Integrated Environmental Assessment and Management — Volume 9999, Number 9999—pp. 1–15
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Complex Watersheds, Collaborative Teams: Assessing Pollutant Presence and Effects in the San Francisco Delta

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(Submitted 23 October 2014; Returned for Revision 19 December 2014; Accepted 23 February 2015)

ABSTRACT

There is a great diversity of sources of chemical contaminants and stressors over large geographic areas. Chemical contaminant inputs and magnitude can potentially exhibit wide seasonal variation over large geographic areas. Together, these factors make linking exposure to monitored chemical contaminants and effects difficult. In practice, this linkage typically relies on relatively limited chemical occurrence data loosely coupled with individual effects, and population- or community-level assessments. Increased discriminatory power may be gained by approaching watershed level assessment in a more holistic manner, drawing from a number of disciplines that target endpoints spanning levels of the biological hierarchy. Using the Sacramento River as a case study, the present study aimed to 1) evaluate the performance of new analytical and biomarker tools in a real world setting and their potential for linking occurrence and effect; 2) characterize the effects of geographic and temporal variability through the integration of suborganismal, tissue, and individual level endpoints, as well as extensive chemical analyses; 3) identify knowledge gaps and research needs that limit the implementation of this holistic approach; and 4) provide an experimental design workflow for these types of assessments. Sites were selected to target inputs into the Sacramento River as it transitions from an agricultural to a mixed but primarily urban landscape. Chemical analyses were conducted on surface water samples at each site in both the spring and fall for pesticides, hormones, and active pharmaceutical ingredients (APIs). Active pharmaceutical ingredients were more often detected across sampling events in the fall; however, at the most downstream site the number of analytes detected and their concentrations were greater in the spring, which may be due to seasonal differences in rainfall. Changes in gene and protein expression targeting endocrine and reproductive effects were observed within each sampling event; however, they were inconsistent across seasons. Larval mortality at the most downstream site was seen in both seasons; however, behavioral changes were only observed in the spring. No clear linkages of specific analyte exposure to biological response were observed, nor were linkages across biological levels of organization. This failure may have resulted from limitations of the scope of molecular endpoints used, inconsistent timing of exposure, or discordance of analytical chemistry through grab sampling and longer term, integrative exposure. Together, results indicate a complicated view of the watershed. *Integr Environ Assess Manag* 2015;1–15. © 2015 SETAC

Keywords: Alternative endpoints Biomarkers Gene expression Integrated assessment Watershed assessment

INTRODUCTION

Densely populated urban areas are known sources of a great diversity of environmental contaminants. These areas are continuing to expand as populations increase, putting sources of contamination in closer proximity to traditionally remote agricultural areas. This has resulted in aquatic life being exposed to a wider array of contaminants that can produce adverse responses individually or in combinations. Consequently, identifying causative agents for ecological impairment has become increasingly difficult. Further adding to the complexity of real world exposures is the temporal variability and seasonal shifts in contaminant load associated with specific sources, such as wastewater treatment plant (WWTP) effluent, and urban storm water and agricultural nonpoint source runoff. This variability has been observed on different temporal scales from daily (Martinovic et al. 2008) to seasonal (Epstein et al. 2000; Loraine and Pettigrove 2006; Sui et al. 2011). The overall complexity of present day watersheds necessitates holistic and/or systemic approaches, which integrate a larger number of disciplines or endpoints to more fully and accurately identify causative stressors.

Traditional toxicological assessments generally rely on one or a few scientific disciplines when trying to establish relationships between the occurrence of chemical stressors and observed impairment within a watershed. Generally, to characterize exposure, these efforts rely heavily on chemical analyses of water samples taken within a watershed of interest. These data are considered within the context of effects, which often consist of broad assessments of ecological quality based

All Supplemental Data may be found in the online version of this article.

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Published online 16 March 2015 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/ieam.1633

on community structure, individual survival, and sublethal effects (e.g., growth or reproduction). A large number of individual stressors or combinations of stressors can produce alterations of these common effects endpoints, often making it difficult to link exposure measurements to observed effects. The addition of alternative, biologically based endpoints may aid in linking contaminant exposures and adverse biological effects but only in as much as they add information on a scale different from that of toxicological measures traditionally relied on.

Endpoints targeting different biological levels may provide clearer pictures of the stressor potential and their resulting impact on the biota, as well other relevant parameters such as the length and intensity of exposure. Through the monitoring of suites of molecular to organismal biomarkers, which complement other diagnostic tools such as analytical chemistry and traditional toxicity endpoints, it may be possible to more closely link exposure to effect. For example, evaluation of effects of environmental stress at several levels of biological organization, from molecular processes up to growth and reproduction, can be related to alterations in population size and community composition (Miller et al. 2007).

Another complicating factor in establishing causal relationships is that sampling, often due to resource limitations, is generally conducted on a limited number of sites, dispersed over a large geographic area, with seasonality frequently ignored as an exposure factor. These types of sampling efforts may be subject to considerable interpretive error, as significant variability in pollutant load is expected when sampling is limited temporally and spatially (Droppo and Jaskot 1995). Observations in both local and deployed organisms may help to avoid errors resulting from temporal and spatial variation in ecological assessments. Although local organisms have the most obvious relevance to chemical occurrence or observed perturbations within a particular watershed, their measurement may lead to confusing results as they have been exposed over their life cycle and may have large habitat ranges. In contrast, the use of deployed model organisms, which are fixed in space, can be used to integrate all biologically relevant exposures that occur during the duration of deployment. These data, in turn, can be used to better understand the toxicological relevance of chemical occurrence measurements. Finally, targeting multiple life stages may also be useful, as early life stages may be more sensitive to chemical stressors than the adult stage.

The current study exemplifies an approach to integrate the exposure and effects of numerous contaminants (over time and space) for both well-known and uncharacterized compounds, such as pharmaceuticals, which lack adequate ecotoxicological information to accurately gauge their potential impact on aquatic organisms. We have employed biological endpoints in model fish in both adult and early life stages that target multiple biological levels. In addition, using chemical analyses, we have catalogued the occurrence and magnitude of contaminants over a wide array of chemical classes including pharmaceuticals, hormones, and pesticides. This study also documented the advantages and difficulties in integrating data from multiple disciplines to provide insight for future studies of complex aquatic ecosystems.

The San Francisco-Delta Estuary, selected as the watershed for the study, is highly complex and variable, with rivers and channels of the Delta being a major source of drinking water for more than 22 million people in California. Climatically, precipitation is unevenly distributed throughout the year with most of the annual precipitation occurring from November through April (wet months). Overlaying this hydrologically complex system are other factors such as infiltration of seawater, phytoplankton growth and decay, runoff from agricultural practices, point source effluent discharges, and storm water inputs. The upper portion of the Delta, the Sacramento Valley, supports a diverse agricultural economy, much of which depends on the availability of irrigation water. More than 2 million acres are irrigated, with major crops including rice, fruits, and nuts, and row crops including tomatoes, sugar beets, corn, alfalfa, and wheat.

In 2004, 4 pelagic fish species declined drastically in numbers compared to historical fluctuations in the San Francisco-Delta Estuary (Delta), termed the pelagic organism decline (POD). The potential role of contaminants, either singly or in combination with other stressors (i.e., habitat deterioration, loss of productivity, invasive species, and hydrology changes) has not been fully characterized. Uncertainty arises from the number and variety of potential stressor sources within the watershed. Significant seasonal toxicity has been observed in the system, especially in the lower Sacramento River (Werner et al. 2010). Changing use patterns of pesticides in the late 1990s and 2000s in the agricultural and urban watersheds of the Delta have continued to point to pesticides as a potential and changing stressor in the decline. Clearly, with these highly complex systems, a need exists to fully characterize exposure and effects.

MATERIALS AND METHODS

Sampling sites

Site selection was designed to capture different contaminants entering into the Sacramento River from identified sources or land use practices throughout the watershed (Table 1). Generally, sampling sites were arranged in a linear fashion so that exposure in more downstream sites was cumulative for chemicals entering the river upstream (Figure 1). Upstream of the Sacramento Metropolitan area, 2 sites were selected, Colusa Basin Drain (CBD) and Veterans Bridge (VB). Colusa Basin Drain was the most upstream site and comprises primarily nonpoint (i.e., agricultural) runoff. It is located 20 miles upstream of the VB sampling site, which was just upstream of influences from the Sacramento metropolitan area. Additionally, the VB site integrates both the inputs from CBD and the Feather River, which are primarily agricultural influences. Both Garcia Bend (GB) and Hood were located on the Sacramento River downstream of the Sacramento metropolitan drainage area. GB and Hood bracket input from a major WWTP effluent (design is 181 million gallons per day). Hood, the most downstream site, should ideally integrate contaminant inputs from all upstream sites, as this site includes all the upstream agricultural and all the urban stormwater runoff and effluent from a major WWTP (~3 miles downstream of the effluent discharge). Most importantly, this site is a California Department of Water Resources (CDWR), real-time monitoring station.

Temperature and discharge data were not available for the selected dates from the Hood monitoring station, thus these data were taken from United States Geological Survey (USGS) monitoring station (station ID 11447650). This station is located between the GB and Hood sites.

Table 1. Site characteristics for the 4 study sites along the Sacramento River, CA

Site (latitude/longitude)	Water sources	Seasonal factors	Chemical analysis	Biological analysis
CBD (38.8121972/ −121.7743111)	Nonpoint integrator of upstream source water	Spring flush	OP; pyrethroids; hormones; pharmaceuticals	
VB (38.6724183/ −121.6250222)	Source water and agricultural drainage and Feather River	Summer/fall dry weather runoff	OP; pyrethroids; hormones; pharmaceuticals	
GB (38.4778916/−121.543325)	66% of urban drainage American River (above major WWTP input)	Summer/fall dry weather runoff	OP; pyrethroids; hormones; pharmaceuticals	
Hood (38.367675/ −121.5205083)	Downstream of Sacramento WWTP, full integration of river drainage	Integrator site	OP; pyrethroids; hormones; pharmaceuticals	Gene expression; plasma vitellogenin; histopathology; larval behavior

CBD = Colusa Basin Drain; GB = Garcia Bend; OP = organophosphate pesticides; WWTP = wastewater treatment plant; VB = Veterans Bridge.
Sites are listed from upstream to downstream.

Chemical analysis

Sampling procedures. Water samples were collected from 4 sites in the Sacramento River watershed via grab sampling. Sampling occurred 6 days after each 1-wk fish exposure was initiated, and continued for the duration of the 4 weekly exposure periods (4 sampling events per season per site). For the organophosphate, pyrethroid, and hormone scans (conducted by the California Department of Fish and Wildlife [CDFW]), new, 1-L, I-Chem amber bottles were used for collection. For the hormones and pharmaceuticals (conducted by the US Environmental Protection Agency [USEPA]), new, 1-liter, silanized and 0.5-liter I-Chem amber bottles were used, respectively. All bottles were rinsed 3 times with sample water and then filled to the top (no head space) with surface water (see Supplemental Data Table S1 for a complete analyte list). When samples could not be collected by submerging the bottle directly in the waterbody, a stainless-steel bucket was used. Before rinsing and filling each bottle, the bucket was rinsed 3 times in sample water. After collection, samples were immediately stored on wet ice. All samples were left on ice and shipped overnight to the analytical facilities. Water chemistry measurements of temperature, pH, dissolved O_2, and specific conductance were taken at each site at the time of collection.

Mean concentrations were derived from all analyses with detectable concentrations. Water samples were analyzed both by USEPA and CDFW independently (see *Materials and Methods*) for the pharmaceuticals gemfibrozil, trimethoprim, sulfamethoxazole, and ibuprofen (March 2009 only). As the independent sets of measurements corresponded well, with the exception of gemfibrozil, all detects were averaged for these compounds for the respective sampling period. For gemfibrozil, results from both laboratories are reported.

CDFW analysis procedures. Determination of organophosphorus pesticides (OPs) and trace level synthetic pyrethroids in water samples was made using CDFW method 52 and 53.2, respectively. Briefly, a measured volume of sample (1000 mL) was extracted with methylene chloride (DCM) using a separatory funnel. The DCM extract was 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 iso-octane. For OP pesticides, the extracts were analyzed by gas chromatography using conditions that permit the separation and measurement of the target analytes in the extracts by Flame Photometric Detectors in phosphorous mode. Pyrethroid pesticides were analyzed using gas chromatography equipped with electron capture detectors.

Pharmaceuticals (Table S3) were extracted by CDFW from water and analyzed by liquid chromatography-tandem mass spectrometry (LC-MSMS) using a modified USEPA Method 1694 (USEPA 2007). Water samples were extracted at pH = 2 and pH = 7 using Solid Phase Extraction (SPE) following a modified EPA 3535A method. Extracts were filtered and analyzed following EPA 1694 guidelines using LC-MSMS (Agilent 6410 triple quadrupole MS/MS system coupled to an Agilent 1200 series LC system). The mass spectrometer was operated with atmospheric pressure electrospray ionization in positive and negative ion modes. The method detection limits range from 0.002 to 0.050 μg/L.

Water samples were extracted for analysis of carbamate pesticides using SPE following a modified EPA 3535A method. Extracts were filtered and analyzed following modified EPA 8318 method guidelines using LC-MSMS (Agilent 6410 triple quadrupole MS/MS system coupled to an Agilent 1200 series LC system).

USEPA ORD analysis procedure: API analysis. Samples were extracted and analyzed for 54 human prescription pharmaceuticals using a previously reported method (Batt et al. 2008). All samples were extracted within 2 days of collection and stored as extracts in silanized glass vials at −10 °C until analysis. A laboratory blank consisting of distilled water, a spiked distilled water control sample, and a matrix spike control sample were also included with each extraction batch along with the wastewater samples. 500 mL sample volumes were filtered through a 0.7 μm filter, 2 mL of a solution

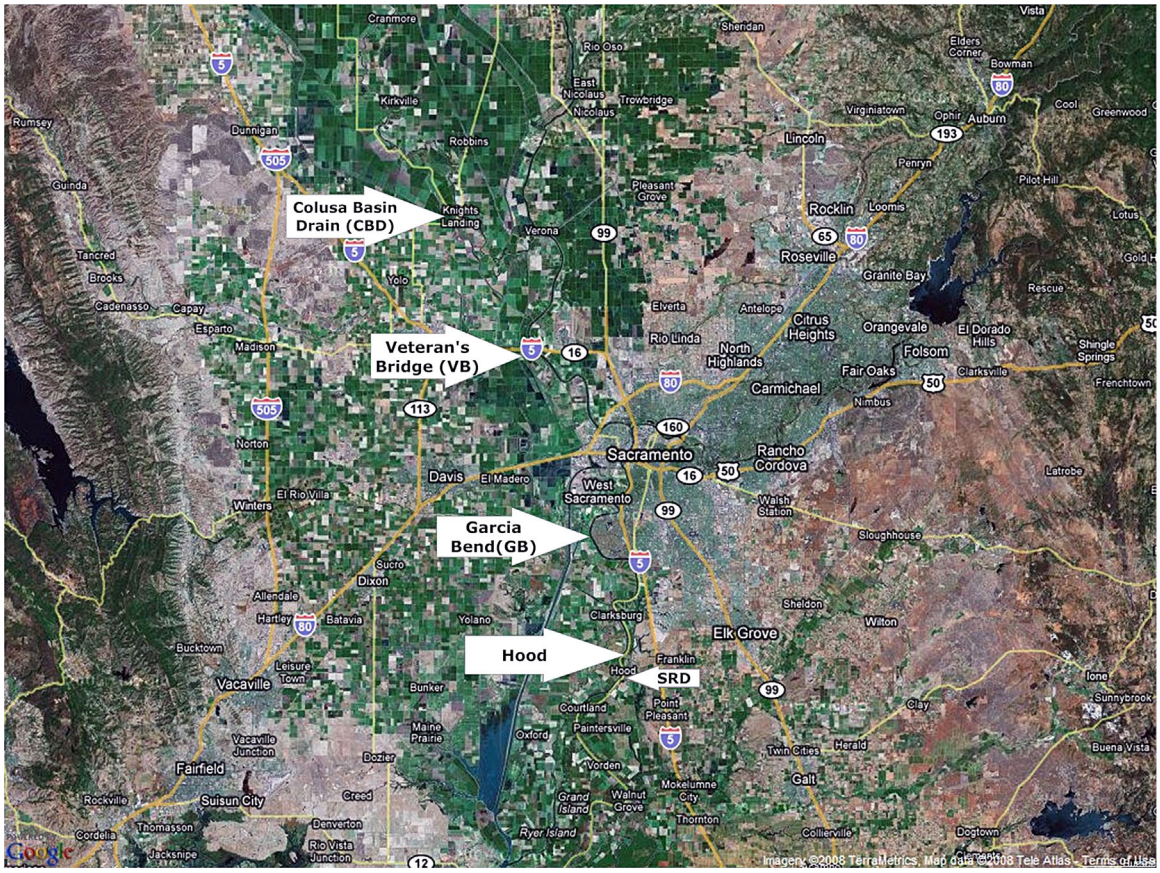

Figure 1. Location of sampling sites within the San Francisco-Delta Estuary.

containing 5.0 g L^{-1} of Na_2 EDTA and 25 mg L^{-1} of ascorbic acid was added, and samples were then spiked with respective isotopically labeled procedural internal standards (at a concentration of 1 μg L^{-1}) before extraction. Samples were passed through 150 mg Oasis HLB MCX cartridges at an unadjusted pH. Acidic and neutral analytes were eluted by acetonitrile and basic analytes were eluted by 95% acetonitrile and 5% ammonium hydroxide into separate silanized glass tubes. The extracts were then concentrated to dryness under a constant flow of N at 40 °C before reconstitution (20% acetonitrile for acidic and neutral analytes; 20% methanol for basic analytes) and reconstituted extracts were then transferred to polypropylene vials for immediate LC-MSMS analysis. Extracts were analyzed using a Waters Aquity ultra performance liquid chromatograph coupled to a Micromass Quattro Micro triple-quadrupole mass spectrometer with an electrospray ionization source operated using multiple reaction monitoring. Analytes were separated on a BEH (ethylene bridged hybrid) C18 column (1.0 × 100 mm 1.7 μm) equipped with 0.2 μm inline filter. Four separate injections were used to cover the range of analytes, with the LC-MSMS conditions being described in detail in (Batt et al. 2008).

USEPA ORD analysis procedure: hormone analysis. Samples were collected as described above. Samples were extracted and analyzed for hormones and steroids using SPE and gas chromatography-MS as described in Esperanza et al. (2004). Briefly, a field blank, laboratory blank, and a solvent blank were included with each batch. The 1-L sample volumes were extracted using superclean Envi-18 (Supelco) cartridges prepacked with 500 mg of solid phase material within 48 h of arrival (typically within 24 h). A total of 1% methanol and surrogates were added to all samples just before extraction. The cartridges were conditioned using 10 mL of methanol and 20 mL of distilled water, and samples were loaded in the cartridges at a flow rate between 5 and 10 mL/min. The cartridges were then washed with 20 mL of distilled water, vacuum dried for 15 min, and 10 mL of methanol were used to elute the analytes. The C-18 extracts were dried under a N stream while the tubes were submerged in a water bath at 40 °C. Extracts were reconstituted in 1 mL of 20% DCM in isooctane and cleaned with a second SPE method. Extracts were cleaned using 1 g SPE neutral alumina (Supelco) cartridges with 1 g of precleaned anhydrous Na_2SO_4 also being placed in the alumina tubes before their conditioning. The alumina cartridges were conditioned with 9 mL of 30% methanol in acetone and 9 mL of 20% DCM in isooctane. The C-18 extracts were transferred to the alumina cartridges, which were then washed with 9 mL of hexane and eluted with 9 mL of 30% methanol in acetone. The cleaned up extracts were then concentrated to approximately 1 mL under N and transferred to 2-mL reaction vials for derivatization with 50 μL of 15% methoxamine in pyridine, followed by 50 μL of pyridine and 100 μL of 10% trimethylchlorosilane in (N,O-bis(trimethylsilyl) trifluoroacetamide (Esperanza et al. 2004).

Biological analysis

Adult fish deployments. Fathead minnow (*Pimephales promelas*, FHM) were selected for this study due to their common use as a toxicological model organism. Animal handling was conducted according to Institutional Animal Care and Use Committee guidelines for the USEPA-AWBERC facility, with the exception of ice being used to anesthetize the fish. Because this work is partly focused on characterization of subtle subcellular changes, it was unclear how the use of the common fish anesthetic agent MS-222 would affect the overall gene expression profiles; thus to minimize interference, ice was used. Before each deployment season, up to 150 6-month-old (≈5 g) male FHMs were isolated in 400 L tanks, away from females for at least 3 weeks to avoid complications from potential background exposure to estrogens. Fish were maintained in isolation for the duration of the exposure period at 25 °C in a 16:8 h light:dark cycle and fed twice daily with dry trout chow and once daily with newly hatched brine shrimp.

Fish were deployed in 4 successive weekly deployments and a side-by-side monthly deployment between November and December 2008 and between March and April 2009, in a flow-through system housed within the Hood field station. Deployments and river access were obtained by the California Department of Water Resources, Environmental Water Quality Estuarine Studies Branch. Weekly, fish were shipped overnight from the USEPA fish culture facility in Cincinnati to California and deployed the next morning on arrival. Fish ($n = 12$–13 for the weekly exposures; $n = 15$ for the monthly) were exposed in a 37.5 L tank for week-long exposures and a 75.7 L tank for 1-mo exposures. Aquaria were housed within the Hood Field Station equipped with a flow-through system. Flow was set at 1.25 L/min at the beginning of each exposure, providing complete water changes every 20 min in the 37.5 L tanks and every 40 min in the 75.7 L tanks. However, depending on the sediment load, flow sometimes dropped to as low as 1.2 L/min by the end of 1 wk. In one instance, flow dropped to 0.8 L/min. Before each week-long exposure, the flow in all tanks was adjusted to 2 L/min and all debris and feces siphoned out. The week and month long exposures were conducted concurrently. Fish were not fed throughout the exposures; however, gut contents suggested that they were eating during the exposure.

Fish processing. Following shipping, time 0 fish ($n = 5$ per week) were immediately sacrificed and processed as described below. These fish represented "reference" fish for means of comparison. For the weekly exposures ($n = 12$–13 per week), fish were removed from the aquaria and mortality was assessed. For the month deployments ($n = 15$), dead fish were removed from the tanks weekly. Live fish were anesthetized on ice and euthanized through cervical dislocation. Brain tissue, half of each liver, and 1 testis were placed, separately, in 1.5 mL snap-cap tubes and flash frozen in liquid N for reverse transcription-quantitative polymerase chain reaction (RT-QPCR or QPCR). The remaining half of the liver and second testis were placed in cassettes and fixed in 10% neutral buffered formalin for 24 h for histological analysis.

Immediately before the sacrifice of the fish from the 1 month exposure, tails were severed posterior to the anal fin and blood was collected from the caudal veins into hematocrit tubes. Once blood flow had stopped, the contents of each tube was transferred to a 1 mL microcentrifuge tube and centrifuged for 5 min at 5000 rpm. After centrifugation, plasma was transferred to a clean tube and flash frozen in liquid N. Plasma samples were analyzed for the vitellogenin protein (VTG).

RT-QPCR. RNA was isolated from liver and vitellogenin (*vtg*) QPCR was conducted as detailed in (Biales et al. 2007). Growth hormone (GH; GI:50980341), insulin-like growth factor-1 (IGF-1; GI:46811843), and thyroid hormone receptor-alpha (THRa; GI:67773310) primers were designed based on existing FHM sequences in the National Center for Biotechnology Information (NCBI) nr database. QPCR for these genes were conducted in brain tissue only. Primer sequences were as follows: GH-Forward 5′–GGGATGTTTGGATGGT-CAAC-3′, GH-Reverse 5′-GCTCTCTCTGAGGCTGTTC-3′; IGF-1-Forward 5′-CAACGGCACACGGACATC-3′, IGF-1-Reverse 5′–CCTCGGCTTGAGTTCTTCTG-3′ THRa-Forward 5′-ATGACCCAGAGAGCGAGAC-3′, THRa-Reverse 5′-CAT-CAGACACCACTCCTAACC-3′, Cyp19-Forward 5′-CATG-CAGAAAAACTCGACCA, Cyp19-Reverse 5′-GCTCCGAC-CAGCTAAAACAG. Genes were selected based on their well-characterized and conserved roles in maintenance, health, and growth in vertebrate species. All genes were normalized to co-amplified 18S rRNA (Universal 18S, Ambion) and converted to relative values as detailed in Biales et al. (2007).

For weekly exposures, 1-sided 2 sample *t* tests were conducted between deployed fish and the paired time 0 fish to determine significance ($p < 0.05$). For *vtg*, a 1-sided *t* test was conducted as a decrease in *vtg* expression would not be expected for male fish. For monthly fish, an ANOVA was first conducted on weekly Time 0 fish to determine if differences existed from week to week. For *vtg* analysis, differences among controls were not observed, so weekly Time 0 values were pooled and a 1-sided *t* test was conducted. All statistical analyses were conducted using SAS statistical software (SAS Institute).

Plasma vitellogenin analysis. Plasma vitellogenin concentrations were measured via a competitive antibody-capture ELISA. The polyclonal anti-FHM vitellogenin (VTG) antiserum (1° antibody, Ab) was provided by Gerald LeBlanc (NC State University). The antiserum was produced in female New Zealand white rabbits by injecting plasma from estradiol-exposed FHMs. Specificity to VTG was obtained by incubating antiserum with plasma from unexposed male FHMs, followed by centrifugation to remove antibodies that recognized other plasma proteins (Parks et al. 1999). Standard VTG was purified by anion-exchange chromatography (Parks et al. 1999) from estradiol-exposed FHM. The standard curve was prepared as a 7-step, 2-fold serial dilution with a range of 4.8 μL/mL to 0.075 μL/mL. Microtiter wells were coated with the purified FHM VTG (200 μL of 600 ng/mL) in a coating buffer of 0.35 M sodium bicarbonate, 0.15 M sodium carbonate, pH 9.6. Plasma samples and standards were pre-incubated in microcentrifuge tubes at a 1:1 sample dilution to 1° Ab (1:20 000 final dilution) at 25 °C for 2 h. Just before the completion of the pre-incubation, microtiter plates were washed 3 times with wash buffer in an automated plate washer, then 200 μL of each preincubation mix (1° Ab + sample or standard) was loaded into the microtiter wells of the assay plate and incubated 1 h at room temperature. Plates were washed and incubated for 1 h at room temperature with 200 μL of horseradish peroxidase-labeled anti-rabbit IgG 2° Ab (Sigma). Plates were again washed and incubated with 200 μL of TMB

substrate (Sigma) for 15 min in the dark. Absorbance was read at 620 nm on a Multiskan EX (Thermo Electron). Standard curves were constructed and sample values calculated using the accompanying Multiskan Ascent software. The standard curves routinely produced r^2 values higher than 0.99. The lowest standard was periodically removed from the curve to maintain linearity. The samples were diluted 1:75, 1:825, and 1:7700 in 0.075 M PBS assay buffer, giving an assay quantitation range of 5.6 μg/mL to 37 mg/mL.

Histology. Following fixation, tissues were dehydrated in a series of ethanol and xylene baths before being embedded in paraffin. Embedded tissues were sectioned at approximately one-third and two-thirds of the depth of the organ (resulting in tissue slices ~100 μm apart) using a Reichert-Jung cassette microtome (4-μm sections). Sectioned tissues were stained using a standard hematoxylin and eosin counter stain protocol modified after Gabe (1976) and Carson (1997). A minimum of 10 histological sections were assessed and ranked on semi-quantitative scales (0–4) for vacuolization of the liver (0 no vacuoles visible, 1 <5% of total area, 2 vacuoles small but throughout image <25% of area, 3 broad presence of large vacuoles 25% to 50% of area, and 4 >50% of area vacuolated) and the presence/absence of eosinic staining/proteinaceous fluid. The developmental stage of the testis and the proliferation of interstitial cells between seminiferous tubules of the testis substructure were also evaluated.

Larval exposures. The larval exposure experiments followed closely published protocols (McGee et al. 2009; Painter et al. 2009). Posthatch FHM larvae (<24 h old) were obtained from the USEPA fish culture facility in Cincinnati. In November 2008, 1 group of 30 larvae ($n = 30$/treatment), and March 2009, groups of 20 larvae in duplicate ($n = 40$/treatment) were exposed for 12 d in 1 L Pyrex glass beakers at 21.6 ± 0.07 °C under constant photoperiod (16:8 h light:dark). Larvae were fed 2 mL of hatched brine shrimp twice daily and allowed to feed ad libitum. The exposure length was chosen to provide the shortest posthatch exposure window that would allow the filming of the animals immediately following exposure (animals were too small to be visible in the filming arena before day 12 posthatch). In November 2008, environmental samples and control well water were mixed to create solutions of 0% (Control), 25%, 75%, or 100% solutions of environmental sample water (pH Control = 8.26 ± 0.01; pH 25% = 8.28 ± 0.0; pH 75% = 8.38 ± 0.01; pH 100% = 8.44 ± 0.02). In March 2009, ammonia tests were performed on environmental samples immediately on arrival to the St. Cloud State University Aquatic Toxicology Laboratory (St. Cloud, MN) (0.23 ± 0.09 mg/L NH3-N, $n = 4$). Then, environmental samples and control well water were aerated for 24 h and then pH adjusted (pH 7.2) before mixing to create solutions of 0% (Control), 25%, 50%, 75%, or 100% solutions of environmental sample water. Static renewals (50%) were performed every 3 days during fall and spring exposure experiments. It should be noted that replacement water was only collected twice, thus was stored for some period of time before being used for renewal. It is unclear how storing samples would be expected to affect the overall level of available toxicants; however, several studies have demonstrated that there is at least some degradation of chemicals toxicants during storage.

After the 12-d exposures, the C-start performance of larvae was measured using a trigger-activated system with a small light-emitting diode (LED) and a vibrating electronic chip attached to the base of the filming arena to provide a stimulus. When activated, the system caused a short vibrational stimulus (<1 s) marked in the field of view by the appearance of the LED light used to determine time zero for data analyses. The filming arena consisted of a 5-cm diameter glass Petri dish positioned on top of a 1-mm grid. The larval escape behavior in the filming arena was recorded using a high-speed digital video camera (Redlake MotionScope M1) at 1000 frames per second. A larva was tested from each treatment in a sequential pattern (solvent control, high, medium, low, solvent control, high, etc.) until the behaviors of all surviving larvae were observed. High-speed video sequences of C-start behaviors were saved as .AVI files and analyzed using ImageJ software from the National Institutes of Health (http://rsbweb.nih.gov/ij/). For each larva, the anterior-most tip of the snout and posterior-most tip of the tail were marked in addition to 2 points representing the 1 mm grid. The resultant coordinate data were exported and used to calculate the time to induction of behavior (latency period), escape velocity (velocity during the first 40 ms after the initiation of an evasive maneuver; body length/ms to exclude any size differences as confounding factors among individual fish), and total escape response [body length/(latency in ms+40 ms)]. Videos were not considered if the latency response was less than 5 ms (false start). The actual sample size for latency period, escape velocity, and total escape response varied slightly between treatments due to factors such as survival, maximum allotted time for filming, and the number of videos not considered due to false starts.

RESULTS

Water quality measures

Water quality measures were not available from the Hood site during the sampling period; the reported measures were taken from a USGS real-time monitoring station located approximately 1 river mile upstream of the Hood site. November 2008 and March 2009 will henceforth be referred to as fall 2008 and spring 2009. During the fall 2008 deployments, temperatures decreased from a maximum daily temperature of 15.5 °C during week 1 to 12.5 °C in week 4 (Table 2). Temperatures increased during the spring 2009 sampling period from an average weekly maximum of 9.38 °C in the first week of sampling to 12.9 °C in the final week. Discharge was considerably less in the fall deployment (maximum weekly average = 9857.1 cubic feet per second [cfs] in week 1) relative to the spring 2009 period (maximum weekly average = 40 700 cfs). Though the discharge during the fall 2008 period was relatively constant across all 4 weeks, a drastic increase was observed from the first week to the second and then a similar drastic decrease from the third to the final week of the spring deployment (Table 2).

Chemical analysis

Concentrations of contaminants were measured 4 times each during fish deployments in fall 2008 and spring 2009. Mean concentrations were derived from all analyses with detectable concentrations summarized in Table 3 (specific data for each chemical method are presented in Tables S1, S2, and S3). Overall, more detections were recorded during the spring 2009 sampling period when compared to the fall 2008 sampling (80 vs 46 detects, counting only those compounds that were measured during both sampling periods). Few

Table 2. Water quality measures taken at USGS sampling site[a]

Week	Average maximum daily temperature (°C)	Average daily discharge (cfs)
1	15.5	9857
2	15.4	8505
3	13.7	7527
4	12.6	7885
5	9.4	28 700
6	12.6	36 942
7	12.1	40 700
8	12.9	22 500

[a]Station ID, 11447650.
cfs = cubic feet per second; USGS = United States Geological Survey.

pharmaceuticals were detected in water samples collected upstream of the City of Sacramento at the CBD and VB sites (Table 3). The number and concentrations of pharmaceuticals detected in Sacramento River samples generally increased at the GB site and were greatest at the Hood location, the integrating site for all downstream urban inputs, upstream agricultural inputs and the WWTP. Pesticide concentrations were low and varied between sites and sampling intervals with no apparent trend related to land use patterns.

Biological analysis

Adult fathead minnow exposures. Survival was high at the Hood sampling site for adult FHMs exposed weekly or for the entire month during the fall 2008 deployment (Table 4). The lowest observed survival rate was 83% following week 2. Similar results were observed during the spring 2009 sampling, with the lowest survival for the weekly exposures being 91% at week 2. For the spring 2009 month-long exposure, 5 of 15 fish died, 2 during week 1 and 3 during week 2, which is consistent with mortality observed in the concurrent weekly exposures.

Gene expression levels for GH, IGF, THR, and Cyp19A, were altered sporadically throughout both sampling periods. Both GH and IGF were shown to be upregulated in week 2 of the fall 2008. IGF was also upregulated during week 4 of this sampling period. Cyp19A was downregulated in the fall 2008 sampling and was not altered during the spring 2009 exposure. During week 4 of the spring deployment GH, IG-F and THRa were all upregulated indicative of potential endocrine effects.

Both vitellogenin protein and mRNA were measured from fish exposed at the Hood site. Unfortunately, the integrity of the fall 2008 plasma samples was compromised, thus no data was reported for this sampling event for VTG protein. In weeks 1 and 2 of the fall 2008 sampling, *vtg* mRNA levels were shown to be upregulated relative to the paired weekly controls. No observable increase in VTG protein levels was observed in the spring sampling for any weekly treatment or the month-long exposure. Few of either the Hood exposed samples or the paired single week controls displayed any VTG protein induction above the detection limit. This was largely reflected in the *vtg* mRNA levels for the same sampling period.

No gross abnormalities of the liver were observed. Vacuolization of hepatocytes were common in liver tissues from the month-long exposures from the Hood site and are suggestive of a high, general pollutant exposure. An analysis of liver tissues of fish exposed in either fall or spring exposures did not produce any statistically significant differences between the sites and/or baseline fish in the prominence of hepatocyte vacuoles (data not shown).

All analyzed gonadal tissues were determined to be male (testis). No gross abnormalities of the testis were observed. There were 2 perceived occurrences of intersex (ovarian tissues embedded in testis tissues). One baseline fish and 1 fish exposed for 1 month at Hood in spring 2009 exhibited tissues perceived as immature oogenic tissues. Some tissue samples from baseline fish, as well as fish exposed for 1 month at Hood, were found to contain inclusions in the testis tissue. The nature of these inclusions could not be determined but is likely of parasitic origin, which is not uncommon in fish exposed to natural waters. The majority of testis examined from both fall 2008 and spring 2009 FHMs exhibited all stages of spermatogenesis as would be expected in a proportional spawner.

Larval survival, growth, and escape performance. Larvae were exposed to 0%, 25%, 50%, 75%, or undiluted river water (100%) collected at the Hood sampling site in both the fall (no 50% treatment) and spring sampling periods. Significant mortality was experienced in the 25% and 100% river water exposures in fall and in the 25% river water during the spring exposures (Table 4, Figure 2).

No changes in growth were observed in larvae exposed to water collected at the Hood field station during the November sampling. However, in the spring sampling, growth was significantly reduced by approximately 10% at all dilutions greater than 25% of Hood water. The reduction in growth was accompanied by a significant trend of a concentration-dependent reduction in total escape performance ($p < 0.01$; Jonckheere–Terpstra test; Table 4, Figure 2) in the March larval exposures.

DISCUSSION

This study aimed to implement and evaluate a multidisciplinary approach for understanding the relationship between exposures and effects in a highly complex watershed. The approach used attempts to link an extensive list of chemical analyses with observations made on multiple biological levels. Biological endpoints consisted of traditional measures exposure and effect (mortality, histology), as well as less commonly used measures (gene expression, behavioral responses). This approach was applied to a real-world sampling situation in hopes of identifying potential contaminants, which vary in space and time, as an underlying cause of the observed decline in fish species. The discussion will focus first on the specific implementation within the Delta and will then discuss strengths and weaknesses with the approach and propose a framework for future characterization on complex watersheds.

POD in the Delta: a case study

Fall 2008 versus spring 2009. Seasonal differences in rainfall (fall flush vs winter/early spring flush events) may complicate identification of stressors, as these differences may shift the distribution and concentration of chemicals into the aquatic environment (Syed et al. 2003). To account for this, exposures to deployed organisms were conducted 2 times, once in fall 2008 and again in spring 2009, at the Hood field station. The

Table 3. Water chemistry analysis

	Fall 2008				Spring 2009			
Compound	**CBD**	**VB**	**GB**	**Hood**	**CBD**	**VB**	**GB**	**Hood**
Gemfibrozil[a] CDFW	<det	32 (4)	30.5 (2)	104 (4)	<det	<det	<det	21 (1)
Gemfibrozil[a] USEPA	<det	<det	<det	<det	<det	<det	<det	56 (2)
Triclosan[a]	<det	<det	<det	30.5 (2)	<det	<det	<det	det
Sulfamethoxazole[a]	<det	<det	<det	28.7 (3)	<det	14 (1)	13 (1)	18.5 (4)
Trimethoprim[a]	<det	<det	<det	22 (5)	<det	<det	det	6.2 (6)
Sulfadimethoxine[a]	<det	<det	<det	41.8 (5)	6 (2)	6.6 (5)	7 (4)	18.6 (8)
Caffeine[a]	NM	NM	NM	NM	det (3)	det (2)	det (2)	det (1)
Ibuprofen[a]	<det	<det	<det	<det	1507 (4)	934 (3)	1375 (2)	956 (2)
Carbadox[a]	NM	NM	NM	NM	<det	<det	5 (1)	<det
Sulfathiazole[a]	NM	NM	NM	NM	<det	<det	det	<det
Sulfamethazine[a]	NM	NM	NM	NM	<det	<det	13 (1)	<det
Oxytetracycline[a]	NM	NM	NM	NM	<det	<det	<det	300 (1)
10-hydroxy-amitriptilyne[a]	<det	<det	<det	<det	<det	<det	<det	0.5 (1)
Atenolol[a]	<det	3 (1)	<det	74 (3)	<det	1.4 (4)	1.4 (2)	34.8 (4)
Triamterene[a]	<det	<det	<det	2.9 (1)	<det	<det	<det	1.9 (1)
Metoprolol%[a]	<det	<det	<det	det	<det	<det	<det	det
Diltiazem[a]	<det	<det	<det	1.5 (3)	1.2 (1)	<det	<det	1.6 (2)
Carbamazepine[a]	<det	2.7 (1)	2.2 (1)	8.2 (1)	<det	2.2 (1)	2.7 (1)	6.4 (1)
Valsartan[a]	<det	<det	<det	98.5 (2)	5.9 (1)	8.1 (1)	12 (1)	88.5 (2)
Hydrochlorothiazide[a]	<det	<det	<det	<det	<det	<det	<det	23.8 (4)
Estrone (E1)[b]	0.8 (4)	NM	NM	0.8 (4)	0.8 (3)	NM	NM	0.5 (4)
17β-estradiol (E2)[b]	<det	NM	NM	<det	<det	NM	NM	<det
Estriol (E3)[b]	<det	NM	NM	<det	<det	NM	NM	<det
17α-ethynylestradiol (EE2)[b]	<det	NM	NM	<det	<det	NM	NM	<det
Progesterone	<det	<det	<det	<det	<det	<det	<det	<det
Testosterone[b]	<det	NM	NM	NM	NM	NM	NM	NM
Chlorpyrifos[c]	NM	NM	NM	NM	det	<det	<det	<det
Fenpropathrin[d]	NM	NM	NM	NM	9 (1)	<det	<det	<det
Bifenthrin[d]	NM	NM	NM	NM	<det	24 (1)	<det	11.5 (2)

[a]PPCP.
[b]Steroid hormone.
[c]OP pesticide.
[d]Pyrethroid.
CBD = Colusa Basin Drain; det = detected, but below quantification limit; GB = Garcia Bend; <det = below detection limit; NM - not measured; VB = Veterans Bridge.
Mean and number of detects (out of 4 measures each; in parenthesis) are given for all detected compounds sampled in November 2008 and/or March 2009. Most compounds were analyzed 4 times during each sampling period. All measurements in ng/L.

Table 4. Summary table of biological endpoints

	Fall 2008				Spring 2009			
Weekly analysis	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
Vtg—mRNA	↑	↑	—	—	—	—	—	—
GH	—	↑	—	—	↓	—	—	↑
IGF	—	↑	—	↑	↑	—	↑	↑
Thra	—	—	↓	—	—	—	↓	↑
Cyp19a	—	—	—	↓	—	—	—	—
Mortality—adult	0%	17%	0%	0%	0%	9%	0%	0%
Monthly analysis								
Vtg—mRNA	<DL	<DL	<DL	<DL	—	—	—	—
Vtg—protein	NM	NM	NM	NM	—	—	—	—
Mortality—adult	0	0	0	0	33%	33%	33%	33%
Mortality—larvae	↑ 25% and 100% effluent	↑ 25% and 100% effluent	↑ 25% and 100% effluent	↑ 25% and 100% effluent	↑ 25% effluent	↑ 25% effluent	↑ 25% effluent	↑ 25% effluent
Histology—liver	—	—	—	—	—	—	—	—
Histology—gonad	—	—	—	—	—	—	—	—
Behavioral (escape)	—	—	—	—	↓ Dose-dependent	↓ Dose-dependent	↓ Dose-dependent	↓ Dose-dependent

DL = at or below detection limit (1.9 μg/mL); NM = not measured.

fall 2008 exposure window was selected to capture fall drainage from the summer growing season and first flush runoff from impervious urban surfaces. The spring window was selected to capture dormant season pesticide applications, which coincides with an increased likelihood of rain events and resulting runoff, which was evidenced by a relatively high daily discharge rate. The Hood sampling site was selected for more intensive sampling because it is the most downstream site and is expected to act as an integrator site making it susceptible to seasonal shifts in runoff from both impervious surfaces and agricultural input, it resides in relatively close proximity to a WWTP discharge, it simplifies sampling logistics as it was available 24 h/d with minimization of vandalism; and it serves as a permanent real-time monitoring station of flow and water quality parameters such as temperature.

Seasonal differences in the number and concentrations of pollutants were observed between the fall 2008 and spring 2009 sampling events (Table 3). As expected, due to its relative position in the watershed, the Hood sampling site appeared to integrate exposure from upstream sources. A noticeable increase in the number of pollutants and their concentrations at the Hood site compared to all 3 upstream sites during both sampling periods was observed with chemical analyses (Table 3). The concentrations of urban contaminants tended to be higher in the fall 2008 sampling, though the overall number of pollutants detected was greater in spring 2009. Taken together, this suggests that the annual spring flush provides additional avenues for a greater number of compounds to enter the aquatic environment through agricultural and impervious surface runoff, whereas contaminant concentrations in fall 2008 sampling suggest a large influence of the seasonal differences in the dilutional capacity of the Sacramento River as a consequence of lower rainfall during the dry season (Table 2).

In addition to season differences associated with rainfall, temperature may also influence biological metabolism and/or toxicity of contaminants. This may be particularly important in understanding the relative role of pesticides. Both the pyrethroids and OP pesticides have been shown to exhibit temperature dependent toxicity and have been implicated as a drivers of aquatic toxicity (Casida et al. 1983; Weston et al. 2005, 2014; Laetz et al. 2014). Maximum daily temperature were on average 2.6 °C higher in the fall 2008 sampling period (Table 2). We observed seasonal differences in the diversity and magnitude of effects in both the successive week-long and corresponding month long exposures for both adults and larvae during the spring 2009 fish exposures relative to the fall 2008 sampling (Table 4). Importantly, we observed a decrease in the larval growth and alterations in larval predator avoidance performance. As the spring sampling coincided with juvenile developmental stages of many endemic fish species, these results may have particular relevance in that they may more directly translate to reduced survival in local populations.

Estrogenic exposures. In the fall 2008 sampling season, the relative impact of estrogenic compounds is unclear. We observed induction of *vtg* mRNA in the first 2 wk of the sampling season; however, using chemical analytical methods, we detected only low levels (0.8 ng/L) of a relatively weak estrogenic compound (E1) during week 1 of this same period. We detected no *vtg* mRNA induction at the month long exposure, which may simply reflect that relevant estrogenic exposures occurred early enough in the 4-wk exposure that *vtg* mRNA levels had returned to baseline (Hyndman et al. 2010).

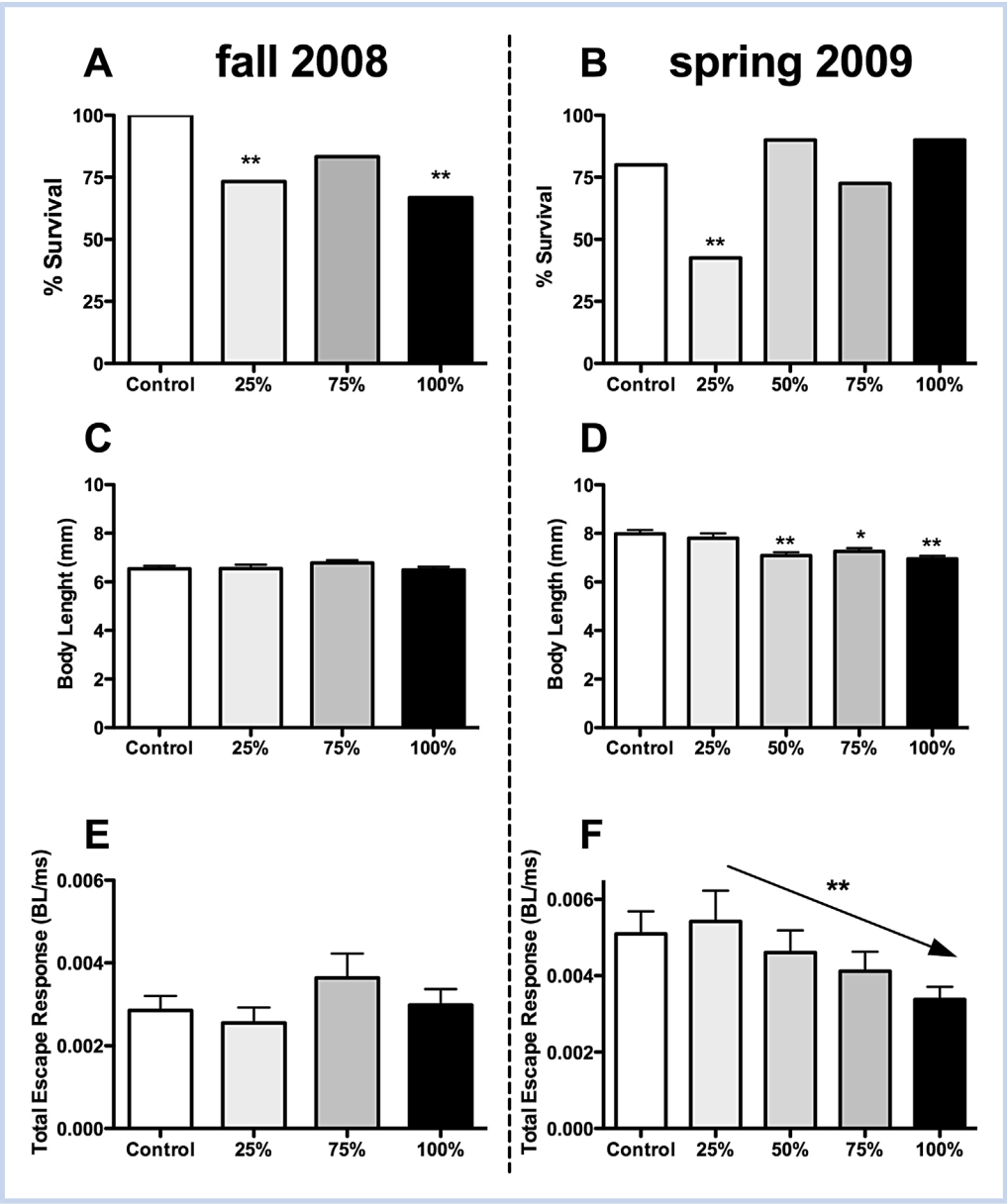

Figure 2. Mortality, growth, and escape responses of larvae exposed to dilutions of river water sampled from Hood sampling site in fall and spring. *Statistical significance at $*p < 0.05$, $**p < 0.01$.

Unfortunately, due to an equipment failure at the field site, we were unable to properly store and measure the vitellogenin protein in fish plasma from this sampling period. In contrast, the spring 2009 sampling provided more consistent results. We observed no induction of vitellogenin mRNA or protein over the spring study period, which is consistent with the relatively low concentrations of estrogenic compounds (E1, E2, and E3) measured. This may be expected, as dilution factors of urban discharge from upstream WWTP will increase with higher flow in the Sacramento River. As laboratory studies have documented *vtg* mRNA induction in estrogen exposed FHMs in as little as 24 hours (Biales et al. 2007), the limited induction of *vtg* mRNA in FHMs in fall 2008, even after a 7-day exposure, suggests that estrogenic activity was also low during this sampling event. However, the relatively low water temperature during both the fall and spring exposures cannot be ignored as a confounding factor, as low temperature has been shown to reduce both the mRNA and protein expression response in fish exposed to estrogens (Brian et al. 2008; Korner et al. 2008).

Histological analysis of livers and reproductive organs of month-long exposed FHMs during both sampling events also failed to identify pathologies previously linked to estrogenic exposure such as a reduction in mature sperm (Vajda et al. 2008) or the presence of ovarian tissues in the male testis (Jobling et al. 1998; Vajda et al. 2008; Hinck et al. 2009). The lack of histopathological findings, paucity of *vtg* mRNA induction, low plasma VTG concentrations in male fathead minnows, and low concentrations of estrogenic compounds in water samples all suggest that estrogenic activity in the Delta and receiving rivers is potentially minimal, at least during the 2, month-long sampling periods of this study.

Larval endpoints. When all endpoints—whether biological or chemical—are considered, a trend emerges where alterations were observed primarily in endpoints targeting larval stages. It

is unclear as to why this is; however, we have identified several potential explanations. Larval exposures consisted of 12-d static renewals, with renewals every 3 d, whereas adults were exposed in stream. Differences in these exposure scenarios make direct comparisons between larvae and adults difficult. Similar difficulties arise when attempting to compare the larval responses to the occurrence data, because larvae exposures did not use the same ambient water samples that were subject to chemical analysis. Larvae may also represent a more sensitive life stage (Kolpin et al. 2002; Panter et al. 2002; van Aerle et al. 2002; Schultz et al. 2012) and thus may respond to levels of toxicants that would not affect adults of the same species. Along these same lines, the behavioral endpoint used here only with the larvae, predator avoidance performance, may be more sensitive than endpoints observed in the adult, such as protein expression or mortality (for example see Schultz et al. [2012]).

Lessons learned—data gaps

In the current study, we have used a wide array of chemical and biological endpoints. Biological measurements targeted a number of biological levels, from single molecules (mRNA and proteins) to histological alterations of important tissues, to organismal level (mortality) and behavioral responses (predator avoidance). Generally, alterations of endpoints from the molecular initiating events through development of an adverse outcome can be related to the duration of exposure, where molecular changes would precede histological changes, which, in turn, would precede organismal changes and so forth. Thus by examining multiple biological levels in a single study, one can estimate both the duration and to some degree the intensity of exposure (Figure 3). Moreover, by considering differences in endpoints, i.e., short versus longer term endpoints, it should be possible to discriminate differences due to intensity from those due to duration, which may aid in identifying causes of impairment or determining risk.

In the current study, measurements were conducted in the context of known population-level effects manifest as declining numbers of 4 native fish species. Despite observations made across the biological hierarchy and the relatively inclusive list of chemical analytes, we were unable to identify a single causative agent for the POD. There are several potential reasons for our inability to identify causative agents, such as our focus solely on the water column. The current work and endpoints primarily focused on chemical stressors; however, the main drivers of fish declines may be nonchemical stressors such as habitat loss or elevated water temperature due to decreasing shade. Alternatively, fish declines could have been due to mixtures of multiple stressors, thus no single stressor would be identified as the driver. Finally, causative exposures may have occurred outside of the physical study area of this work. Even with those potential explanations, we have identified some clear data gaps that if addressed would greatly augment the application of this type of multidisciplinary approach to environmental issues. Below is a discussion of these data gaps as well as recommendations for future work.

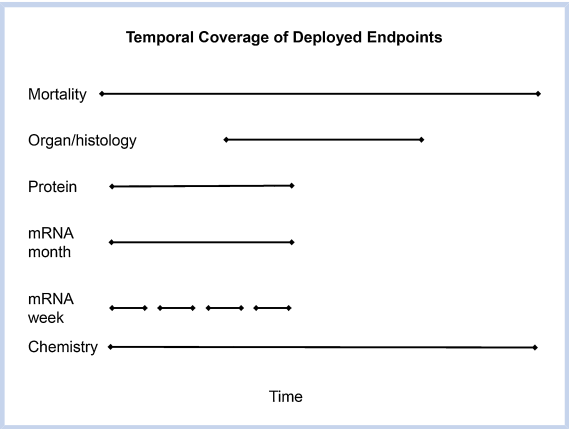

Figure 3. Relative time scales accounted for through endpoint analysis targeting different levels of biological hierarchy.

Need for additional validated molecular markers—increase number of chemicals and classes. In the case study, we assessed changes in gene and protein expression in hopes of identifying exposures and effects in important biological pathways. Although *vtg* mRNA is generally considered a reliable biomarker of estrogenic exposure, and there are several other single gene biomarkers of exposure to stressors, such as metallothioneins for metal exposure (Viarengo et al. 1999) and Cyp proteins for PAH exposures (Lee and Yang 2008), the total list of biomarkers relative to the number of stressors found in the environment is limited. To more effectively identify important exposures, there exists a great need to cover a larger portion of the total chemical stressor space with additional biomarkers. Further work is needed not only to increase the number of chemicals and chemical classes for which biomarkers exist, but to identify which chemicals are of greatest importance for biomarker development. Groupings of chemicals by mode of action (MOA), chemical class, or important biological axis may be an efficient way to quickly cover the total stressor space as opposed to a more traditional chemical by chemical approach. Moreover, by developing molecular markers targeting MOA or biological axes it will be increasingly possible to ascribe biological relevance to chemical data, as alterations of these types of biomarkers will potentially account for the total biological activity of mixture constituents and their interactions even if they were not anticipated based on structural analysis.

The increased sensitivity, technical reliability and reduced costs associated with global transcriptional platforms, such as microarrays, suggests that future molecular biomarkers will switch from single genes to suites of genes that change reliably in response to exposure of a given toxicant or class of toxicant. A number of manuscripts have been published in the recent years using microarrays or other global analysis platforms (Wang et al. 2008; Biales et al. 2011), which attempt to establish signatures of exposure for a number of toxicants. The use of microarrays in complicated field studies has been traditionally avoided due to the perceived high cost of these experiments; however, as additional multigene and single gene biomarkers are developed and validated, the relative cost of the microarray decreases proportionally, because there is no net cost increase with each additional biomarker. Thus, it is likely that ecological assessments in the future will include molecular-level biomarkers. Examples of where these global platforms are being used in field settings already exist (Sellin Jeffries et al. 2012).

An alternative approach to developing biomarkers for a specific chemical or group of chemicals would be to develop site-specific biomarkers. Here, contrasts can be conducted between reference and impaired sites or target known sources. These types of analyses can be used in combination with chemical analyses and land use information making it possible

to identify impairments or relevant exposures based on the expression profiles associated with each site. Ecological relevance of this type of approach could be validated through the use of chemical manipulations of the environmental samples as is conducted in either effects directed analysis or toxicity identification evaluations (Burgess et al. 2011).

Need for validated molecular markers in additional species. Microarrays have been developed for an increasing number of model and nonmodel species, making them more applicable to a wider range of environmental conditions. Having this tool available in a number of species will aid in understanding the nature of the exposure and for making linkages between the model, deployed organisms, and the native fish. That being said, the majority of nonmodel species lack sufficient characterization on the molecular level to be useful for the development microarrays in the short term. Although, with recent advances and decreasing costs associated with next generation sequencing (NGS), this number is likely to increase rapidly. Moreover, the emerging use of NGS for transcriptional profiling may side step the issue of poor characterization entirely, as this experimental platform does not rely on the a priori development of hybridization probes (Wang et al. 2009).

Use of local fish. In the present study, we looked for biological responses in deployed fish only. Although an effort was made to make deployments as comprehensive as possible, accounting for seasonal differences as well as integrating exposure over varying time scales, it is still unlikely that these captured a true representation of all of the exposures that occur over an organism's lifetime. Deployed fish are spatially fixed and may simply miss an exposure, as it has been shown that exposures can be heterogeneous within a waterbody even over a small geographic range. Including native fish in the assessment of a waterbody may reduce the uncertainty resulting from the identified limitations of deployed fish and may help relate observations within the deployed fish with observed ecological impairment.

Several factors should be considered when selecting a native fish for inclusion in an assessment. One practical aspect that must be considered is the local abundance of the native fish; if a fish is relatively rare in an area, then removing them may have unintentional negative impacts. Similarly, the selected fish must be present in a high enough abundance that it is possible to collect enough samples to achieve sufficient statistical power to make clear conclusions. Another factor to consider is the relative sensitivity of the native and the model organisms. If sensitivities between selected model and native organisms differ greatly, it will make it difficult to reconcile differences in results (discussed below). Finally, understanding life history traits of the native species is important in the interpretation of results. If a limited spatial area is being targeted within a watershed, it reasons that a relatively stationary species is selected. Likewise, if a particular habitat is expected to be impacted then selecting a native species that would spend most of its time in that habitat would be reasonable.

Establish causal linkages between biological levels and developmental stages: Demonstrating that an exposure has occurred using molecular biomarkers does not require an understanding of the role of the altered mRNA in mediating the exposure. However, demonstrating where genes exhibiting altered expression lie in the response pathway may be a requirement if the goal is to link biomarker expression to apical endpoints evident at higher biological levels (Ankley et al. 2010). This linkage is critical for biomarkers to be used predictively; however, it is a much more difficult task than using gene expression as indicators of exposure. Recently, several studies have demonstrated that it is possible to link biomarker expression to predictive models of impairment. For example, Miller et al. (2007) demonstrated that altered levels of VTG protein in female FHM could be used as a predictive and quantitative measure for reduced population size. Watanabe et al. (2011), using the adverse outcome pathway concept, linked domoic acid exposure to alterations at higher biological levels. The linkage of expression data to adverse outcomes will greatly enhance the interpretability of the array of data resulting from multidisciplinary approaches.

The establishment of causal linkages may also be aided by monitoring biological endpoints in developmental stages, as these life stages may be more sensitive to toxicants and overall disturbance. The importance of inclusiveness in terms of endpoints and developmental stages was demonstrated in the current case study, as larval endpoints were found to be more sensitive to exposure. Moreover, due to their small size and corresponding resource requirements, the use of early life stages in these types of assessments opens up additional exposure and effect tools, such as the behavioral assays used here. Although behavioral endpoints are not often used in ecological assessments, swimming, as a measure of performance in fishes, is a key factor in linking an organism's phenotypic character (e.g., physiology, anatomy) with its use of environmental resources (e.g., food, O_2, nesting sites) for the overall reproductive output and survival of the individual and population (Wainwright 1994). Thus inclusion of these types of endpoints could have important implications in identifying causative agents, as they have clear ecological relevance.

Better chemical sampling methods. It is often desired to link biological measurements with the occurrence of chemicals in the watershed. However, with few exceptions this has been largely unsuccessful. One possible reason for the low success rate in linking biological responses and occurrence may be due to technical issues associated with the way water bodies are sampled. Typically, because of the high cost and effort of taking chemical samples, which stem from not just analyzing the samples but also from having to collect water samples at sometimes distant sites, a chemical sample is most often a grab sample. It has been shown that even when samples are collected at relatively short intervals, they can be highly variable (Droppo and Jaskot 1995). Although often multiple samples are taken and composited, each still represents a single moment in time, whereas as native or deployed fish are integrating tens to thousands of stressors over longer durations. Therefore, it is not surprising that there is often a disconnect between chemical analyses and biological endpoints. This was observed in the current study, where low levels of estrogenic compounds were found in both fall and spring sampling events, though *vtg* mRNA expression was only seen in the fall. Deployed or native organisms are exposed to dynamic mixture of contaminants over longer periods of time and respond to only the biologically available fraction of chemicals. Linking chemical and biological endpoints may be addressed through the use of passive sampling technologies, which are able to

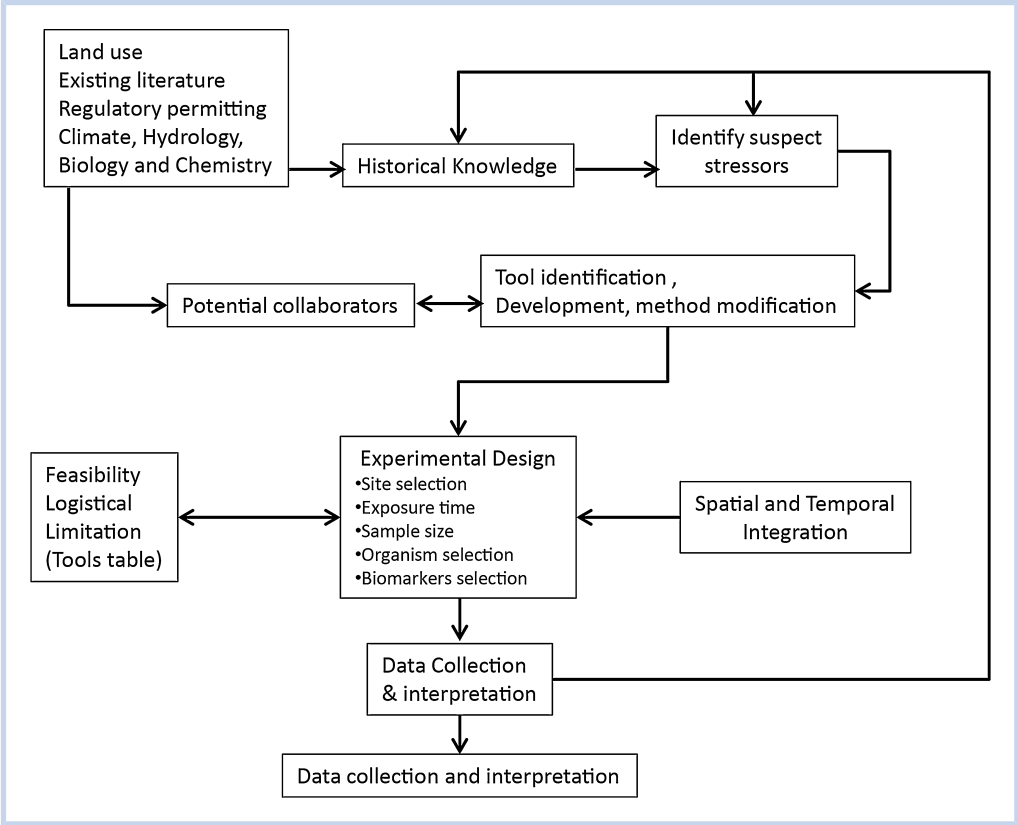

Figure 4. Logical workflow for the planning and implementation interdisciplinary watershed scale assessment.

measure ongoing exposure in situ for extended durations. Unfortunately, it is difficult to relate chemical concentrations measured within the sampling device to concentrations in the water column. There is a clear need for further advancement of passive sampling technologies that can be used to not only detect exposures but also estimate instream concentrations. These could be deployed side-by-side with caged organisms thus making the linkage between chemistry and biology less problematic and greatly aiding in the interpretation of the results.

Aligning to species sensitivity distribution. Ideally, when characterizing a particular waterbody, all measurements would be conducted in resident fish or using deployed organisms of a local species, as these fish will be more representative of a specific location. The ability to use many of the tools used here in local fish species is dependent on the availability of specific resources, such as electroshocking equipment or boats. Moreover, significant upstream experimental effort may be required to establish new or modify existing molecular biomarkers for new fish species, and this is not a practical approach if multiple watersheds are to be characterized. In light of this, using model organisms may be the only or best option when conducting these types of studies. To more easily extrapolate responses observed in the model organism to resident organisms in a particular waterbody, the sensitivity of the model organism must be calibrated to the organisms of concern. This can be accomplished either by direct comparison between the model organism and the organism of interest, which may require additional experimentation, or a through comparison to a closely related surrogate species. Acute and chronic toxicity data are not available for most species, thus predictive models may be employed to allow comparisons across taxa. One such tool that can be used to predict acute toxicity between the organism of interest and the model organism is the Web-based Interspecies Correlation Estimate (Web-ICE) (http://www.epa.gov/ceampubl/fchain/webice/). This tool correlates species sensitivity to chemicals with biological parameters to estimate acute toxicity. Although there will be some degree of uncertainty surrounding the predicted value, it is often the most pragmatic option and provides at least an estimate of relative sensitivities.

Environmental compartment. This case study focused largely on exposures that occur in the water column and ignored those that may have occurred within other environmental compartments, such as sediment. Focusing only on a single compartment may lead to false negatives, concluding an exposure has not occurred when it has. This may be especially true in this particular case, as the pyrethroid pesticides, which are known to be a problem in this watershed, are highly lipophilic and thus are predicted to adsorb strongly to the organic component of sediment particles (Gan et al. 2005). Thus, sole reliance on effects-based biomarkers for organisms that predominantly reside in the water column may miss these exposures altogether or suggest lower exposure levels as exposures in these species may be indirect through trophic interactions. In fact, California has recently implemented a statewide Stream Pollution Trends (SPoT) monitoring program that includes a sediment toxicity testing component (Hinck et al. 2009).

Understanding of logistical limitations and upfront analysis of hydrology and land use for site selection. With the assumption that these types of large multidisciplinary studies are necessary

to truly understand the total picture of exposure and effects within a watershed, and that research dollars and time are limited and projected to remain that way, consideration must be given as to which tools to apply and where to use them in a given watershed. Essentially, it is not possible or pragmatic to simply look for everything everywhere. Furthermore, too much data may cloud interpretation of results. Understanding the logistical limitations of sampling is required for the most efficient use of resources. Figure 4 represents a logical flow through experimental design and implementation. Initially, watershed characteristics should be considered. For example, land use should be a primary consideration when selecting the types of potential inputs and pathways, as this will drive the selection of analytical and biomarker tools to be used to identify specific types of chemical and nonchemical stressors. Knowing a priori what sources (WWTPs, agriculture) and practices (e.g., management practices) are present in a watershed will facilitate the efficient use of resources. This can be addressed to some extent by mining publicly available databases, such as the California Department of Pesticide Regulation Pesticide Use Reporting database (http://www.cdpr.ca.gov/docs/pur/purmain.htm). Understanding the likely chemical contaminants and timings of their occurrence in the context of the hydrology of the system will greatly enhance study design and may be used to identify critical integrator sites that may require more intense sampling efforts. For example, in the case study outlined above, by understanding the hydrology, we were able to partition sources and shifts in contaminant profiles seasonally with changing flows. Ideally, this type of approach could be used to apportion the total experimental resource allocation among sites within a given watershed. Moreover, this may guide the selection of potential collaborators with specific knowledge of the watershed. Once potential stressors have been identified, appropriate analytical tools may be selected. The need for particular expertise may also guide selection of collaborators. Design of the experimental effort must be considered in the context of hydrological and climate information (seasonality and flow) as well as the feasibility in terms of resource sampling limitations. Once finalized, implementation will result in new data that can feed into the historical knowledge of the particular watershed and help to further prioritize potential stressors.

CONCLUSIONS

The current study suggested a seasonal variability of chemical presence and biological effects in the Sacramento River watershed. Although several a priori hypotheses were corroborated by multiple independent data sets, the lack of congruence across data sets speaks to the complexity of the challenges faced when assessing environmental pollution in complex aquatic ecosystems. Assessment of these systems requires spatial and temporal integration and needs to target multiple levels of biological complexity and environmental chemistry. Consequently, these efforts exceed the resources and expertise of any one entity and require extensive collaboration among governmental agencies, academic institutions and watershed stakeholders. The value of a real-time monitoring station, like the Hood Field Station is critical for several reasons: it allows for constant exposures, side stepping issues associated with grab samples of water; in this case, it serves as a key integrator location and an upstream boundary of the Bay-Delta; and it provides a location for multiple collaborators to explore new biomarker endpoints, while learning more about the complex watershed conditions.

Additionally, models should be used hand-in-hand with monitoring data to better evaluate where and when to monitor within a watershed (Hoogeweg et al. 2012). Models can be used to identify what chemicals and where to monitor them, waterbody reaches of highest risk, and where to target management practices and mitigation measures. These studies in conjunction with model output information provide risk assessors with a "weight-of-evidence" approach for regulatory decision-making. This is especially important in the characterization of highly complex watersheds with a large array of contaminants, over significant geographical distance, which is potentially highly resource intensive for an individual program.

Acknowledgment—We would like to thank field biologists in California and students at St. Cloud State University who assisted with sample collection and analysis.

Disclaimer—The views expressed in this article are those of the authors and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency.

SUPPLEMENTAL DATA

Table S1. US EPA API Chemistry Results
Table S2. CDFW Chemistry Results
Table S3. US EPA Hormone Chemistry Results

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