In vitro* bioassay response versus water discharge in the Sacramento River sites: (a) Lower Sacramento River (site 6), (b) Sacramento River in Delta (site 8).

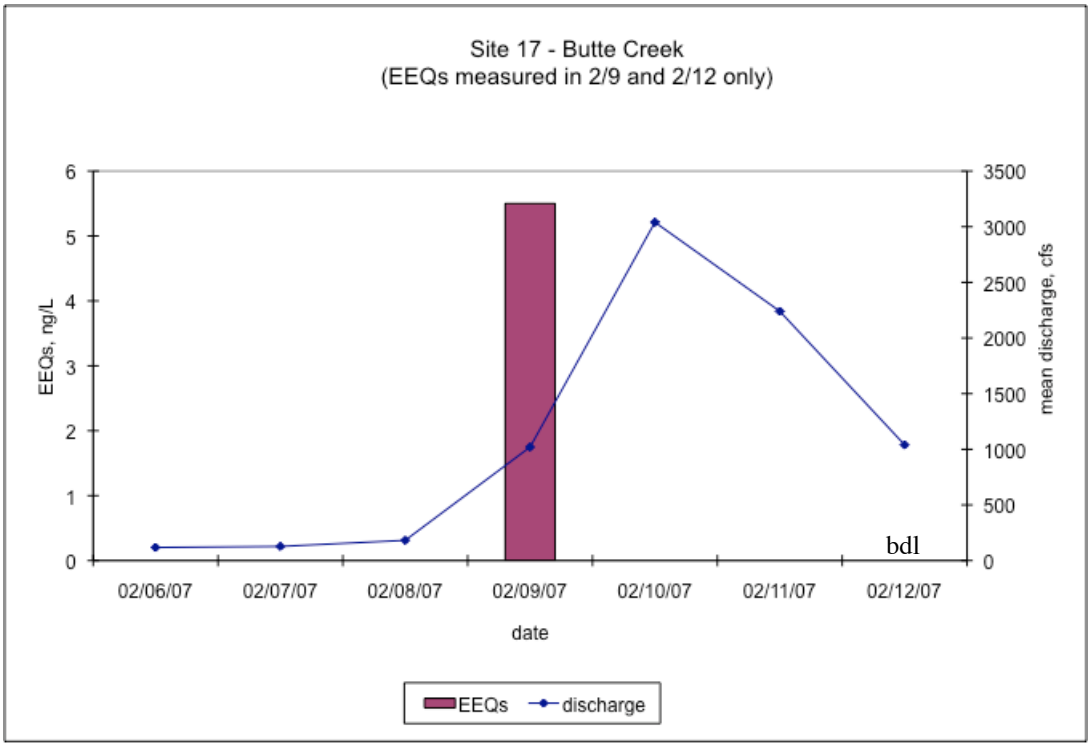


Figure 4. *In vitro* bioassay response versus water discharge in Butte Creek (site 17). bdl: below detection limit.

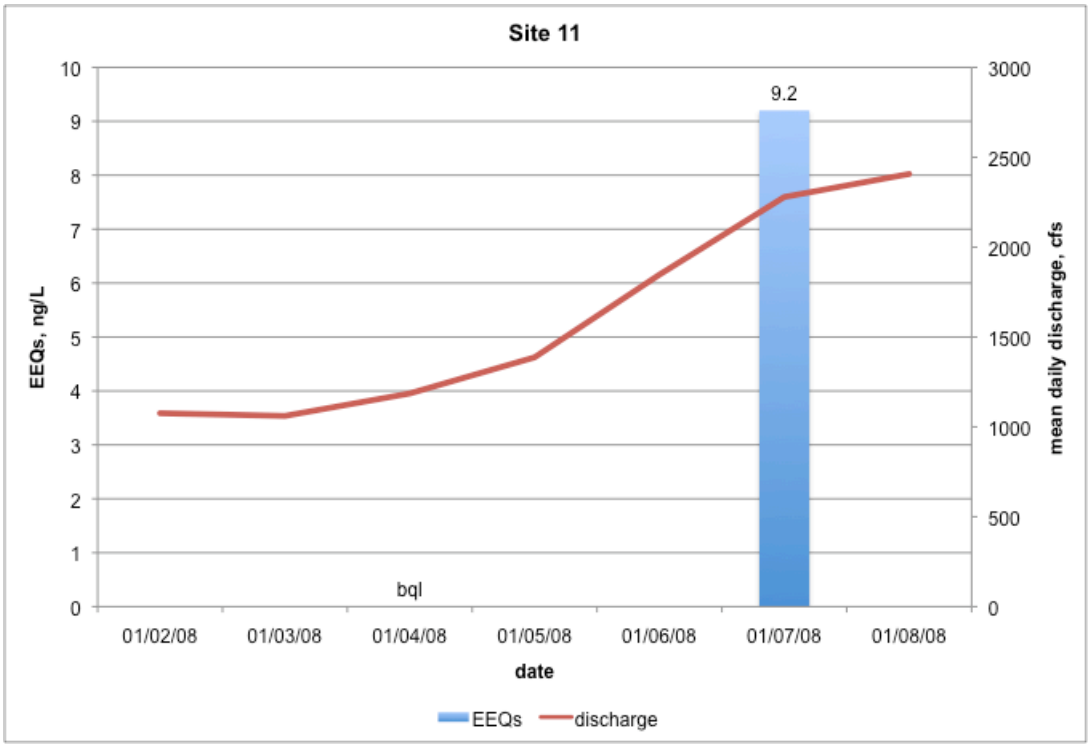


Figure 5. *In vitro* bioassay response versus water discharge in the San Joaquin River site (site 11). bdl: below quantitation limit.

Targeted Sampling (Task 2.1)

A first attempt to sample additional sites in the Napa River was conducted in August 2007, but the river upstream from the usual sampling site (Site 14) was completely dry at several points; at the other sites, water levels were low and appeared stagnant. Therefore, sampling of the Napa River was postponed until the wet season. In January 2008, the usual sampling site in the Napa and 3 more sites upstream were sampled during a major storm (Sites 14, N-11, N-26, and N-28). All of the samples were analyzed for hormones and APEs by GC/MS, and for VTG using both *in vivo* and *in vitro* bioassays.

All analytes were below detection limits in the Napa River sites during the storm in January 2008 (Table 10). The only exceptions were NP, OP, and NP1EO, which were present in quantifiable amounts at Site 14 (i.e., < 1 μg/L). The *in vitro* response (Table 11) tended to increase downstream: the site furthest upstream—close to Kimball Canyon Dam—had non-detectable levels; the next site, slightly more than 2 km downstream, had detectable levels, albeit below quantitation limits; the 3rd site, near Yountville, showed the highest response (168 ng/L EEQs); finally, site 14 had a lower response at 16 ng/L. The order-of-magnitude decrease in response did not appear to be a result of dilution because water discharge only approximately doubled between the two sites. As in our previous sampling events, the compounds measured in the GC/MS analysis cannot explain the high estrogenicity in the bioassays, and no correlation was found between estrogen equivalents calculated from the concentrations of the estrogenic compounds (chem EEQs) and the estrogen equivalents calculated with the VTG bioassay (bio EEQs). However, the location of the 2 downstream sites, in the region of the Napa Valley with the highest pesticide use, suggested a correlation between pesticide use or agricultural practices and bioassay response.

A second sampling event in the Napa River was conducted under dry weather conditions in August 2008. Water was present in all sites except for the site furthest upstream. In addition to the Napa, the Tuolumne River was selected for a more extensive sampling event in August 2008 because samples from the site in this river (Site 12) consistently produced the highest responses in the *in vitro* tests, although the *in vivo* response was consistently low. In addition to the usual site (Site 12), two more sites were sampled: one upstream at Old La Grange, and one downstream, close to the confluence of the Tuolumne with the San Joaquin River and downstream from Modesto. Additionally, a sample was taken from Ceres Canal near Modesto; water from this canal originates in the Tuolumne River, upstream from the site in Old La Grange.

Results of the APE and hormone analysis from the sites in the Napa and Tuolumne rivers fell within previous observations (Table 10): all the hormones were below detection limits or present in very low concentrations; i.e. under the thresholds for rainbow trout feminization. The APEs were all present, but also in relatively low concentrations (under 100 ng/L in all cases). Results from the bioassays were also low, below 0.5 ng/L EEQ *in vitro* and below 0.5 μg/kg EEQ *in vivo*. The only exception was the site in the Tuolumne River at Old La Grange, with 30 ng/L EEQ *in vitro* (Table 11).

Table 10. Steroid hormones and APEs in the Napa and Tuolumne rivers.

Site Date	17α-E2 (ng/L)	17β-E2 (ng/L)	E1 (ng/L)	E3 (ng/L)	P (ng/L)	MP (ng/L)	T (ng/L)	AD (ng/L)	NP (ng/L)	NP1EO (ng/L)	NP2EO (ng/L)	OP (ng/L)	OP1EO (ng/L)	OP2EO (ng/L)
Site N-28: Napa River nr Kimball Canyon dam														
January 2008	bdl	bdl	bdl	na	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
August 2008	na	na	na	na	na	na	na	na	na	na	na	na	na	na
Site N-26: Napa River at Myrtledale Rd														
January 2008	bdl	bdl	bdl	na	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
August 2008	nd	0.058	0.71	nd	nd	nd	nd	1.9	97	31	12	2.7	5.6	3.2
Site N-11: Napa River at Yount Mill Rd														
January 2008	bdl	bdl	bdl	na	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
August 2008	nd	0.10	0.79	nd	nd	nd	nd	nd	99	32	19	2.1	4.0	4.0
Site 14														
January 2008	bdl	bdl	bdl	na	bdl	bdl	bdl	bdl	459	165	bdl	33	bdl	bdl
August 2008	nd	0.13	3.3	nd	nd	nd	nd	nd	41	18	6.5	1.4	11	3.9
Site 12														
August 2008	nd	0.050	0.31	0.22	nd	nd	nd	nd	75	36	21	1.5	5.6	6.6
Ceres Canal near Modesto														
August 2008	nd	0.033	0.16	0.46	nd	nd	nd	1.9	70	12	3.4	1.7	2.3	1.0
Tuolumne River at Old La Grange														
August 2008	nd	0.053	0.28	nd	nd	nd	nd	nd	62	13	6.8	1.9	5.2	5.1
Tuolumne River dowstream Modesto														
August 2008	nd	0.060	0.50	0.47	nd	nd	nd	nd	93	16	6.4	2.9	3.5	2.0

bdl: below detection limit. na: not analyzed. **17α-E2**: 17α-estradiol. **17β-E2**: 17β-estradiol. **E1**: estrone. **E3**: estriol. **P**: progesterone. **MP**: medroxyprogesterone. **T**: testosterone. **AD**: androstenedione. **NP**: nonylphenol. **NP1EO**: nonylphenol monoethoxylate. **NP2EO**: nonylphenol di-ethoxylate. **OP**: octylphenol. **OP1EO**: octylphenol monoethoxylate. **OP2EO**: octylphenol di-ethoxylate.

Table 11. EEQs in Napa River by *in vivo* and *in vitro* bioassays (mean ± SD).

Site **Date**	**EEQ *in vitro* (ng/L)**	**EEQ *in vivo* (µg/kg wet wt.)**
Site N-28: Napa River nr Kimball Canyon dam		
January 2008	bdl	0.32 ± 0.27
August 2008	na	na
Site N-26: Napa River at Myrtledale Rd		
January 2008	bql	0.37 ± 0.32
August 2008	0.35 ± 0.011	0.474 ± 0.083
Site N-11: Napa River at Yount Mill Rd		
January 2008	168 ± 164	bdl
August 2008	bdl	0.22 ± 0.002
Site 14		
January 2008	16 ± 6.1	0.33 ± 0.28
August 2008	0.025 ± 0.005	bdl
Site 12		
August 2008	0.013 ± 0.007	bdl
Ceres Canal near Modesto		
August 2008	bdl	bdl
Tuolumne River at Old La Grange		
August 2008	30 ± 18	0.45 ± 0.034
Tuolumne River dowstream Modesto		
August 2008	bdl	bdl

bdl: below detection limit. na: not analyzed.

A more extensive sampling in the Sacramento/San Joaquin Delta in collaboration with Prof. Ingeborg Werner from UC Davis occurred in April 2008. Prof. Werner studies pelagic organism decline in the Delta, and her group monitors a series of sites in the Delta (Table A4) for organic pollutants, including pyrethroids, and they also conduct toxicity tests using delta smelt and inland silversides. GC/MS analysis of the samples found no or low concentrations of the steroid hormones and the APEs at most of the Delta sites (Table A5). The highest levels for the hormones (~2 ng/L E1) and APEs (~150 ng/L NP, 220 ng/L NP1EO, 370 ng/L NP2EO, 13 ng/L OP, 28 ng/L OP1EO, 40 ng/L OP2EO) were found at the mouths of the Sacramento and Napa Rivers, consistent with our previous observations. *In vitro* VTG analysis showed very low activity; all samples were under 0.032 ng/L EEQs (Table A6). Note that the use of qPCR for the analysis of this set of samples lowered the detection limit from 0.12 to 0.004 ng/L EEQs. In contrast, *in vivo* analysis (Table A6) showed elevated levels of estrogenicity in sites 405 (26 µg/kg EEQ) and 711 (13 µg/kg EEQ), in the Carquinez Strait and the Sacramento River respectively.

Further attempts to identify unknown xenobiotics in runoff samples involved the sampling additional sites along the Napa River and Delta in December 2009 and February 2010 indicated the presence of low concentrations of steroid hormones (less than 2 ng/L for each) and APEs (~50 ng/L NP,~ 200 ng/L NP1EO, ~5 ng/L NP2EO, ~5 ng/L OP, ~10 ng/L OP1EO, ~10 ng/L OP2EO). These concentrations were similar to those previously measured and were insufficient to explain the observed estrogenic signals in the bioassays. Analysis of these samples with the *in vitro* trout liver hepatocyte assay did not show evidence of estrogenic compounds in these samples. These findings were somewhat surprising because previous analyses from these same sites almost always showed significantly elevated concentrations of estrogenic substances.

Toxicity/(xeno)estrogen identification evaluation (TIE) (Task 2.2)

Water samples from the Napa, Sacramento, and Mokelumne rivers (sites 14, 8, and 9 respectively) were obtained in June 2007 for TIE analysis. The sites in the Napa and the Sacramento River were chosen because they have consistently shown high levels of estrogenicity, both *in vivo* and *in vitro*. The sample from the Mokelumne River, which never showed estrogenicity in our past sampling events, was spiked with 100 ng/L of E2 as a positive control.

The spiked sample exhibited the highest *in vitro* and *in vivo* estrogenicity (Figure 6) in the 60% methanol fraction (*in vivo* activity was also observed in the 80% fraction). GC/MS analysis of the different fractions confirmed the presence of E2 in the estrogenic fractions of the spiked sample (Mokelumne). For the Sacramento River Delta sample, *in vitro* and *in vivo* estrogenicity was centered on the 60% methanol fraction, which corresponds to compounds with intermediate polarity, including E2. For the Napa River sample, most of the estrogenicity observed in the bioassays was detected in the 100% methanol fractions, indicating that the compounds

responsible for estrogenicity were relatively hydrophobic. As previously observed for the whole samples, E2 was not detected in any of the fractions from the Sacramento and Napa rivers.

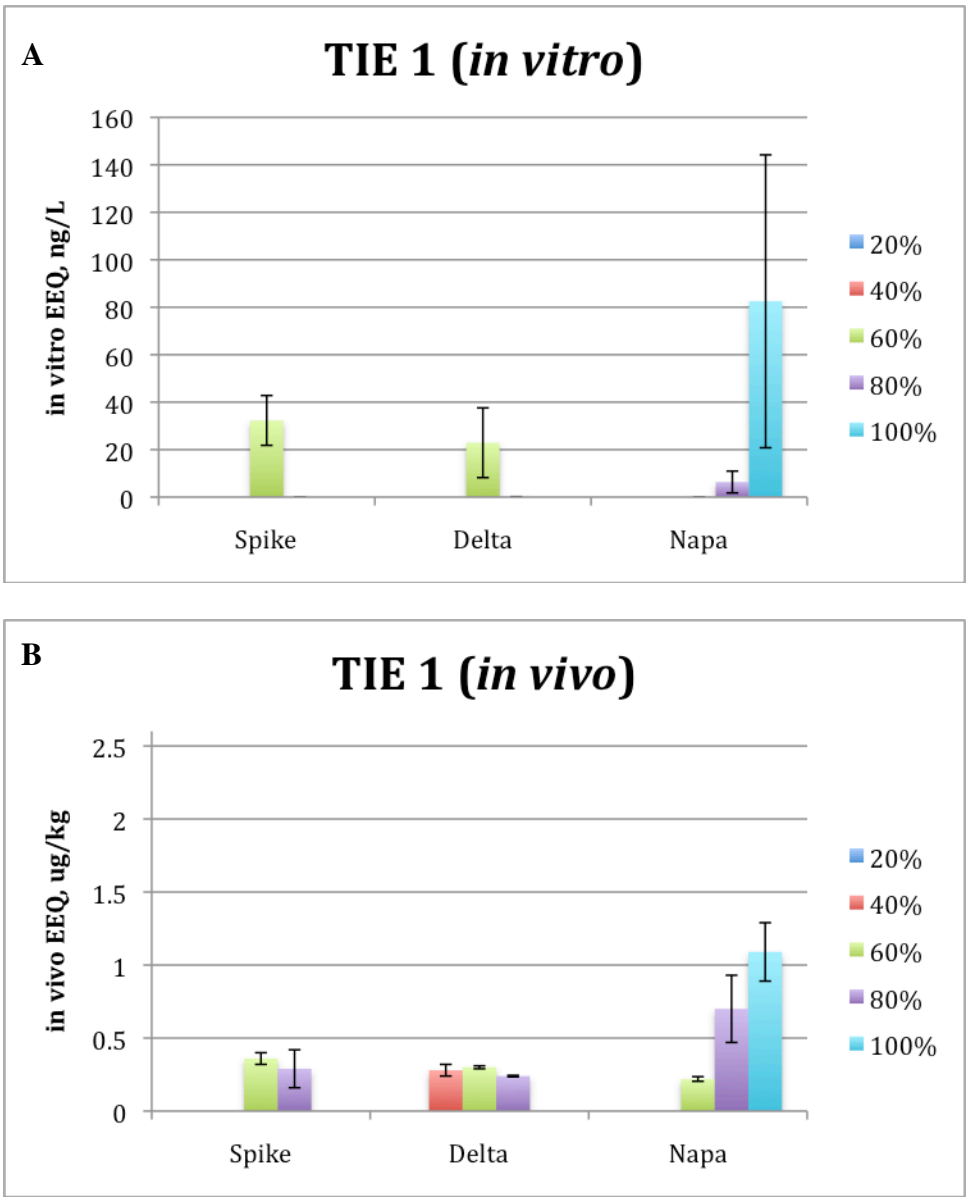


Figure 6. Toxicity identification evaluation (TIE) response (A) *in vitro*, and (B) *in vivo*. Percentages refer to the proportion of methanol in the eluting mixture.

The fractions exhibiting the highest estrogenic activity (i.e., the 60% methanol fractions from the Sacramento River Delta and the spiked Mokelumne sample, and the 100% methanol fraction from the Napa River) were subjected to a more precise separation using high-performance liquid chromatography (HPLC). HPLC fractionation was performed in a C18 reversed-phase column

using the following gradient: 0-3 min, 40% methanol; 3-30 min, 40-100% methanol. 3-mL (or 3 min) samples were collected and analyzed using the *in vitro* and *in vivo* bioassays.

The highest *in vitro* activity (Table 7) was observed in fraction 9 of the spiked control (Figure 7), where E2 was expected to elute (confirmed with a separate E2 injection); additionally, activity was also observed in fractions 8 and 10 of the spiked sample, but the source of the activity is unknown. From the non-spiked samples, only fraction 8 of the Sacramento River (Figure 8) and fraction 6 of the Napa (Figure 9) showed *in vitro* activity and it was present at relatively low levels (under 5 ng/L); the source of the activity is also unknown. However, Napa fractions 7 and 8 might have been diluted more than the rest and this might explain the lack of activity in these samples. As for the *in vivo* assays, most fractions were below the detection limit, except for three of Napa's fractions (1, 2 & 7) that showed slight activity (Figure 10). The lack of activity in the HPLC fractions and the high response observed in the original SPE fractions suggested that dilution and/or losses during sample processing occurred. A mass balance of the E2 recovered in the spiked control extraction points towards incomplete recovery of the analyte in the process. The activity observed in Napa River sample's early fractions (Figure 10) was unexpected and the causes unknown, but this phenomenon had been observed in samples from a wastewater treatment plant effluent and sediment extracts collected close to wastewater treatment plant outfalls [26].

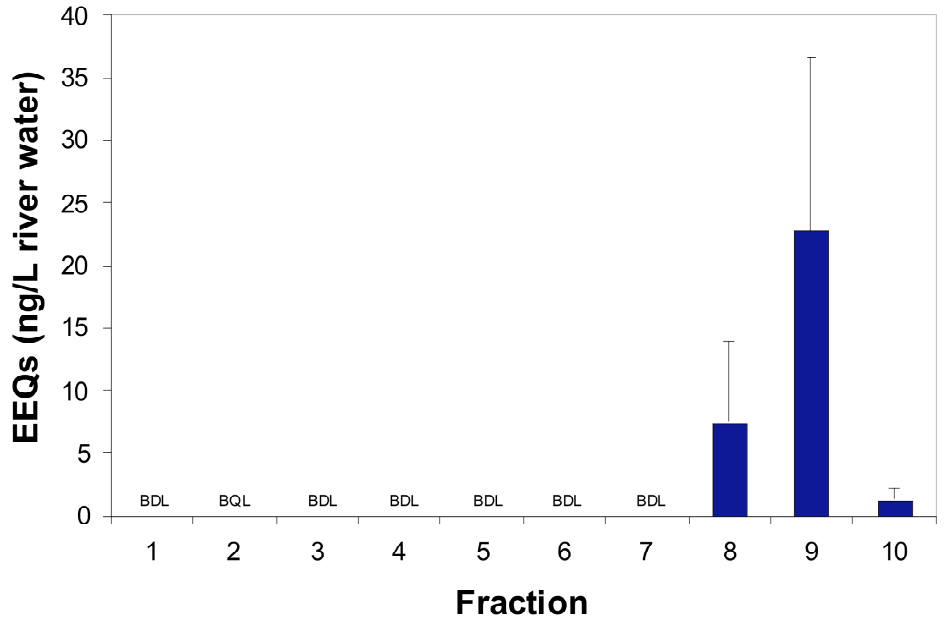


Figure 7. *In vitro* estrogenic activity of the HPLC fractions from the 60% SPE fraction of the TIE analysis for the spiked Mokelumne sample.

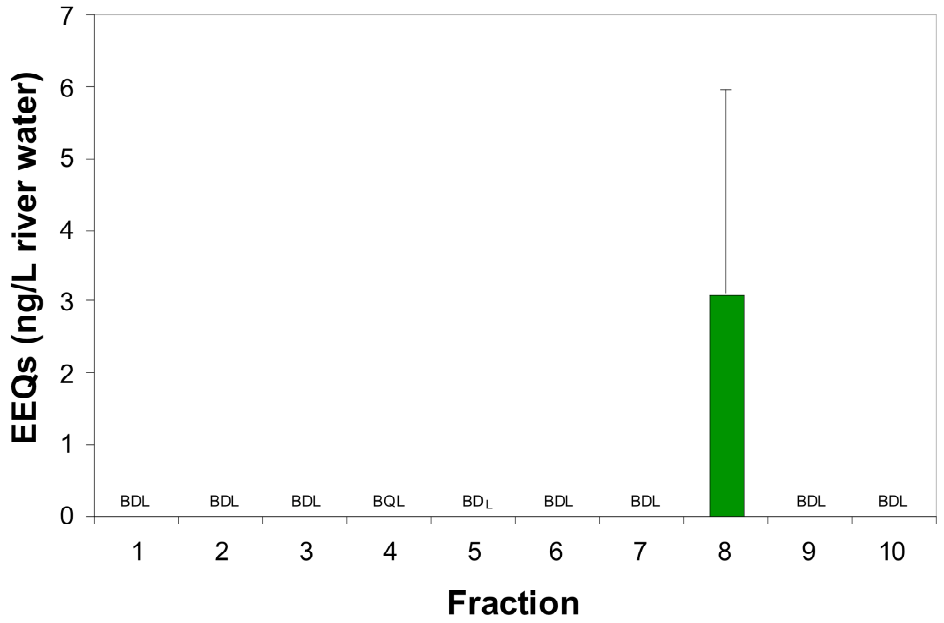


Figure 8. *In vitro* estrogenic activity of the HPLC fractions from the 60% SPE fraction of the TIE analysis for the Sacramento River Delta sample.

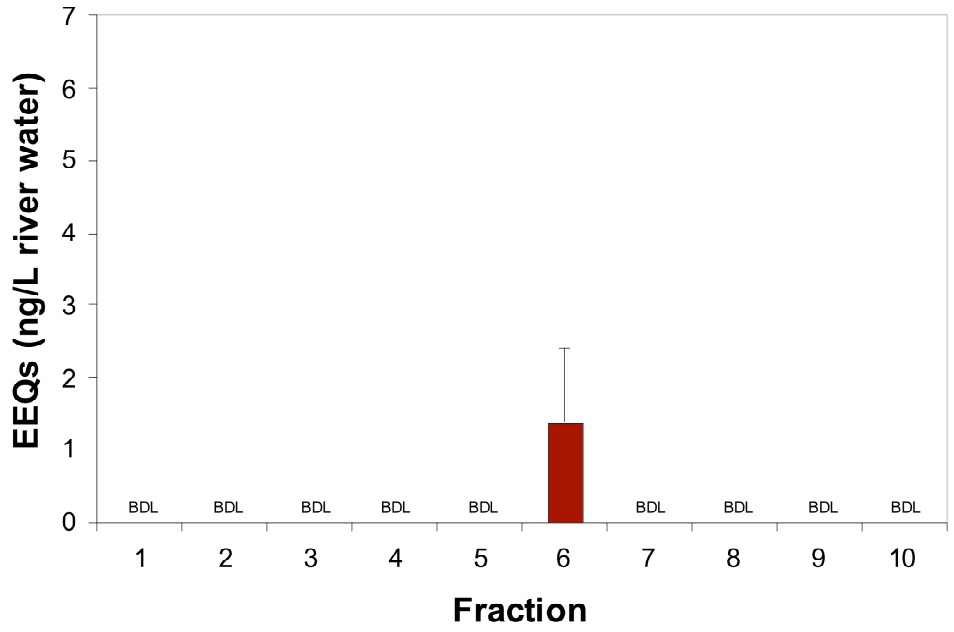


Figure 9. *In vitro* estrogenic activity of the HPLC fractions from the 100% SPE fraction of the TIE analysis for the Napa River sample.

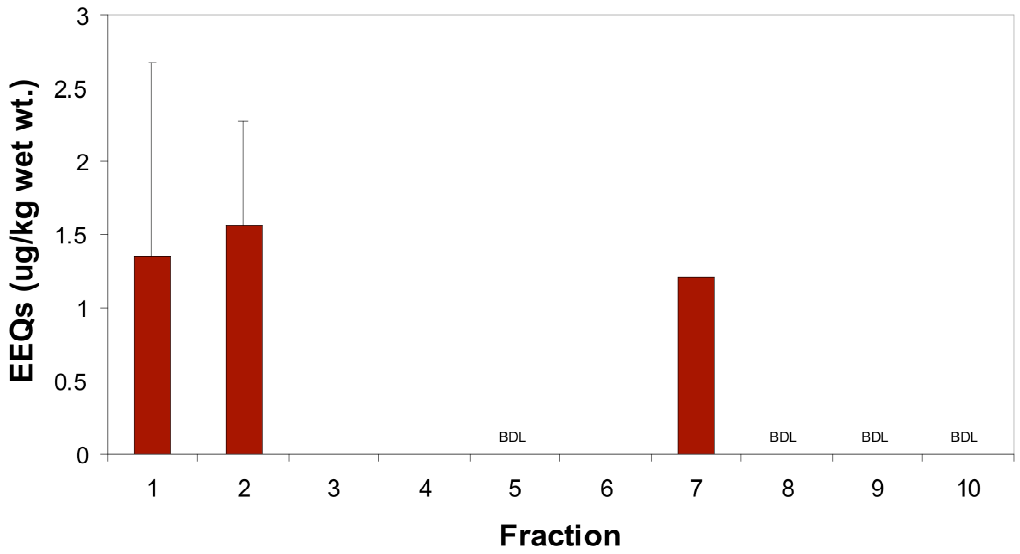


Figure 10. *In vivo* estrogenic activity of the HPLC fractions from the 100% SPE fraction of the TIE analysis for the Napa River sample.

Selected HPLC fractions were analyzed for a suite of pharmaceuticals, personal care products, phytoestrogens, pesticides (including pyrethroids) and hormones. E2 was detected in fractions 2, 7, 8 & 9 of the spiked control, suggesting possible cross contamination or the presence of E2-complexes with NOM that were able to elute early in the HPLC run. However, the low concentrations detected cannot explain the estrogenicity observed. Low concentrations (< 5 ng/L) of some pharmaceuticals were also reported (atenolol, trimethoprim, meprobamate, and gemfibrozil).

A second round of TIE analysis was conducted to verify the findings from the first round. The two sites discussed above (14 and 8) and site 12 in the Tuolumne River were chosen because of the consistently high *in vitro* response shown in the survey; additionally, dechlorinated tap water spiked with E2 (30 ng/L) was used as positive control. In this TIE round, larger sample volumes (10 L) were used in order to improve sensitivity, and aliquots of all TIE samples were extracted and stored for LC/QToF analysis to identify unknowns in the samples showing activity. These samples were eluted sequentially with increasing methanol concentrations as described above, and then tested for VTG with both bioassays.

Similar to the first TIE round, the spiked sample exhibited the highest *in vitro* and *in vivo* estrogenicity in the 60% methanol fraction, but in this case *in vitro* activity was also observed in the 80% fraction and *in vivo* activity in the 100% fraction (Figure 11). The sample from the Sacramento River Delta site (8) also showed similar results to the first TIE: *in vitro* and *in vivo* estrogenicity was centered on the 60% methanol fraction, which corresponds to compounds with intermediate polarity, including E2 (Figure 11). The Napa River sample showed most of the *in vitro* estrogenicity in the 80% fraction (versus 100% in the first TIE); the *in vivo* activity was

mainly in the 100% fraction, as it was in the first TIE (Figure 11). The Tuolumne fractions did not show detectable activity in any of the bioassays.

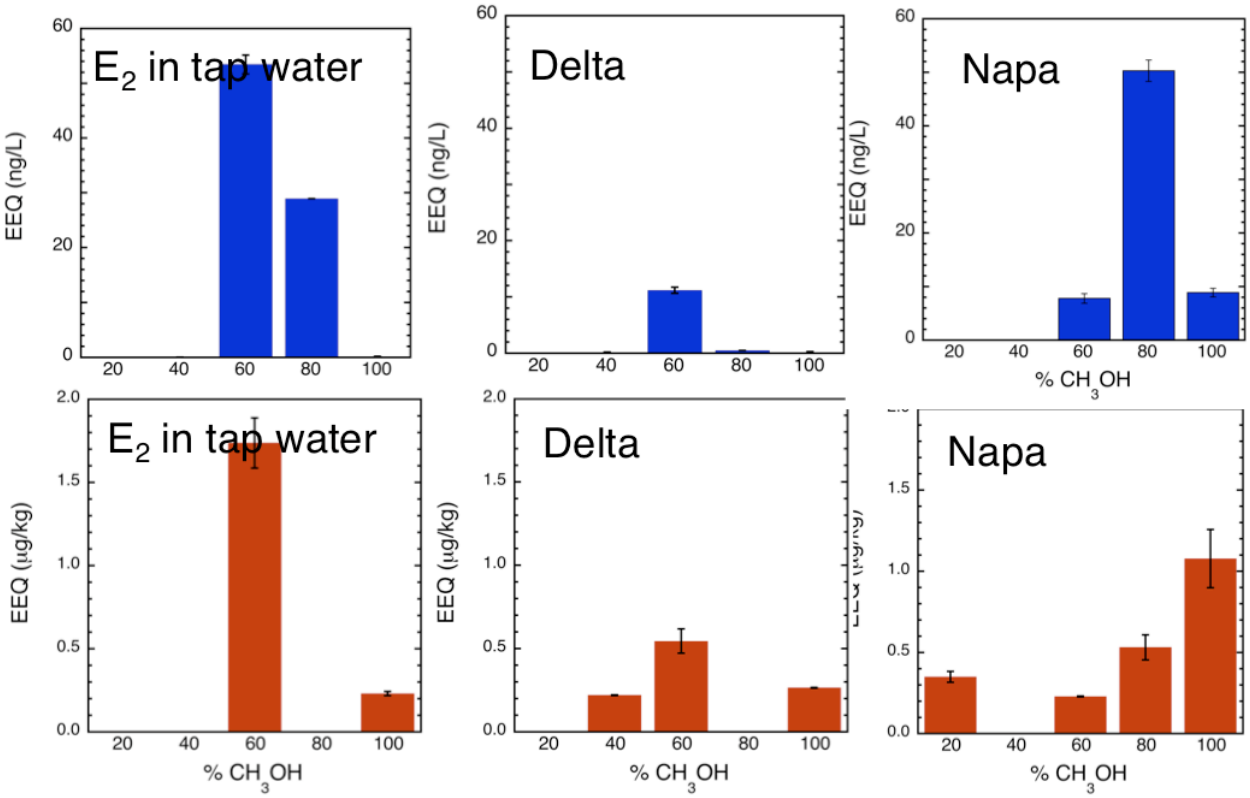


Figure 11. Estrogenic activity observed in fractions eluted from solid-phase extraction during the second round of TIE analysis.

The Napa and Sacramento fractions with the highest activity were subjected to the HPLC fractionation as described above followed by analysis with the two bioassays. HPLC fractionation of the 60% methanol fraction from the Sacramento River site showed *in vitro* activity in only one fraction (fraction 7) while the *in vivo* bioassay showed activity in fractions 6 to 10. For reference, fraction 7 corresponds to the retention time for 17β-estradiol. Therefore, it is likely that the activity at the Sacramento River site was attributable to a compound with chemical properties similar to that of estradiol (i.e., a moderately hydrophobic compound). HPLC fractionation of the 100% methanol fraction from the Napa River showed a distinctly different pattern, with activity in both assays clustered around an early eluting hydrophilic fraction and a late-eluting hydrophobic fraction. The explanation for this phenomenon is unclear, but it suggests that multiple compounds may be responsible for the observed activity.

The SPE fractions showing the highest activity (80 and 100% from Napa, 60 and 80% from the Sacramento), and the Old La Grange sample described above were sent to the California Department of Fish and Game for pesticide analysis and to SNWA for LC/QToF analysis for unknowns. Along with these, 4 of the archived samples from the survey with the highest *in vitro* and/or *in vivo* activity were also sent for analysis. Most of the pesticides were not detected with the notable exception of diuron, which was present in all of the samples (a list of analytes and

detection limits is included in Table 12). On the basis of calibration curves in a surface water matrix, we estimated the concentrations were less than 10 ng/L. The occurrence of diuron in samples from these locations is not surprising because it is one of the most heavily used herbicides in California [28], but it is noteworthy that it was present in relatively high concentrations in the samples with the highest bioassay responses. The Department of Fish and Game analyzed a second set of archived samples to better evaluate the correlation between the presence of diuron and bioassay response. Again, concentrations of pesticides were low, with no compound detected at concentrations high enough to explain the observed estrogenicity.

Table 12. Detection limits for pesticides analyzed in TIE samples.

Compound	Detection limit (μg/L)*
Triazines (LC-MS/MS)	
Ametryn	0.005
Atraton	0.005
Atrazine	0.005
Cyanazine	0.005
Deisopropyl-atrazine	0.005
Desethyl-atrazine	0.005
Desmetryn	0.005
Dipropetryn	0.005
2-Hydroxyatrazine	0.005
Molinate	0.005
Prometon	0.005
Prometryn	0.005
Propazine	0.005
Secbumeton	0.005
Simazine	0.005
Simetryn	0.005
Terbuthylazine	0.005
Terbutryn	0.005
Thiobencarb	0.005
Organophosphate pesticides (GC-FPD)	
Chlorpyrifos	0.050
Diazinon	0.020
Miscellaneous compounds (LC-MS)	
Imidacloprid	0.050
Myclobutanil	0.050
Oryzalin	0.050
Oxyfluorfen	0.050
Tebuconazole	0.050
Surfactants (LC-MS)	
Nonylphenol	2.00
Nonylphenol ethoxylate	2.00

The LC/QToF analysis performed at SNWA did not reveal any unsuspected compounds either, except for different series of ethoxylated compounds (possibly alcohol ethoxylates and polyethylene glycol) that were present in high concentrations in a sample from Site 5 in the Lower Feather River. Otherwise, the LC/QToF confirmed the presence of the compounds detected in the pesticide analysis, but identification of other compounds was hindered by the presence of natural organic matter in the samples.

Whole Fish Bioassays (Tasks 1.3, 2.3 and 2.4)

Salmonid Bioassays (Tasks 1.3 and 2.3)

The Chinook salmon dose/response relationship to E2 was tested *in vitro* and *in vivo* in fish obtained from Nimbus hatchery in the American River. Chinook salmon was found to be only slightly more sensitive than rainbow trout to E2 (Figure 12); therefore, we concluded that rainbow trout was a good surrogate for Chinook salmon in these tests. Given the logistical problems associated with acquiring and transporting juvenile salmon, the research team decided that subsequent *in vivo* testing would employ rainbow trout instead of Chinook salmon.

To conduct the experiments with whole rainbow trout, water samples collected during February 3, 2010 were shipped to UC Riverside where they were used for an exposure experiment with juvenile fish. Unfortunately, the water samples were acutely toxic to the trout. The majority of the fish cultured in either sample died within two days.

To obtain information on the estrogenic activity of the whole water samples, Japanese medaka (*Oryzias latipes*), was used as a test organism. This species is generally more tolerant to poor water quality than rainbow trout and we have had considerable experience culturing the fish and evaluating their response to estrogenic compounds.

As part of the bioassay, water from the Napa River and Central Delta sites was collected in Teflon containers and shipped to UCR for in 7-day *in vivo* exposures with Japanese medaka. Three sexually mature males were housed per 500 mL beaker with 300 mL exposure water. Water changes/feeding occurred every other day. The negative control used dechlorinated tap water and the positive control was 100 ng/L of 17β-estradiol in dechlorinated tap water. Liver samples were collected on Day 7, homogenized in PBS and centrifuged at 10,000 x g. Aliquots of the supernatant were used for ELISA using VTG antibodies for Japanese medaka (Biosense, Bergen Norway). Protein was normalized by the Bradford assay.

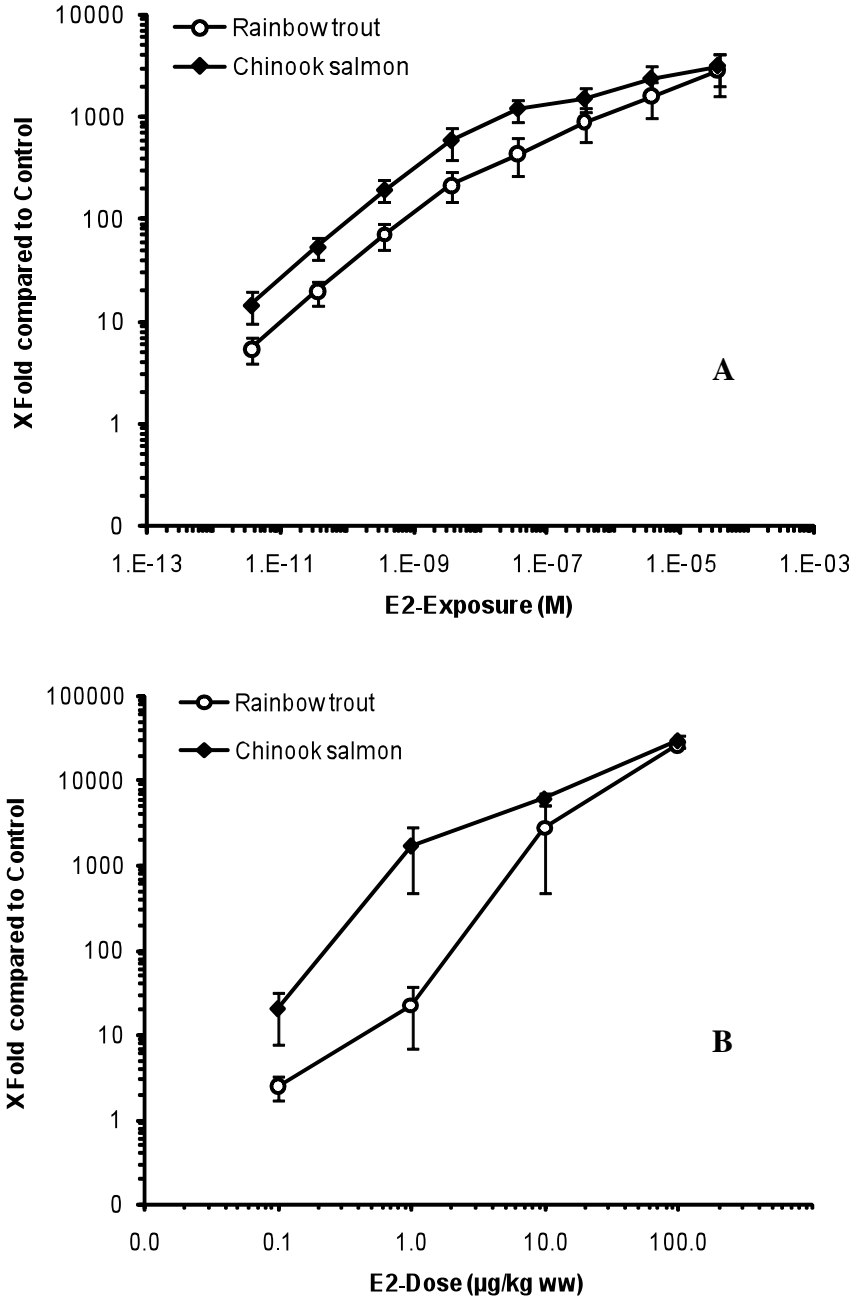


Figure 12. VTG induction in response to E2 exposure. A) VTG mRNA induction in primary hepatocyte bioassay from rainbow trout (*Oncorhynchus mykiss*) and chinook salmon (*Oncorhynchus tshawytscha*). B) VTG protein induction in whole animal assay. Data are presented as mean ± SD (n=3-4 in hepatocyte bioassay and 3-5 in whole animal bioassay).

Results indicated a significant elevation in vitellogenin production, relative to control, at the Napa River site (Figure 13). The Sacramento River Delta sample, collected at Walnut Grove (site 8), showed vitellogenin concentrations slightly below the control sample (Figure 13). Overall, the increase of protein-normalized vitellogenin concentrations in the Napa River sample

(i.e., 226 ng/mg) was approximately half of the value observed for the 100 ng/L 17β-estradiol positive control (i.e., 389 ng/mg with a background of 56 ng/mg for the control sample). These findings confirm our earlier findings of estrogenic substances in water extracts in the Napa River but not in the Walnut Grove sample.

Analysis of these same samples using the *in vitro* hepatocyte assay (Figure 14) did not indicate the presence of estrogenic compounds in either sample. The explanation for this discrepancy is unclear.

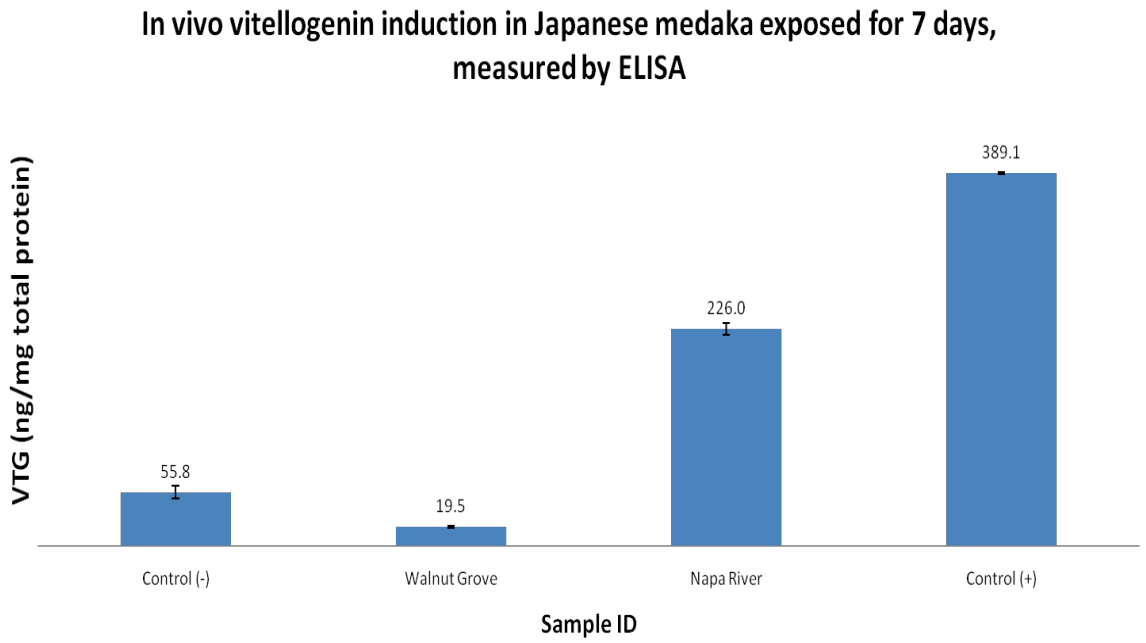


Figure 13. *In vivo* vitellogenin induction in Japanese medaka after a 7-day exposure to water from sites 8 (Sacramento River in Delta at Walnut Grove) and 14 (Napa River).

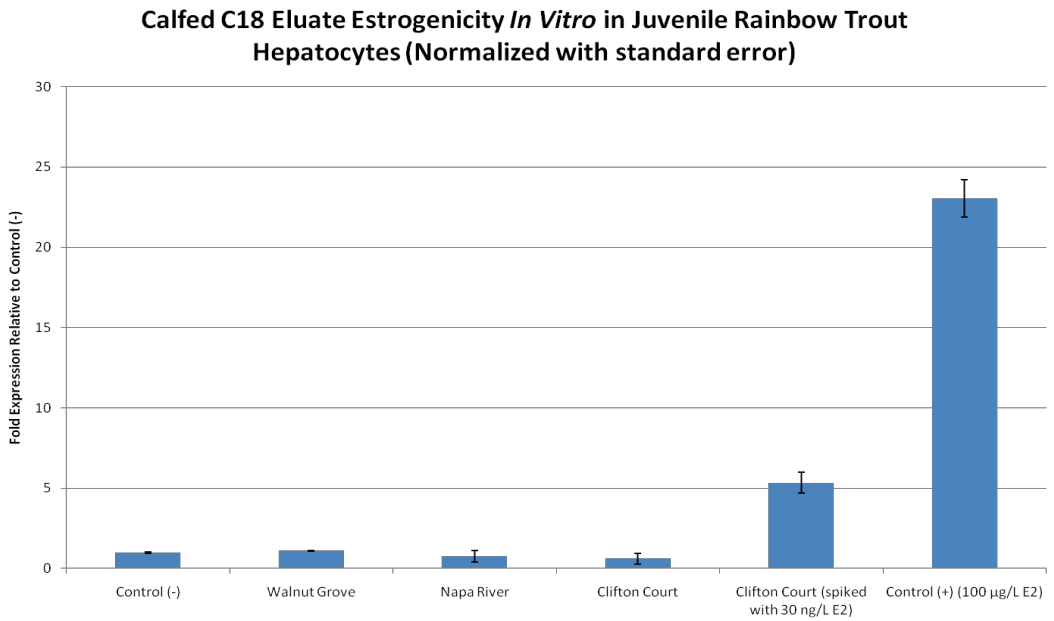


Figure 14. *In vitro* estrogenicity response in juvenile rainbow trout hepatocytes exposed to water extracts from sites 8 (Sacramento River in Delta at Walnut Grove) and 14 (Napa River).

Choriogenin Bioassays (Task 2.3 and Task 2.4)

A screening study was conducted in February 2007 in the Delta and San Francisco Bay. Eight Delta fish species were captured during this event. Most of the fish analyzed for the presence of plasmatic choriogenins gave negative results; the exceptions were 1 (out of 10) threadfin shad, 4 (out of 4) Sacramento splittail, and the 2 longfin smelt captured. The latter, however, were egg-bearing females and therefore expected to test positive for these proteins. Additionally, several fish were sexually immature and were not appropriately sexed; therefore, additional sampling had to be conducted for sexually mature fish. The low number of fish captured during sampling limited the assessment of choriogenin and prevented us from drawing any inferences about the data.

Attempts to collect fish in the Delta in late 2008 and spring 2008 were unsuccessful for the most part. All sampling in the Lower Sacramento was halted in December 2007 by the CDF&G because of the extremely low number of Delta smelt in the system. Sampling of outmigrating salmon smolts was not possible either because of the USFWS prohibition on any "take" of salmon due to the low return of adult salmon to the Delta in 2007. The only species sampled was American shad, taken near Antioch near the convergence of the Sacramento and San Joaquin rivers. None of the serum samples of 40 individuals tested positive for choriogenin, although a true positive control is needed to rule out suspected nonspecific binding of the antibodies.

Due to the lack of suitable numbers of fish for choriogenin analysis, the research team, working in consultation with our advisory group decided to focus our attention on inland silverside (*Menidia beryllina*), a native fish species that can also be cultured in the laboratory. To assess the potential for feminization of *Menidia* and other native species two strategies were employed. First, wild fish were sampled from locations near the sites in the Napa River and Sacramento Delta. In addition, whole water samples collected from the same sites plus a reference site near Clifton Forebay were used for laboratory exposures of fish. Details of these experiments are summarized below.

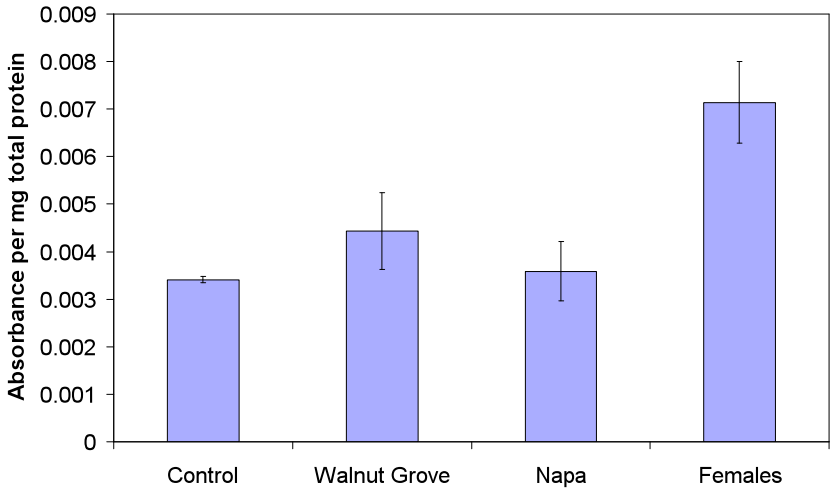
During December, 2009 three scientists spent eight days surveying for suitable sites and collecting wild *Menidia*. Field collection from the Napa and Sacramento Rivers was conducted and fish were returned to the Bodega Marine Lab, where sampling of blood plasma, livers and gonads from male, female and juvenile *Menidia* was performed. Fish collected at the Napa site were 49-79 mm long and weighed 0.5-1.7 grams. Fish collected at the Walnut Grove site were 33-54 mm long and weighed 0.14-0.76 grams. Blood samples were collected individually in different volumes of buffer depending on fish length, spun in a centrifuge for 5 minutes at 5000 RPM to separate plasma, which was then stored at -80 ^{o}C for future analysis of choriogenin levels. Liver and gonads were collected and stored at -80 ^{o}C for future histological analysis.

During February 2010, water samples were collected from both the Napa and Sacramento River sites. Site water exposures were performed by exposing 5 replicates of 10 naive fish each (50 fish per site, 150 total fish in 15 aquaria) for 14 days to control, Napa or Sacramento River water. Exposures required daily water changes and water chemistry assessment of dissolved O_2, nitrogen, pH and salinity. Fish mortality was low in controls (1 fish out of 50) and highest in

Sacramento River water at ~30%. Upon completion of the tests, the fish were analyzed for choriogenin to assess the potential for feminization upon exposure to this water sample.

Choriogenins in plasma from resident *Menidia* collected on a single day in December 2009 at both sites were compared. We found that there was no significant difference between choriogenin levels in plasma pooled from 3 male and/or juvenile fish collected from either of the sites and depurated controls. As validation of the assay, choriogenin/chorion levels in pooled plasma from resident female *Menidia* were higher than that of the experimental samples. Choriogenins detected in plasma were approximately 1 pg per mg of total protein in male fish, and only slightly higher in females.

Choriogenin levels in whole-body homogenate were assayed in naïve fish exposed for two weeks to water collected directly from each site in January 2010. We found that water from neither of the sites elicited a significant increase in choriogenin levels compared to controls (Figures 15 A and B). This finding parallels that of the survey of choriogenin levels in resident fish, and both were taken at similar times of year.



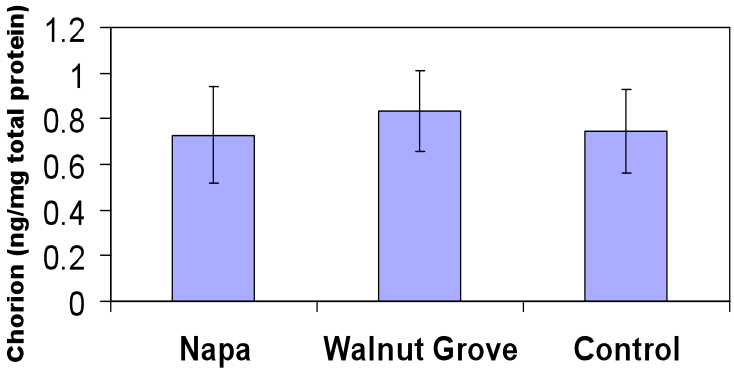


Figure 15. Choriogenin abundance compared to controls during assays performed during the winter of 2009/2010. A) Plasma choriogenin levels from resident male or juvenile *Menidia* collected from each site. B) Choriogenin abundance in whole-body homogenate following a two week incubation of juvenile fish in water collected at each site.

Outplants

Naïve fish were outplanted in flow-through buckets at both sites for a period of two weeks. Afterword, choriogenin levels were quantified in whole-body homogenate. We found that fish outplanted at both sites had significantly higher levels of choriogenin than controls (Figure 16). In fish from the Sacramento River, the induction of choriogenin was equivalent to 1.9 ng per mL chorion per mg total protein, or an exposure to approximately 1 ng/L EE2. All three replicates from the Sacramento River had similar choriogenin levels.

Only two replicates were retrieved from the Napa River due to tampering during the course of the outplant deployment. There was a large difference between these two replicates. Instead of an average choriogenin level, these data are displayed separately. As shown in Figure 16, Napa-1 had slightly higher choriogenin levels than the Sacramento River outplants, at 2.3 ng chorion per mg total protein. Napa-2 had a much stronger response at nearly 30 ng of chorion per mg total protein – the equivalent of *Menidia*'s response to treatment with approximately 25 ng/L of EE2.

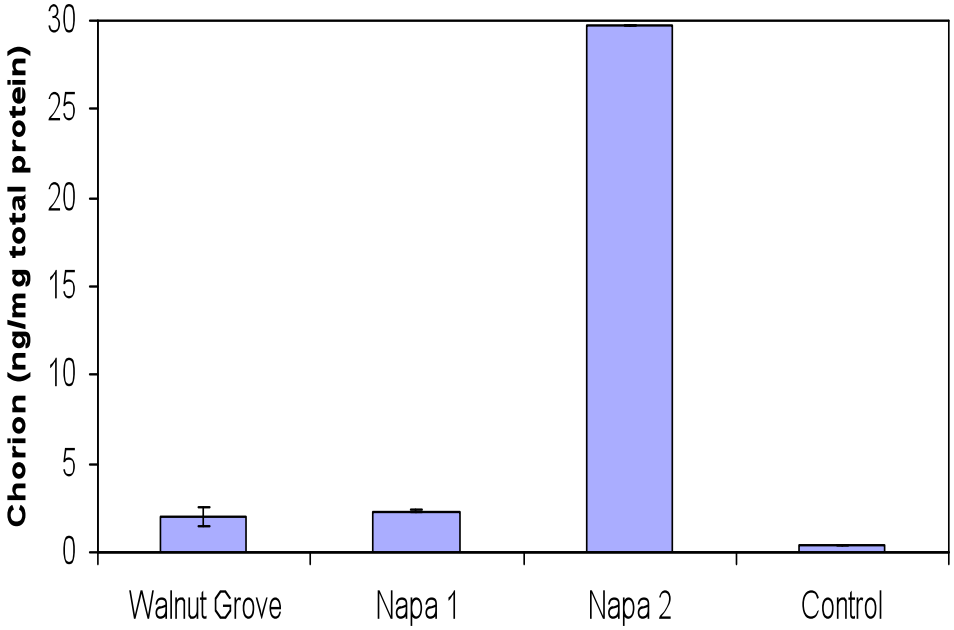


Figure 16. Choriogenin abundance in whole body of fish following a two week outplant in flow-through buckets at each of the sites. The two Napa replicates are shown separately because of the large discrepancy between the values. The Walnut Grove site is an average ± standard deviation of three replicates.

Comparison of Western blot to ELISA for choriogenin analysis

There are many advantages to using the ELISA method instead of Western Blots for detection of choriogenins. Perhaps the most important justification is that the sensitivity with ELISA is much greater. ELISA can detect down to 90 ng chorion per mL in whole-body homogenate, whereas

Western can only detect down to 17 µg per mL – nearly 1000-fold greater sensitivity with the ELISA. This allows a larger number of replicates with the same volume of sample, thus facilitating greater accuracy and precision.

Another significant advantage is the high-throughput nature of ELISAs. The ELISAs described in this report were performed in 96-well plates, but the method is easily adapted to 384-well plates, which would use even smaller sample volumes. One 96-well plate is equivalent to 6 Western Blots, but uses a smaller amount of reagents than a single blot, thus saving both money and time.

Finally, quantification of choriogenins compared to known quantities of chorion is more repeatable and more accurate with ELISA than Westerns. ELISA measures the production of a soluble colored product of an enzymatic reaction (TMB reacting with horse-radish peroxidase, in this case) with great fidelity. Westerns can be quantified, but it requires performing computer-aided densitometry of band darkness that is less sensitive and accurate. It is important to note, however, that Western Blots provide more information (most notably the molecular weight) of the bands detected. This information is integral to interpretation of ELISA results, so both methods are required for assay validation.

One weakness to both methods is that the *Menidia* antibody was developed against purified chorion, whereas it is being used to detect the presence of choriogenins. Additionally, both methods use chorion protein for creating a standard curve for choriogenin quantification. It is not known what, if any, specific differences in binding occur with these two proteins. Nevertheless, the quantification of choriogenins presented here are unlikely to be an exact representation of the amount of choriogenin present. However, because the relative choriogenin-to-chorion ratios are consistent between ELISAs for the samples, the quantification can be used for comparisons between fish. It is for this reason that, wherever possible, we have reported the choriogenin induction response as compared to the more relevant exposures to known concentrations of EE2.

When normalized to total protein, the level of choriogenins detected in whole-body homogenate was almost 1000-times greater than those detected in plasma. This could be because plasma is relatively rich in protein compared to whole-body homogenate, which would contain more lipids, tissue, and DNA. This could also be an indication that choriogenins are not circulating through the bloodstream, even though they may be present in other organs. Further research is required to differentiate these possibilities.

Estrogenicity of local rivers

The two sites surveyed generally displayed similar levels of estrogenicity. At both sites the assays performed during the winter (i.e. resident fish collection and site-water exposure) showed no difference from controls. Similarly, the outplant experiment, which occurred during the summer, showed increased abundance of choriogenins at both sites. The three Sacramento River replicates all had similar abundances of choriogenin, corresponding to a treatment with ~1 ng/L EE2. Unfortunately there was a large discrepancy in abundance of choriogenins between the two recoverable Napa replicates that made it through tampering during the outplant. The lower-

responding replicate was similar to the Sacramento River samples. The other had a choriogenin abundance that correlated to treatment with ~25 ng/L EE2. It is unknown what caused such a difference, but it may be that stress levels (potentially both hypoxia and heat stress) differed between fish in the replicates following the tampering. It is also possible, however, that the tampering artificially decreased the response in the lower-responding replicate.

The two sites chosen for this assay were previously identified as being among the most likely places in the San Francisco Bay and Sacramento/San Joaquin Delta for presence of estrogenic EDCs. As such, they were predicted to act as a source of positive signal for validation of the assay. Of the 3 assays performed, this prediction only held true in the outplant experiment. This assay took place in early-August, which tends to be a hot, dry time of year in the Bay/Delta area. The other two assays were both during the winter of 2009/10, which was characterized by particularly heavy and consistent rains. Temperature, oxygen levels, estrogenic EDCs, and other stressors are all possible contributors to these differences between the timepoints. Once again, further research is required to determine what, if any, affect seasonal weather patterns have on levels of endocrine disruption in area fishes.

Future work should be performed using standardized outplant and laboratory assays throughout the year to verify these findings and determine what effect seasonal weather has on this induction.

Contributions and Deliverables

A website including a description of the project objectives and results was created and can be found at: http://www.ce.berkeley.edu/~sedlak/CALFEDwebsite.htm>

Platform presentation. Kolodziej, E.; Sedlak, D.: Agricultural sources of steroids hormones in surface waters. 4th Biennial CALFED Science Conference. Sacramento, CA. October 2006.

Platform presentation. Lavado, R.; Loyo-Rosales, J. E.; Kolodziej, E. P.; Sedlak, D. L.; Schlenk, D.: Evaluation of steroid estrogen and estrogenic activity in surface waters from Central California. Southern California SETAC Annual Meeting. Los Angeles, CA. April 2007.

Poster presentation. Lavado, R.; Loyo-Rosales, J. E.; Kolodziej, E. P.; Sedlak, D. L.; Schlenk, D.: Evaluation of steroid estrogen and estrogenic activity in surface waters from Central California. PRIMO (Pollutant Responses in Marine Organisms) 14th International Symposium. Florianopolis, Brazil. May 2007.

Platform presentation. Kolodziej, E. P.; Loyo-Rosales, J. E.; Sedlak, D. L.: Steroid hormone sources and fate in agricultural watersheds. Micropol and Ecohazard Conference. Frankfurt, Germany. June 2007.

Poster presentation. Lavado, R.; Loyo-Rosales, J. E.; Kolodziej, E. P.; Sedlak, D. L.; Schlenk, D.: Evaluation of steroid estrogen and estrogenic activity in surface waters from Central California. SETAC 28th Annual Meeting. Milwaukee, WI. November 2007.

Platform presentation. Loyo-Rosales, J. E.; Sedlak, D. L.; Lavado, R.; Schlenk, D.: Estrogenic activity in Central Valley rivers. 18th Annual Meeting of the Northern California Regional Chapter of SETAC. Berkeley, CA, May 2008.

Platform presentation. Floyd, E.; Lavado, R.; Loyo-Rosales, J.; Kolodziej, E.; Sedlak, D.; Snyder, S. A.; Vanderford, B. J.; Schlenk, D.: Detection of estrogenic activity in surface waters from the Central Valley of California. Platform presentation. 2008 Annual Meeting of the Southern California Regional Chapter of SETAC. Dana Point, CA, May 2008.

Platform presentation. Loyo-Rosales, J. E.; Lavado, R.; Floyd, E.; Schlenk, D.; Sedlak, D. L.: Identifying the causes of feminization of Chinook salmon in the Sacramento and San Joaquin river system. 5th Biennial CALFED Science Conference. Sacramento, CA, October 2008.

Peer reviewed publication. Lavado R., Loyo-Rosales J.E., Floyd E., Kolodziej E.P., Snyder S.A., Sedlak, D.L. and Schlenk D. (2009) Site-specific profiles of estrogenic activity in agricultural areas of California's inland waters *Environ. Sci. Technol* 43(24): 9110-9116.

Problems or Delays Encountered

A substantial delay in the project was encountered, as the official contract and Notice of Award was not completed until April 11, 2006. Therefore, a delay of approximately 15 weeks occurred after the official start date of January 1, 2006, before work on the project could actually begin. For this reason, all work on the project is approximately 15 weeks behind schedule. No samples were collected during the winter, wet season of 2006 as originally anticipated in the grant proposal. Also, given the uncertainty surrounding the completion date of the work contract, a delay in hiring project personnel at UC Riverside was experienced, somewhat delaying Tasks 1.2 and 1.3.

Additionally, the delay of the contract also stalled the purchase of the GC/MS system. An existing GC system was used for method development and the analysis of the first round of samples. However, this instrument required considerable repairs to achieve the sensitivity needed for trace steroid hormone analysis.

Chemical analysis of the samples was delayed during the second semester of 2006 due to the lack of a working instrument. Despite extensive repair work on the old GC/MS system, it was not possible to make it perform to the required levels; it was finally decided to halt the use of this instrument due to the high cost of further repairs. The new GC/MS system was installed in September 2006, but adapting the analytical methods to this instrument required more involved work than originally planned; additionally, due to a faulty electrical connection, the instrument did not perform optimally for several months.

Because of the sampling ban in the Delta and the low availability of fish, it was not possible to fulfill the original objective of Task 2.4, namely, to analyze the extent of feminization in non-salmonid fish in the watershed by collecting samples from a trawl. As discussed in the previous section, we have focused our attention on the collection of a native fish species, Mississippi Silverside, *Menidia beryllina*, which is easily caught near our sampling sites.

The stop work order, which was issued in December 2009 placed significant constraints on the project. After resumption of the project funding, considerable effort was needed to reestablish momentum because key team members either left for other jobs (i.e., Dr. Loyo-Rosales) or were assigned to other research projects (Dr. Lavado).

Personnel Changes:

Ramon Lavado joined the project in October 2006 as a post-doctoral researcher in UC Riverside. Dr. Lavado has been working in the adaptation and/or development of the *in vitro* and *in vivo* bioassays for the project.

Starting in January 2007, management of the project shifted to Jorge E. Loyo-Rosales, who joined the group in July 2006 as a post-doctoral researcher in UC Berkeley to work in the chemical analysis of (xeno)estrogens. The original manager, Dr. Edward P. Kolodziej, is now an Assistant Professor at the University of Nevada, Reno. Dr. Kolodziej, however, is still involved in the project as an external advisor. Dr. Loyo-Rosales left the University in December 31, 2008

for a faculty position at the Monterrey Institute of Technology in Mexico. Following his departure, laboratory activities at UC Berkeley became the responsibility of two doctoral students (D. Scott Mansell and Erika Houtz). Professor Sedlak is serving as the project manager.

Emily Floyd joined the project in November 2007 as a post-doctoral researcher in UC Riverside. Dr. Floyd conducted the bioassays for the project with Dr. Lavado. Emily Floyd left the project in the summer 2008 and Dr. Lavado continued as the main post-doctoral researcher at UC Riverside.

Scott Mansell, Erika Houtz and Suzanne Brandcer assisted on the project after the work resumed in 2009.

Contract Modifications

An internal transfer of funds ($38,000.00) within the grant was requested and approved in November 2007. The funds were transferred from the amount assigned to the sub-contractor conducting the sampling (AMS) to UC Riverside for additional laboratory analysis. This transfer allowed us to replace routine monitoring of all the sites with targeted sampling and additional TIE studies to identify the chemical agent(s) responsible for the estrogenicity observed in the bioassays and to provide additional information on the source of these compounds.

A budget readjustment was requested and granted in April 2010. The reallocation was made to provide resources to conduct outplant experiments and continue TIE experiments.

A no-cost extension was requested and granted in June 2010 to provide time to complete the *Menidia* outplants and complete analyses of bioassay samples.

Notes/Other

<u>Collaboration with parallel CALFED projects</u>

The tasks for this CALFED funded project are being conducted in collaboration with those of Dr. Bernie May, the principal investigator for the research proposal, "Are 'Apparent' Sex Reversed Chinook Salmon a Symptom of Genotoxicity?", which has been funded by CALFED. Dr. Kolodziej and Dr. Sedlak have been in contact with Drs. May and Williamson to discuss the details of research results regarding apparent sex-reversal of Central Valley Fall Chinook and other fish species. Unfortunately, during to scheduling constraints, Kevin Williamson was unable to attend the June 13, 2006 meeting. Dr. Williamson has been in contact with Dr. Kolodziej and has received a copy of the minutes of our first advisory board meeting.

Additional meetings of Dr. Kolodziej and Dr. Loyo-Rosales with Dr. Williamson occurred during the 4th Biennial CALFED Science Conference in Sacramento. Dr. Williamson's recent research supports their hypothesis that the 'apparent' sex-reversed salmon observed in the Sacramento-San Joaquin river system is a result of a genetic mutation rather than alteration of sexual differentiation caused by early exposure to (xeno)estrogens in the water.

Dr. Williamson was also present at the 2nd meeting with all the project's personnel. At this meeting, he presented the latest results from his research on the genetic causes of the apparent XY-female Chinook salmon in California. His results suggest that the altered sexual differentiation observed in the Chinook cannot be explained by exposure to endocrine disrupting chemicals. Its origin is either recombination of the Y-chromosome markers from the Y to the X chromosome, or even the complete loss of the markers from the Y chromosome.

A second collaboration involves the doctoral research of Susanne Brander, who is supervised by Professor Gary Cherr of UC Davis' Bodega Marine Laboratory. Professor Cherr is a member of our external advisory committee. Ms. Brander was awarded a CALFED fellowship to study endocrine disruption in the inland silverside (*Menidia beryllina*) using choriogenin production in males as a biomarker for estrogenic activity. As part of her research, Ms. Brander conducted laboratory testing of the response of the silverside to estrogenic contaminants, such as E2, and exposed the fish to water extracts from several of the sites where we have observed estrogenic activity. She also exposed silversides to water in the field by placing juvenile fish in mesh bags anchored at different locations in the watershed.

After the end of the stop-work order, we increased the collaboration with the Bodega Marine Lab group through our sub-contract to AMS. As part of these efforts, Susanne Brander collected samples from two of the sites where we had observed high signals in our fish bioassays. These collaborative efforts are detailed in the current progress report.

Collaboration with other groups

Besides the collaboration with Prof. Inge Werner from UC Davis mentioned above (Project Status - Task 2.1), our group also collaborated with Prof. P. Lee Ferguson at the University of South Carolina. Prof. Ferguson has developed receptor-affinity extraction methods for the analysis of (xeno)estrogens in water. In this technique, recombinant nuclear hormone receptors are immobilized on a solid phase; these receptors capture their respective ligands when water is passed through the solid phase. These ligands, (xeno)estrogens in this case, are then eluted and analyzed by mass spectrometry. Prof. Ferguson attempted to analyze samples from the Sacramento River in the Delta and the Napa River (sites 8 and 14; Fig. 1). However, his findings were inconclusive.

After the CALFED Science Conference in October 2008, our group started collaboration with Dr. David Crane's Water Pollution Control Laboratory at the California Department of Fish and Game. Their group analyzed approximately 30 of our archived samples, including the most estrogenic, for a series of over 50 different compounds including carbamate pesticides, phenylurea herbicides, organophosphate pesticides, surfactants, and triazines, among others.

REFERENCES

1. Purdom, C.E., et al., *Estrogenic effects of effluents from sewage treatment works*. Chemistry and Ecology, 1994. **8**(4): p. 275-285.
2. Harries, J.E., et al., *A survey of estrogenic activity in United Kingdom inland waters*. Environmental Toxicology and Chemistry, 1996. **15**(11): p. 1993-2002.
3. Jobling, S., et al., *Widespread sexual disruption in wild fish*. Environmental Science & Technology, 1998. **32**(17): p. 2498-2506.
4. Desbrow, C., et al., *Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening*. Environmental Science & Technology, 1998. **32**(11): p. 1549-1558.
5. Snyder, S.A., et al., *Identification and quantification of estrogen receptor agonists in wastewater effluents*. Environmental Science & Technology, 2001. **35**(18): p. 3620-3625.
6. Devlin, R.H. and Y. Nagahama, *Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences*. Aquaculture, 2002. **208**(3-4): p. 191-364.
7. Afonso, L.O.B., et al., *Y-chromosomal DNA markers for discrimination of chemical substance and effluent effects on sexual differentiation in salmon*. Environmental Health Perspectives, 2002. **110**(9): p. 881-887.
8. Huang, C.H. and D.L. Sedlak, *Analysis of estrogenic hormones in municipal wastewater effluent and surface water using enzyme-linked immunosorbent assay and gas chromatography/tandem mass spectrometry*. Environmental Toxicology and Chemistry, 2001. **20**(1): p. 133-139.
9. Hanselman, T.A., D.A. Graetz, and A.C. Wilkie, *Manure-borne estrogens as potential environmental contaminants: A review*. Environmental Science & Technology, 2003. **37**(24): p. 5471-5478.
10. Kolodziej, E.P., T. Harter, and D.L. Sedlak, *Dairy wastewater, aquaculture, and spawning fish as sources of steroid hormones in the aquatic environment*. Environmental Science & Technology, 2004. **38**(23): p. 6377-6384.
11. Kolodziej, E.P. and D.L. Sedlak, *Rangeland grazing as a source of steroid hormones to surface waters*. Environmental Science & Technology, 2007. **41**(10): p. 3514-3520.
12. Routledge, E.J., et al., *Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach*. Environmental Science & Technology, 1998. **32**(11): p. 1559-1565.
13. Jobling, S., et al., *Inhibition of testicular growth in rainbow trout (Oncorhynchus mykiss) exposed to estrogenic alkylphenolic chemicals*. Environmental Toxicology and Chemistry, 1996. **15**(2): p. 194-202.
14. Tyler, C.R., et al., *Metabolism and environmental degradation of pyrethroid insecticides produce compounds with endocrine activities*. Environmental Toxicology And Chemistry, 2000. **19**(4): p. 801-809.
15. Donohoe, R.M. and L.R. Curtis, *Estrogenic activity of chlordecone, o,p'-DDT and o,p'-DDE in juvenile rainbow trout: Induction of vitellogenesis and interaction with hepatic estrogen binding sites*. Aquatic Toxicology, 1996. **36**(1-2): p. 31-52.
16. Carlson, D.B. and D.E. Williams, *4-hydroxy-2 ',4 ',6 '-trichlorobiphenyl and 4-hydroxy-2 ',3 ',4 ',5 '-tetrachlorobiphenyl are estrogenic in rainbow trout*. Environmental Toxicology and Chemistry, 2001. **20**(2): p. 351-358.
17. Hamers, T., et al., *Estrogenic and esterase-inhibiting potency in rainwater in relation to pesticide concentrations, sampling season and location*. Environmental Pollution, 2003. **123**(1): p. 47-65.
18. Xie, L.T., et al., *Evaluation of estrogenic activities of aquatic herbicides and surfactants using an rainbow trout vitellogenin assay*. Toxicological Sciences, 2005. **87**(2): p. 391-398.
19. Weston, D.P., J. You, and M.J. Lydy, *Distribution and toxicity of sediment-associated pesticides in agriculture-dominated water bodies of California's Central Valley*. Environmental Science & Technology, 2004. **38**(10): p. 2752-2759.
20. Debruyn, A.M.H., M.G. Ikonomou, and F. Gobas, *Magnification and toxicity of PCBs, PCDDs, and PCDFs in upriver-migrating Pacific salmon*. Environmental Science & Technology, 2004. **38**(23): p. 6217-6224.
21. Kolodziej, E.P., J.L. Gray, and D.L. Sedlak, *Quantification of steroid hormones with pheromonal properties in municipal wastewater effluent*. Environmental Toxicology And Chemistry, 2003. **22**(11): p. 2622-2629.
22. Arsenault, J.T.M., et al., *Effects of water-borne 4-nonylphenol and 17 beta-estradiol exposures during parr-smolt transformation on growth and plasma IGF-I of Atlantic salmon (Salmo salar L.)*. Aquatic Toxicology, 2004. **66**(3): p. 255-265.

23. Milston, R.H., et al., *Short-term exposure of chinook salmon (Oncoryhnchus tshawytscha) to o,p '-DDE or DMSO during early life-history stages causes long-term humoral immunosuppression*. Environmental Health Perspectives, 2003. **111**(13): p. 1601-1607.
24. APHA, *Standard methods for the examination of water and wastewater,*, ed. A. Public, A.W.W.A. Health Association, Water, and E. Federation. Vol. 19. 1995, Washington, DC.
25. Carlson, D.B. and D.E. Williams, *Sex-specific vitellogenin production in immature rainbow trout*. Environmental Toxicology and Chemistry, 1999. **18**(10): p. 2361-2363.
26. Schlenk, D., et al., *In vivo bioassay-guided fractionation of marine sediment extracts from the Southern California Bight, USA, for estrogenic activity*. Environmental Toxicology and Chemistry, 2005. **24**(11): p. 2820-2826.
27. Rodriguez-Fuentes, G., et al., *Osmotic regulation of a novel flavin-containing monooxygenase in primary cultured cells from rainbow trout (Oncorhynchus mykiss)*. Drug Metabolism and Disposition, 2008. **36**(7): p. 1212-1217.
28. Green, P.G. and T.M. Young, *Loading of the herbicide diuron into the California water system*. Environmental Engineering Science, 2006. **23**(3): p. 545-551.

APPENDIX

Table A1. Water quality data for dry-weather water samples.

Site Month	pH	T (°C)	TSS (mg/L)	TOC (mg/L)	NO_3-N (mg/L)	DO (mg/L)	Conductivity (µS/cm)
Site 1							
July 2006	9.0	14.4	2.0	3.1	bdl	8	81
September 2006	8.0	13.5	9.6	4.0	bdl	8	92
November 2006	5.5	10.5	bdl	1.1	0.06	4	124
January 2007	6.0	10.0	0.2	1.1	0.05	11	104
March 2007	6.0	10.8	3.5	1.3	bdl	7	130
April 2007	6.0	12.4	3.2	1.1	bdl	7	109
August 2007	6.0	13.5	bdl	2.1	0.05	7	180
Site 2							
July 2006	6.5	20.8	2.0	2.6	bdl	7	112
September 2006	8.0	19.0	2.4	1.9	bdl	7	138
November 2006	7.5	8.7	7.8	1.1	0.08	8	123
January 2007	6.0	9.7	1.0	0.9	0.12	7	118
March 2007	6.0	11.5	2.5	1.2	0.15	7	111
April 2007	6.0	15.5	4.6	1.0	bdl	8	120
August 2007	6.0	21.2	3.9	2.2	bdl	8	157
Site 3							
July 2006	6.5	17.8	1.0	3.7	bdl	10	66
September 2006	7.5	17.0	3.2	2.7	bdl	6	67
November 2006	7.5	11.7	5.2	1.4	0.02	5	75
January 2007	6.0	10.4	0.8	1.5	bdl	9	70
March 2007	5.0	11.5	0.8	1.7	bdl	7	70
April 2007	6.0	15.0	1.3	1.1	bdl	8	74
August 2007	8.0	18.4	bdl	2.8	bdl	9	192
Site 4							
July 2006	7.5	20.5	2.2	3.2	bdl	10	62
September 2006	7.5	21.5	2.0	2.0	bdl	6	85
November 2006	7.8	7.0	7.8	0.9	bdl	6	71
January 2007	6.0	9.6	2.0	1.2	0.04	9	63
March 2007	5.0	12.0	3.2	1.9	bdl	8	72
April 2007	5.0	15.8	3.1	0.9	bdl	7	72
August 2007	6.0	21.2	2.9	3.4	bdl	7	81
Site 5							
July 2006	6.5	20.5	11.2	3.3	0.06	8	85
September 2006	7.5	18.0	12.6	2.7	bdl	6	80
November 2006	na	na	na	na	na	na	na
January 2007	na	na	na	na	na	na	na
March 2007	5.0	10.0	23.4	2.0	0.11	4	78
April 2007	na	na	na	na	na	na	na
August 2007	na	na	na	na	na	na	na
Site 6							
July 2006	6.5	20.5	29.2	2.7	0.09	7	118
September 2006	9.0	20.0	48.8	3.5	0.07	6	390
November 2006	N/A	7.7	15.2	2.5	0.18	3	149
January 2007	6.0	7.0	19.4	1.6	0.22	7	159
March 2007	6.0	11.3	31.6	1.5	0.25	7	158
April 2007	6.0	16.2	15.7	1.0	0.14	7	144
August 2007	6.0	18.5	18.2	2.2	bdl	7	125

bdl: below detection limit. na: not analyzed. **T**: temperature. **TSS**: total suspended solids. **TOC**: total organic carbon. **NO_3-N**: nitrates (as nitrogen). **DO**: dissolved oxygen.

Table A1. Water quality data for dry-weather water samples (cont'd)

Site Month	pH	T (°C)	TSS (mg/L)	TOC (mg/L)	NO_3-N (mg/L)	DO (mg/L)	Conductivity (µS/cm)
Site 7							
July 2006	7.5	18.3	1.4	2.9	bdl	8	35
September 2006	7.5	16.5	0.6	2.3	bdl	7	39
November 2006	8.0	9.8	3.0	37.8	0.02	7	46
January 2007	5.0	8.0	2.0	1.4	bdl	6	47
March 2007	na	na	na	na	na	na	na
April 2007	5.0	12.0	2.1	1.1	bdl	7	550
August 2007	5.5	19.2	3.1	3.4	bdl	7	52
Site 8							
July 2006	8.5	24.4	27.0	3.7	0.09	6	127
September 2006	8.5	20.0	7.8	3.6	0.08	6	166
November 2006	8.5	10.0	4.6	3.0	0.15	8	160
January 2007	6.0	8.0	14.0	2.1	0.23	8	154
March 2007	6.0	10.5	24.5	2.4	0.19	8	120
April 2007	6.0	16.8	17.5	1.2	0.16	7	133
August 2007	6.0	21.1	16.8	3.9	0.02	6	148
Site 9							
July 2006	6.5	18.3	7.8	3.8	bdl	7	41
September 2006	7.5	15.5	4.8	2.8	0.04	5	28
November 2006	7.5	12.7	31.4	1.9	bdl	4	30
January 2007	5.0	9.8	2.0	1.7	bdl	6	28
March 2007	5.0	10.5	8.8	1.9	bdl	6	30
April 2007	5.0	12.5	2.6	1.2	bdl	6	32
August 2007	5.5	16	3.8	4.6	bdl	6	36
Site 10							
July 2006	7.5	16.4	9.6	3.3	0.04	8	44
September 2006	7.5	16.0	4.0	2.8	bdl	7	50
November 2006	7.5	11.5	1.2	1.7	0.07	4	48
January 2007	5.0	9.0	0.2	1.8	0.08	7	51
March 2007	5.5	11.9	2.6	2.2	0.15	6	65
April 2007	5.0	12.5	0.9	1.2	bdl	6	48
August 2007	5.5	15.3	1.7	3.9	0.065	7	48
Site 11							
July 2006	8.5	25.5	81.0	4.8	0.47	8	410
September 2006	9.0	22.0	48.4	3.9	1.66	6	496
November 2006	8.0	11.2	31.2	3.8	2.30	5	898
January 2007	6.0	7.5	21.0	3.5	2.33	5	889
March 2007	7.0	15.6	60.4	4.0	2.02	7	928
April 2007	7.0	17.5	46.7	1.7	1.94	6	963
August 2007	7.0	21.9	52.5	9.4	3.2	7	1071
Site 12							
July 2006	8.0	17.5	1.6	3.0	bdl	8	34
September 2006	8.0	19.0	0.8	2.6	0.04	9	35
November 2006	7.5	11.5	2.8	1.6	0.04	6	37
January 2007	5.0	8.5	0.2	1.6	0.04	5	40
March 2007	5.0	13.0	2.6	1.6	bdl	6	45
April 2007	6.0	14.0	3.0	1.1	bdl	7	47
August 2007	7.0	24.5	2.8	3.8	bdl	7	60
Site 13							
July 2006	8.0	24.7	14.4	3.6	1.90	5	171
September 2006	8.0	22.0	3.6	2.8	0.64	6	79
November 2006	8.5	11.5	2.8	1.7	2.38	9	197
January 2007	5.0	7.0	0.2	1.7	2.09	5	143
March 2007	6.0	15.0	11.0	2.3	1.55	6	150
April 2007	6.5	17.3	14.5	1.2	2.18	7	178
August 2007	8.0	22.5	3.5	5.5	3.1	7	325

bdl: below detection limit. na: not analyzed. **T**: temperature. **TSS**: total suspended solids. **TOC**: total organic carbon. **NO_3-N**: nitrates (as nitrogen). **DO**: dissolved oxygen.

Table A1. Water quality data for dry-weather water samples (cont'd)

Site Month	pH	T (°C)	TSS (mg/L)	TOC (mg/L)	NO_3-N (mg/L)	DO (mg/L)	Conductivity (µS/cm)
Site 14							
July 2006	8.5	21.6	23.0	3.8	0.11	7	470
September 2006	8.5	21.0	23.6	6.0	bdl	6	>1999
November 2006	8.0	10.0	29.6	8.6	0.45	3	>1999
January 2007	6.0	8.2	4.2	2.4	0.80	6	425
March 2007	5.0	12.5	9.8	2.3	0.63	6	216
April 2007	7.0	15.0	4.3	1.4	0.26	7	343
August 2007	7.6	20.6	38.0	12.7	na	7	>1999
Site 15							
July 2006	8.0	24.4	15.4	4.5	0.28	5	190
September 2006	9.0	22.5	8.6	3.3	0.21	6	254
November 2006	9.0	11.5	8.2	2.6	0.56	7	341
January 2007	6.0	7.0	16.6	3.5	0.84	9	549
March 2007	7.0	14.0	6.2	5.1	0.96	8	383
April 2007	7.0	16.3	5.2	2.0	0.86	7	334
August 2007	7.0	21.2	10.7	5.6	0.23	6	481
Site 17							
July 2006	na	na	na	na	na	na	na
September 2006	na	na	na	na	na	na	na
November 2006	5.5	8.1	1.6	1.3	bdl	8	97
January 2007	6.0	7.5	0.0	0.9	bdl	7	79
March 2007	6.0	11.9	3.0	1.2	bdl	7	83
April 2007	6.0	14.9	5.4	0.9	bdl	7	79
August 2007	6.0	25.3	6.3	2.4	bdl	7	117

bdl: below detection limit. na: not analyzed. **T**: temperature. **TSS**: total suspended solids. **TOC**: total organic carbon. **NO_3-N**: nitrates (as nitrogen). **DO**: dissolved oxygen.

Table A2. Water quality data for storm event samples (northern sites)

Site Date	pH	T (°C)	TSS (mg/L)	TOC (mg/L)	NO_3-N (mg/L)	DO (mg/L)	Conductivity (µS/cm)
Site 3							
February 9, 2007 (before)	5.0	9.5	3.6	1.6	bdl	8	70
February 12, 2007 (after)	6.0	9.8	4.8	1.8	bdl	6	68
December 19, 2007 (before)	7.4	9.4	2.8	0.94	bql	6	88
December 21, 2007 (after)	7.8	6.5	4.1	1.2	bql	na	83
Site 4							
February 9, 2007 (before)	5.0	10.0	2.2	1.2	bdl	8	83
February 12, 2007 (after)	6.0	9.0	21.4	2.8	0.12	7	66
December 19, 2007 (before)	na	na	na	na	na	na	na
December 21, 2007 (after)	7.8	6.3	8.3	na	0.07	9	74
Site 6							
February 9, 2007 (before)	6.0	11.5	13.2	1.4	0.14	7	169
February 12, 2007 (after)	5.0	12.5	241.4	4.3	0.16	8	78
December 19, 2007 (before)	8	9.6	23	1.1	bdl	3	180
December 21, 2007 (after)	8.1	8.6	36	1.8	bdl	7	170
Site 8							
February 9, 2007 (before)	6.0	11.0	11.2	1.7	0.12	8	177
February 12, 2007 (after)	6.0	10.5	144.0	2.4	0.13	7	166
December 19, 2007 (before)	7.6	8.5	13	1.9	bdl	7	195
December 21, 2007 (after)	7.7	7.8	10	3	bdl	8	199
Site 17							
February 9, 2007 (before)	5.0	9.5	57.2	1.9	0.07	7	84
February 12, 2007 (after)	6.0	9.5	5.4	2.5	0.03	6	61
December 19, 2007 (before)	8	7.8	8.7	2	bql	4	93
December 21, 2007 (after)	8.2	3.8	3.7	1.4	bdl	4	86

bdl: below detection limit. na: not analyzed. **T**: temperature. **TSS**: total suspended solids. **TOC**: total organic carbon. **NO_3-N**: nitrates (as nitrogen). **DO**: dissolved oxygen.

Table A3. Water quality data for storm event samples (southern sites)

Site Date	pH	T (°C)	TSS (mg/L)	TOC (mg/L)	NO_3-N (mg/L)	DO (mg/L)	Conductivity (µS/cm)
Site 10							
January 4, 2008 (before)	6	9.5	1.1	1.2	bdl	5	52
January 7, 2008 (after)	6	9.3	20	2.7	0.25	5	55
Site 11							
January 4, 2008 (before)	6	8.6	23	2.7	1.25	7	1074
January 7, 2008 (after)	6	7.5	130	5.5	0.84	5	691
Site 12							
January 4, 2008 (before)	5	9.5	3.2	1.4	bql	8	56
January 7, 2008 (after)	5	9.5	46	4.6	0.38	5	81
Site 13							
January 4, 2008 (before)	6	8.4	3.4	1.4	0.21	5	53
January 7, 2008 (after)	6	8.8	520	10	0.17	5	89
Site 15							
January 4, 2008 (before)	7.9	8	27	2.3	0.24	8	656
January 7, 2008 (after)	na	na	na	na	na	na	na

bdl: below detection limit. na: not analyzed. **T**: temperature. **TSS**: total suspended solids. **TOC**: total organic carbon. **NO_3-N**: nitrates (as nitrogen). **DO**: dissolved oxygen.

Table A4. Sampling sites in the Sacramento/San Joaquin Delta

Site ID	*Location*	*Latitude*	*Longitude*
340	Napa River @ Delta	38-05'-51"N	122-15'-43.9"W
405	Carquinez Strait, just west of Benicia army dock.	38-02'-22.9"N	122-09'-01.8"W
508	Suisun Bay, off Chipps Island, opposite Sac. North ferry slip.	38-02-'43.8"N	121-55'-07.7"W
602	Grizzly Bay, northeast of Suisun Slough at Dolphin.	38-06'-50.4"N	122-02'-46.3"W
711	Sacramento River at the tip of Grand Island.	38-10'43.7"N	121-39'-55.1"W
815	San Joaquin River		
902	Old River at mouth of Holland Cut.	38-01'-09.1"N	121-34'-55.9"W
915	Old River-Western arm at railroad bridge.	37-56'-33"N	121-33'-48.6"W

Table A5. Steroid hormones and APEs in the Sacramento/San Joaquin Delta sites.

Site	17α-E2 (ng/L)	17β-E2 (ng/L)	E1 (ng/L)	E3 (ng/L)	P (ng/L)	MP (ng/L)	T (ng/L)	AD (ng/L)	NP (ng/L)	NP1EO (ng/L)	NP2EO (ng/L)	OP (ng/L)	OP1EO (ng/L)	OP2EO (ng/L)
340	bdl	bql	2.0	bdl	bdl	bdl	bdl	bdl	15	39	39	4.8	6.2	4.3
405	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	45	bdl	bdl	4.6	bdl	bdl
508	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	51	bdl	bdl	5.7	bdl	bdl
602	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	46	123	bdl	4.9	20	bdl
711	bdl	bql	bql	bql	bdl	bdl	bdl	bdl	bdl	bdl	bdl	13	bdl	bdl
815	bdl	bdl	bql	bdl	bdl	bdl	bdl	bdl	90	227	379	4.7	23	61
902	bdl	bdl	bql	bdl	bdl	bdl	bdl	bdl	49	bdl	bdl	3.6	bdl	bdl
915	bdl	bdl	bql	bdl	bdl	bdl	bdl	bdl	45	bdl	bdl	3.9	bdl	bdl

bdl: below detection limit. na: not analyzed. **17α-E2**: 17α-estradiol. **17β-E2**: 17β-estradiol. **E1**: estrone. **E3**: estriol. **P**: progesterone. **MP**: medroxyprogesterone. **T**: testosterone. **AD**: androstenedione. **NP**: nonylphenol. **NP1EO**: nonylphenol monoethoxylate. **NP2EO**: nonylphenol di-ethoxylate. **OP**: octylphenol. **OP1EO**: octylphenol monoethoxylate. **OP2EO**: octylphenol di-ethoxylate.

Table A6. EEQs in in the Sacramento/San Joaquin Delta sites by *in vitro* and *in vivo* bioassays (mean ± SD).

Site	**EEQ *in vitro* (ng/L)**	**EEQ *in vivo* (µg/kg wet wt.)**
340	0.032 ± 0.005	0.90 ± 0.03
405	0.022 ± 0.007	26 ± 4.0
508	0.014 ± 0.004	bdl
602	0.022 ± 0.003	1.0 ± 0.10
711	bdl	13 ± 1.7
815	0.033 ± 0.006	0.80 ± 0.12
902	0.032 ± 0.019	bdl
915	bdl	2.0 ± 0.32

bdl: below detection limit.