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RESEARCH

Chemical and Toxicological Effects on Cache Slough after Storm-Driven Contaminant Inputs

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ABSTRACT

Chemical and toxicological testing in the Cache Slough complex (the slough) of the North Delta indicated the aquatic biota are exposed to a variety of wastewater-derived food additives, pharmaceuticals, and personal care products in highest concentration during dry periods, and many insecticides, herbicides and fungicides with peak concentrations after winter rains. The insecticide groups currently known to be of greatest toxicological concern are the pyrethroids and the fiproles (i.e., fipronil and its degradation products). After stormwater runoff enters the system via Ulatis Creek, both pesticide groups attained concentrations that posed a threat to aquatic life. When the commonly used testing species, *Hyalella azteca*, was placed in Cache Slough, toxicity — and, at times, near total mortality — was seen over at least an 8-km reach of Cache Slough that extended from the uppermost end almost to the junction with the Deep Water Ship Channel. Previous work over many years has shown similar results after other winter storms. However, when *H. azteca* that carried a mutation providing resistance to pyrethroid pesticides were also deployed in the slough, no ill effects were observed, which provided strong evidence that pyrethroids were responsible for toxicity to the non-resistant strain. Abundant resident *H. azteca* in Cache Slough carry any of four mutations that provide resistance to pyrethroids. They also carry a mutation that provides resistance to organophosphate pesticides, and likely carbamate pesticides as well. After many years of exposure, sensitive genotypes have been nearly eliminated from the system, and replaced by a population unaffected by many insecticides now in common use. We offer a variety of reasons why this shift to a population with mutant genotypes

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is of considerable concern, but also note that society has yet to fully consider the ecological and regulatory ramifications of the evolutionary attainment of pollutant resistance.

KEY WORDS

pesticides, pyrethroids, fipronil, *Hyalella*, pesticide resistance, Cache Slough

INTRODUCTION

The Cache Slough complex (the slough) in the northwest Sacramento-San Joaquin Delta is a network of tidal freshwater sloughs bounded by levees, with the surrounding uplands used for irrigated agriculture. The area provides a wide variety of habitat types, from tidal marshes to deep, open water. The area is of particular significance as habitat for the Delta Smelt (*Hypomesus transpacificus*), an endemic fish that has suffered dramatic declines in numbers, and is now listed as endangered under the California Endangered Species Act and as threatened under comparable federal legislation (CDFW 2019). The Cache Slough complex is a major spawning area for the species (Murphy and Hamilton 2013), and one of the few locations in the Delta with a year-round resident population (Sommer and Mejia 2013). The region has been designated by the state of California as a high-priority area for ecosystem restoration (DSC 2013).

In view of the significance of Cache Slough and associated waterways, it is concerning that its waters have been shown to be toxic to a commonly used testing organism, the amphipod, *Hyalella azteca*. Monitoring during winter storms often demonstrated its paralysis and inability to swim — and, on one occasion, mortality — after laboratory exposure to Cache Slough waters (Weston et al. 2014). Pyrethroid pesticides were suspected as the cause of this toxicity, based largely on their concentration in comparison to known effect thresholds. The same study also documented one occasion when the organophosphate pesticide, chlorpyrifos, entered nearby Lindsey Slough via agricultural return flow from surrounding croplands, and caused mortality in *H. azteca* when slough waters were tested.

Many likely contaminants potentially enter Cache Slough, the vast majority of which have never been analyzed in its waters. Urban runoff from the city of Vacaville flows approximately 20 km down Alamo and Ulatis creeks, potentially providing a mixture of street runoff and urban-use pesticides to Cache Slough. The city of Vacaville's Easterly Wastewater Treatment Plant discharges tertiary-treated municipal wastewater to Old Alamo Creek — a tributary within the Ulatis Creek watershed — that in turn enters Cache Slough. Finally, the surrounding agricultural lands represent a potential source of a wide variety of herbicides, fungicides, and insecticides.

Our study, by incorporating notable enhancements to earlier work, was intended to provide a deeper understanding of the chemical and biological responses of Cache Slough to the runoff that accompanies storms. First, to obtain a comprehensive contaminant profile of Cache Slough waters, our chemical analyses included not only targeted compounds known to be associated with runoff toxicity in the Delta — including pyrethroids, chlorpyrifos, and fipronil (Weston and Lydy 2014; Weston et al. 2014) — but, in addition, we screened for nearly 4,000 non-target compounds. Second, we again monitored for toxicity to the amphipod, *H. azteca*, but this time using an *in situ* method. A novel Toxicity Identification Evaluation (TIE) approach using *H. azteca* that bear a pesticide-resistance mutation was used to identify the toxicant responsible for mortality (Weston et al. 2018). Finally, we tested for pesticide resistance in resident *H. azteca* throughout the Cache Slough complex to determine which chemicals are providing persistent selective pressures on resident invertebrate populations.

MATERIALS AND METHODS

Study Sites

We established five sites for chemical monitoring, with four of these also used for concurrent *in situ* toxicity testing (Figure 1; Appendix A). We used site UB (chemical monitoring only; no toxicity testing) to characterize the water that enters Cache Slough from Ulatis Creek; the site was located 5 km upstream of its junction with Cache

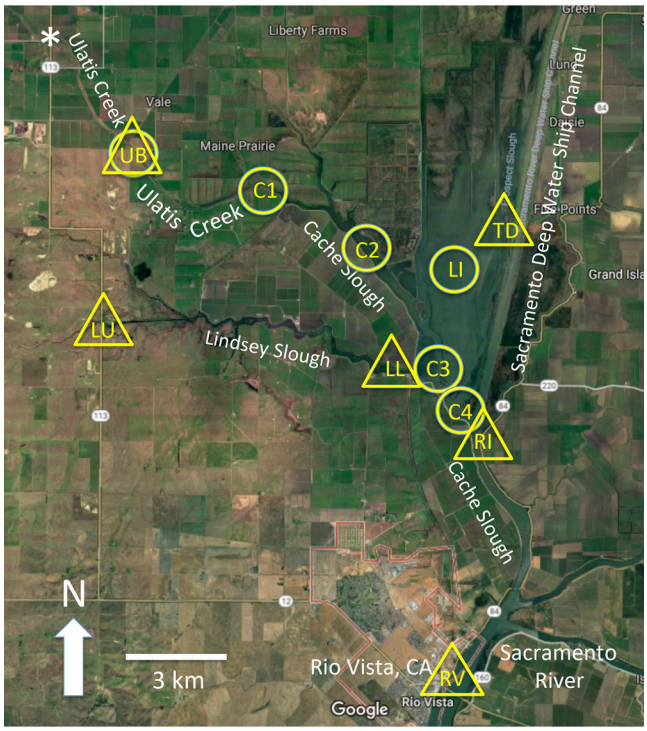

Figure 1 Map of the study area north of Rio Vista, CA, showing location of all sampling points. *Circles* indicate locations for the chemistry and toxicology studies (except chemistry only at UB); *triangles* indicate locations for collection of resident *H. azteca* used for genetic sequencing. The location of the Highway 113 drain discussed in the results is shown by an *asterisk*. Source: Image from Google Earth.

Slough at the most downstream road-accessible site on the creek. We established four sampling sites along the length of Cache Slough, with increasing distances from its western end where Ulatis Creek enters. Site C1 was at the mouth of Ulatis Creek, and sites C2, C3, and C4 were 4, 8, and 10 km farther seaward, respectively. Site LI (Liberty Island) served as a control site for *in situ* toxicity testing. In previous sampling (Weston et al. 2014), no pyrethroids were detected at the LI site after storms, and chlorpyrifos concentrations were consistently low (typically <2 ng L^{-1}) and never exceeded 18 ng L^{-1}.

We collected resident *H. azteca* for genetic sequencing from six sites located so as to geographically cover the Cache Slough complex: Ulatis Creek (site UB as described above); upper and lower Lindsey Slough (sites LU and LL); the eastern margin of Liberty Island in the Toe Drain, which is heavily influenced by agricultural runoff (site TD) at Ryer Island (site RI); and at Rio Vista along the Sacramento River just below its confluence with Cache Slough (site RV).

Water Sampling

Sampling at the five chemical monitoring sites occurred before, during, and after two storms in early 2016. The first storm was January 4-6, during which 7.3 cm of rain fell (based on Vacaville, California precipitation records), and the second storm was March 4-7, during which 9.4 cm of rain fell. Each storm had been preceded by a dry period of approximately 2 weeks. Our general approach was to initiate water sampling 8 to 12 h before the rain began, then sample daily throughout the rain — and for an additional 2 d as runoff moved through the system. Thus, we collected samples daily from January 4-8 and March 4-9, though during each rainstorm there was 1 day when hazardous storm conditions prevented sample collection (January 6, March 5).

We sampled a third storm only for chemical analysis (no *in situ* toxicity testing) only at sites UB and C4. Rainfall began in the evening of April 6, 2017, and ended on April 7, with a total accumulation of 6.1 cm. Again, the antecedent dry period was approximately 2 weeks. We took no pre-storm sample, but sampled daily from April 7-9.

Depending on the site, we accessed sampling locations either by car or by boat. We filled 1-liter glass bottles (pre-cleaned for pesticide analyses) just below the water surface, and then returned them to the laboratory for their extraction later the same day. We collected another bottle in the same manner to use for total suspended solids analysis. We took surface measurements for temperature, conductivity, pH, and dissolved oxygen.

Chemical Analyses

We extracted and analyzed the water samples by both gas chromatography (GC) coupled to a high-resolution time-of-flight (QTOF) mass spectrometer (MS) and liquid chromatography (LC) coupled to QTOF-MS. Full details of the analytical methods are described in Moschet et al. (2017).

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We analyzed semi-polar to non-polar pesticides using an Agilent 7200B GC-QTOF-MS, separately analyzing particulate matter recovered by filtration as well as the filtered water, but summing the fractions for all data reported herein. The particulate matter was sonication-extracted using hexane and acetone. The filtered water was extracted by solid-phase extraction using an Oasis HLB cartridge (Waters, Milford, MA). We performed the GC-QTOF analysis in negative chemical ionization mode as well as in electron ionization mode.

We analyzed polar to semi-polar pesticides on an Agilent 6530 LC-QTOF-MS. In brief, filtered surface water samples were extracted by solid-phase extraction using a multi-layered cartridge that contained Oasis HLB, Strata XAW, Strata XCW (Phenomenex, Munich, Germany) and Isolute ENV+ (Biotage, Uppsala, Sweden), to enrich neutral, cationic, and anionic species with a broad range of physico-chemical properties. We analyzed the samples using electrospray ionization in both positive and negative mode.

Our approach distinguishes between "target" and "suspect" compounds. The "target" compounds were nearly 50 pesticides that we specifically sought to quantify, including many of the high-use pesticides in the Cache Slough region, and several that had been previously linked to aquatic toxicity in the area. For these compounds, we used an authentic reference standard for proper quantification. By GC-QTOF-MS, we quantified 12 target pyrethroids — as well as chlorothalonil, chlorpyrifos, fipronil, and five fipronil degradates — with reporting limits of 0.1 ng L^{-1}. By LC-QTOF-MS, we quantified 28 target pesticides with reporting limits between 0.5–15 ng L^{-1}. Appendix B provides a list of all target compounds.

Analysis for the second group, the "suspect" compounds, was enabled by a key advantage of the high-resolution MS detection: the method can screen for thousands of potential contaminants without the need for authentic reference standards. We screened the GC-QTOF-MS data using a library that contained 750 pesticides (Agilent GC/Q-TOF – Pesticide Personal Compound Database and Library [PCDL]). The library contained mass spectra as well as retention times for most of the pesticides. Using the LC-QTOF-MS data, we screened suspect compounds using two libraries that together contained over 3,100 water-related contaminants, including pesticides, pharmaceuticals, personal care products, and industrial chemicals (Agilent Pesticide PCDL; Agilent Water Contaminants PCDL). We screened the acquired data for the accurate masses of the molecular ion, the most abundant isotopes, and the tandem MS fragments of the chemicals in the library. We first tentatively identified with high confidence the detected compounds from the suspect screening. For a full confirmation, we purchased a reference standard. We determined the concentrations of these compounds retrospectively. Therefore, their reported concentrations are considered semi-quantitative. Details about the data evaluation are described in Moschet et al. (2017).

Toxicity Testing

We used four populations of the amphipod, *H. azteca* for *in situ* toxicity testing. The first two populations were laboratory-cultured *H. azteca* from the strain widely used throughout the U.S. for testing. During the January storm, animals came from a culture maintained at the University of California, Berkeley (UCB). During the March storm, the Berkeley culture was not able to provide sufficient animals, so we used animals from a culture maintained at Southern Illinois University (SIU). The two groups are comparable since (1) the origins of both can be traced to a culture begun at a U.S. Fish and Wildlife Service laboratory in Columbia, Missouri, (2) both groups have been shown to be equally sensitive to pyrethroid insecticides, and (3) both fall in the same clade within the *H. azteca* species complex (Weston et al. 2013).

The other two populations of *H. azteca* we used came from the American River (Sacramento, CA) or Mosher Slough (Stockton, CA) — both water bodies with a history of pyrethroid exposure (Weston and Lydy 2010; Weston and Lydy 2012; Major et al. 2018). Mutations have arisen in wild populations that dramatically increase their resistance to pyrethroid. Using sensitivity to the

pyrethroid cyfluthrin as an example, reported 96-h LC50s (concentration of a substance that is lethal to 50% of the population exposed to it for the stated test duration) for the standard laboratory-cultured animals are 1-5 ng L^{-1} (Weston et al. 2013). They are 52-379 ng L^{-1} for the American River population, and 99-268 ng L^{-1} for the Mosher Slough population (Weston et al. 2018). Both populations have been shown to have an amino acid substitution at position 925 of the voltage-gated sodium-channel protein, the target of pyrethroid insecticides. Instead of leucine at the 925 position in wild-type *H. azteca*, many American River individuals possess an isoleucine, and all the Mosher Slough individuals possess either an isoleucine or valine (Major et al. 2018). The isoleucine substitution has been reported in insects to inhibit binding of pyrethroids to the target site (O'Reilly et al. 2006), and we presume the valine substitution functions similarly. Our intent was to concurrently expose both the laboratory-cultured, non-resistant *H. azteca*, and the pyrethroid-resistant, wild-collected individuals to waters from Cache Slough. If the non-resistant individuals showed toxicity, but none was seen in the resistant individuals, it would provide strong evidence that a pyrethroid was the responsible toxicant. The potential for cross-resistance is an important consideration when applying this approach (i.e., if the mutation for pyrethroid resistance also confers resistance to other toxicants). However, this potential is considered low because the mutation causes a very specific effect — a conformational change in the voltage-gated sodium-channel protein — so that the toxicity of any substance with a mechanism of action independent of the sodium channel should remain unaffected. Furthermore, we have demonstrated that even resistance to DDT — a pesticide that does act upon the voltage-gated sodium channel just as pyrethroids do (Davies et al. 2007) — is not enhanced in *H. azteca* that bear the leucine/isoleucine substitution at position 925 (Weston et al. 2018). We have previously published additional validation of this approach using resistant organisms to identify responsible toxicants (Weston et al. 2018), and additional details can be found there.

One day before deploying amphipods in Cache Slough for both the January and March 2016 storms, we collected fresh Mosher Slough animals, and sieved them through stacked screens to obtain animals of a uniform size/age class, using those that could pass through a 1,000-µm screen but were retained on a 600-µm screen. They were held in the laboratory overnight at the temperature of their home site and of Cache Slough (11-13 °C). American River animals had been maintained in culture at UCB for approximately 1 year, and there had been no apparent loss of pyrethroid resistance (Weston et al. 2018). These animals, as well as the non-resistant UCB or SIU populations routinely used for testing, were temperature acclimated before use, and similarly size-fractionated. We exposed all populations of *H. azteca in situ* using 150-ml polyethylene containers, with 5-cm openings in the container top and bottom, and covered with 500-µm nylon mesh to prevent escape. The containers were suspended from an anchored sub-surface buoy, and held approximately 1-2 m below the water surface and 2 m above the bottom. At each site, we deployed five replicate containers for each population, with 10 individuals per container. The animals were put in place just before heavy rain began, and left in place for 4–5 d throughout the rainstorm and subsequent runoff. We then transported the containers to the laboratory in site water for processing later the same day. The surviving amphipods were scored for both the number alive, and the number alive but paralyzed and unable to perform coordinated swimming movements. Because many insecticides are neurotoxins, in past work we have often found some *H. azteca* to remain alive after a 4-d exposure, but paralyzed, often with no movement other than a faint twitch of a single appendage (Weston and Lydy 2010; Weston et al. 2014).

We used CETIS (Comprehensive Environmental Toxicity Information System™; Tidepool Scientific Software, McKinleyville, CA) to statistically analyze the toxicity data. We compared effects at each of the Cache Slough sites to the Liberty Island control site, using t-tests if the data met parametric assumptions, and Wilcoxon Rank Sum tests if they did not.

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Resident *H. Azteca*

H. azteca is abundant in Cache Slough and nearby waterways, and we collected resident populations from several sites (UB in January 2018; TD in April 2018; all others in May 2018). We collected them by sweeping a D-net through aquatic vegetation. We then set aside specimens in 100% ethanol for later gene sequencing, as described below.

We collected resident animals from UB in January and July 2018 and used them the day after collection to determine chlorpyrifos and pyrethroid (i.e., cyfluthrin) LC50s. We used individuals that passed through a 1,000-µm screen, but were retained on a 600-µm screen, for testing, which is a somewhat larger size class than is often tested, but sufficient smaller individuals were not available in the wild population at collection times. The compounds were obtained from ChemService (West Chester, PA), dissolved in acetone, and spiked into test waters held in 100-ml beakers. Solvent concentrations never exceeded 50 µL L^{-1}, and were kept constant across all treatments by the addition of pure acetone, as needed. We established a dilution series with concentration steps of 2X, and used three replicates per step. We also established a control treatment of water without pesticide (groundwater from Davis, CA) and a solvent control treatment. We placed a 1-cm^{-2} piece of nylon screen in each beaker to provide a substrate to which the amphipods could cling. The beakers were held under a 16:8 h light cycle, in a water bath at a temperature of 19 °C. After 48 h, we removed approximately 80% of the water in each beaker and replaced it with freshly prepared solutions. The animals were not fed during the test. After 96 h, we stopped the test and scored the animals for both paralysis and survivorship. We used CETIS software to determine the EC50 (the concentration causing a sublethal effect to 50% of the exposed population; in this instance, inability to swim) and LC50, using the Spearman-Karber method. We analyzed water from one concentration step near the mid-point of the range, using a composite of the solutions prepared at time 0 and at the 48-h water change. We used the difference between the nominal and actual concentration (actual equal to 77% of nominal for chlorpyrifos, 61% for cyfluthrin) to adjust the point estimates, and report data based on actual concentrations.

DNA Extraction

Individuals of *H. azteca* preserved in ethanol were first examined under magnification to determine sex. We preferentially selected males for DNA extraction. However, when we had to use gravid females, we made an effort to dissect and remove embryos to avoid potential contamination from offspring DNA. We extracted genomic DNA from 10-20 individual *H. azteca* from each collection site using the Qiagen DNeasy® Blood & Tissue Kit (Qiagen, Germantown, MD) with slight modifications. To fully macerate and homogenize the tissue, we placed each individual in a 2-ml micro-centrifuge tube with 180 µL Buffer ATL (Qiagen), 20 µL Proteinase K (Qiagen), and one 3.2-mm stainless steel bead. We homogenized tubes in the TissueLyser LT (Qiagen) for 10-20 minutes at a rate of 50 oscillations/minute. After maceration, we incubated the micro-centrifuge tubes 16-24 h at 56 °C. We added homogenized sample to DNeasy columns and purified it using their standard protocol. We used a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA), to measure genomic DNA for purity (260/280 ratio) and nucleic acid concentration.

Resistance Mutation Genotyping Analysis

We previously reported that some natural populations of *H. azteca* elsewhere in California have evolved resistance to two different classes of pesticides: the pyrethroid and organophosphate insecticides (Weston et al. 2013; Major et al. forthcoming). These populations harbor point mutations that result in amino acid substitutions in the target site proteins for the insecticides: the voltage-gated sodium channel (Vgsc) for pyrethroids and the acetylcholinesterase (AChE) for organophosphates. To determine the genotypes of the Cache Slough-collected *H. azteca*, we used a genotyping assay for *vgsc*, the locus for the Vgsc gene (Major et al. 2018) and *ace-1*, the locus for the AChE gene (Major et al. forthcoming). We genotyped 10 individuals at the *vgsc* and *ace-1* loci to detect resistance mutations at a frequency of 5% or greater within the population.

To perform the genotyping assay for *vgsc*, we amplified a 543-bp segment of the *vgsc* using the primers AGGGTGTTCAAGCTCGCTAA (forward) and ACATGCTCTCGATCCACTCC (reverse) and the Phusion Hot Spot II High-Fidelity Green Taq Polymerase Master Mix (Thermo Fisher Scientific), with 5 µl of individual *H. azteca* gDNA. Thermocycler settings included an initial melting phase at 98 °C for 30s, then 35 cycles consisting of 98 °C for 10s, 64.2 °C for 30s, 72 °C for 30s, and a final extension phase at 72 °C for 10min. After we confirmed all bands on an agarose gel, we cleaned them with the QIAquick PCR Purification Kit (Qiagen) with a 40-µl elution volume. We sent between 200 and 300 ng of cleaned PCR product to the Massachusetts General Hospital DNA Core (Cambridge, MA) for sequencing on an ABI3730XL 96-capillary DNA Analyzer with internal reverse primer: GGCCGTCTTGAGACCATTT. We used a similar approach to amplify and sequence individuals at the *ace-1* locus using the primers TTCCGAAACCGAGACCTACC (forward) and TGACGTTGCAAGTGAAGTGG (reverse) for PCR amplification (producing a 906-bp segment) and the internal reverse sequencing primer: GATTGGGACAAACGGGAAGT. All custom DNA oligos were synthesized by Integrated DNA Technologies (Coralville, IA).

After a sequence of sufficient quality was obtained for each individual, all sequences were trimmed and aligned with GeneStudio Professional Edition and manually scored at the M918, L925, and F936 loci for the *vgsc* genotypes and at the G119 loci for the *ace-1* genotypes. Because both alleles were sequenced simultaneously for each individual, homozygotes presented as a singular peak, while heterozygotes presented as two approximately equal peaks at the same locus. Secondary peaks less than 30% percent of the primary peak height at a locus were not recognized as true heterozygotes (Major et al. 2018), because small secondary peaks can indicate baseline noise or contamination (i.e., true contamination or offspring alleles). Most calls were clear; we discarded any ambiguous sequences and repeated the assay for that individual.

Species Determinations

The amphipod *H. azteca* is generally recognized to be a species complex, with the members typically differentiated by gene sequencing (Witt and Hebert 1999). We created a 326-bp *vgsc* alignment of over 200 *H. azteca* individuals, including animals from wild population surveys (those from Major et al. [2018]) and those collected in the current study using MUltiple Sequence Comparison by Log-Expectation (MUSCLE) in Molecular Evolutionary Genetics Analysis (MEGA) v 7.0 (Kumar et al. 2016). After alignment, we used PhyML (Guindon et al. 2010) (*http://www.atgc-montpellier.fr/phyml/*) to generate a maximum likelihood tree. We retained branch supports of greater than 90% (1,000 bootstrap replicates) and displayed them on branches of an unrooted cladogram (not shown). We overlaid species determinations, based on analysis of the mitochondrial gene cytochrome ***c*** oxidase subunit I (COI) from Major et al. 2018, onto the *vgsc* cladogram. Based on these distinctions, we used the highly-supported branches of the *vgsc* trees to infer species affiliation for the individuals in the current study that were not sequenced at COI.

RESULTS

General Storm-Driven Patterns of Water Quality

The heavy rainfall from the storms resulted in large pulses of turbid waters entering Cache Slough via Ulatis Creek (Figure 2). Before the rain, background total suspended solids (TSS) concentrations were approximately 10 mg L^{-1} at all sites. During heavy rains, TSS concentrations increased 10- to 50-fold. In the early stages of the runoff event, the elevated TSS levels remained limited to the upper portions of Cache Slough, reaching only as far as C2. With time, however, the turbid waters continued to move seaward, eventually reaching C3 and C4 in the March storm. Two days after the rain ceased, Ulatis Creek continued to carry elevated TSS concentrations, though turbidity was substantially reduced from the earlier peak, and the input was not sufficient to affect anything but the uppermost reaches of the Slough. Throughout the entire storm, Liberty Island waters remained unaffected by the TSS input of Ulatis Creek,

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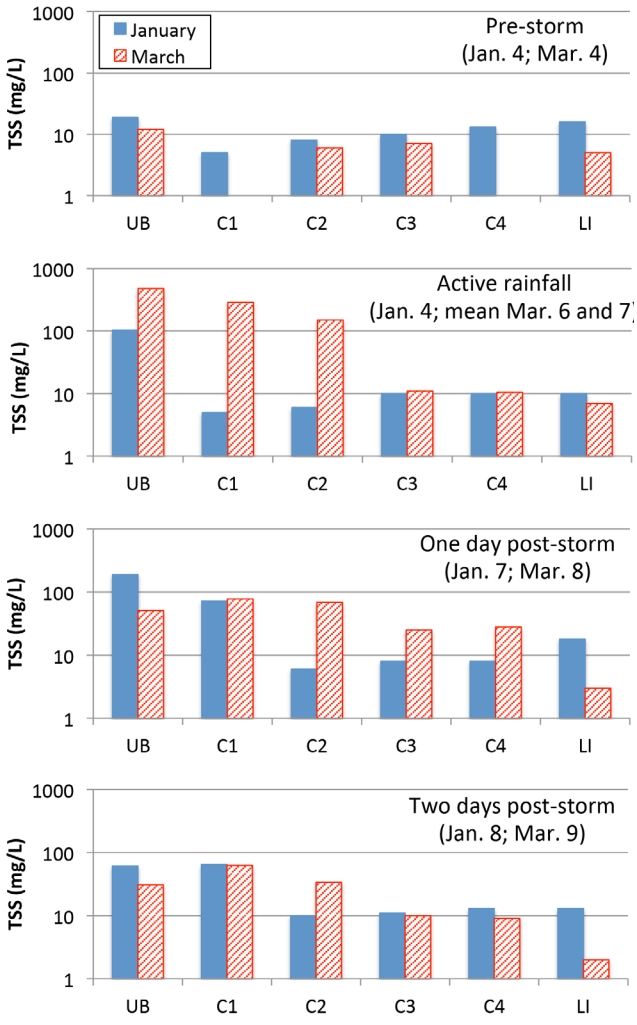

Figure 2 Total suspended solids (TSS) concentrations (log scale) at all sites over the January and March 2016 storms (April 2017 data set incomplete and not shown)

because they are separated from Cache Slough by a berm with only a few narrow gaps.

The most frequently detected compounds were largely pesticides, either with agricultural or urban applications. Of the 48 target pesticides or degradation products, we detected 41 of them in at least one sample, and we detected seven (2,4-D, boscalid, diuron, fipronil, fipronil amide, fipronil-sulfone, and fipronil-desulfinyl) in all 57 samples analyzed. The ubiquity of fipronil and its degradation products (together referred to as the fiproles) was particularly notable, and because there are no registered agricultural uses of the pesticide in California, their presence indicated the urban influence of Vacaville or Dixon.

We detected an additional 72 compounds by suspect screening in at least one sample, including pharmaceuticals, flame retardants, perfluorinated compounds, and additional pesticides (see Moschet et al. 2017). Compounds with the highest observed environmental concentrations (>1,000 ng L^{-1}) were among those identified by suspect screening rather than among the target pesticides. The Vacaville wastewater treatment plant was the likely source for these high-concentration compounds, given their uses. These include sucralose (artificial sweetener), iohexol (contrast agent in X-ray imaging), metformin (diabetes medication), tolytriazole (corrosion inhibitor), and the flame retardants TCPP (tris(1-chloro-2-propyl) phosphate) and TDCPP (tris(1,3-dichloroisopropyl) phosphate).

The target pesticides were of particular interest because of their potential association with aquatic toxicity. The total concentration of all target pesticides, derived by summing the component compounds (Figure 3), exhibited noteworthy spatial and temporal patterns. First, it is clear that winter rains, and the subsequent runoff, play a significant role in introducing pesticides into the Cache Slough complex. Just before the rains, the total target pesticide concentrations were less than 200 ng L^{-1}. During and after the rain, concentrations rose rapidly, increasing approximately 7-fold to near 1,500 ng L^{-1}. The only sample set that superficially appeared to contradict this pattern (April 2017 data of Figure 3) lacked a pre-storm sample, so the first sampling point on April 7 was taken after 2.5 cm of rain had already fallen. Substances with the highest concentrations differed across storms, with only the herbicide pendimethalin being among the highest-concentration compounds in all three events. The herbicides metolachlor and 2,4-D (2,4-dichlorophenoxyacetic acid) were among the highest concentrations in two storms (January/April and March/April, respectively).

Second, consistent with our expectation of Ulatis Creek as the primary pesticide source, the highest concentrations tended to be in the creek or at

upper Cache Slough stations nearest the creek's mouth (Figure 3). During the January 2016 storm, concentrations were generally highest at the Ulatis Creek station (UB), and next highest at the first Cache Slough station downstream (C1). The target pesticides were never at noticeably elevated concentrations elsewhere in Cache Slough during the January storm. During the March 2016 storm, concentrations were again highest at sites in or near Ulatis Creek (UB, C1 and C2), though lesser-affected waters could be detected further seaward. Liberty Island (LI) remained unaffected.

Third, it is apparent that the runoff-derived contamination introduced via Ulatis Creek can extend far down the length of Cache Slough. The March rainstorm caused an increase in concentrations at least as far as station C4, a distance of 10 km from the mouth of Ulatis Creek, and a distance of over 30 km from Vacaville, where many of the contaminants originated. The data show a lag in attainment of peak concentrations with increasing distance down Cache Slough, as would be expected. After the March storm, concentrations at UB and C1 peaked on March 6 and 7 as the rainfall ceased. However, peak concentrations at C2, C3, and C4 were not observed until March 8 and 9.

Finally, the total target pesticide concentrations were, at some sites, highest and often still rising on the last day of sampling, suggesting that a more extended monitoring period may have been necessary to capture the maximum aqueous concentrations. Though our last samples were approximately 48 h after the rain ceased, the data suggest a couple more days of additional sampling would have been preferable. Extended monitoring would have been valuable at all sites, but especially at the more seaward sites in Cache Slough, such as C3 and C4, given the time lag for runoff-derived contaminant input to reach that area.

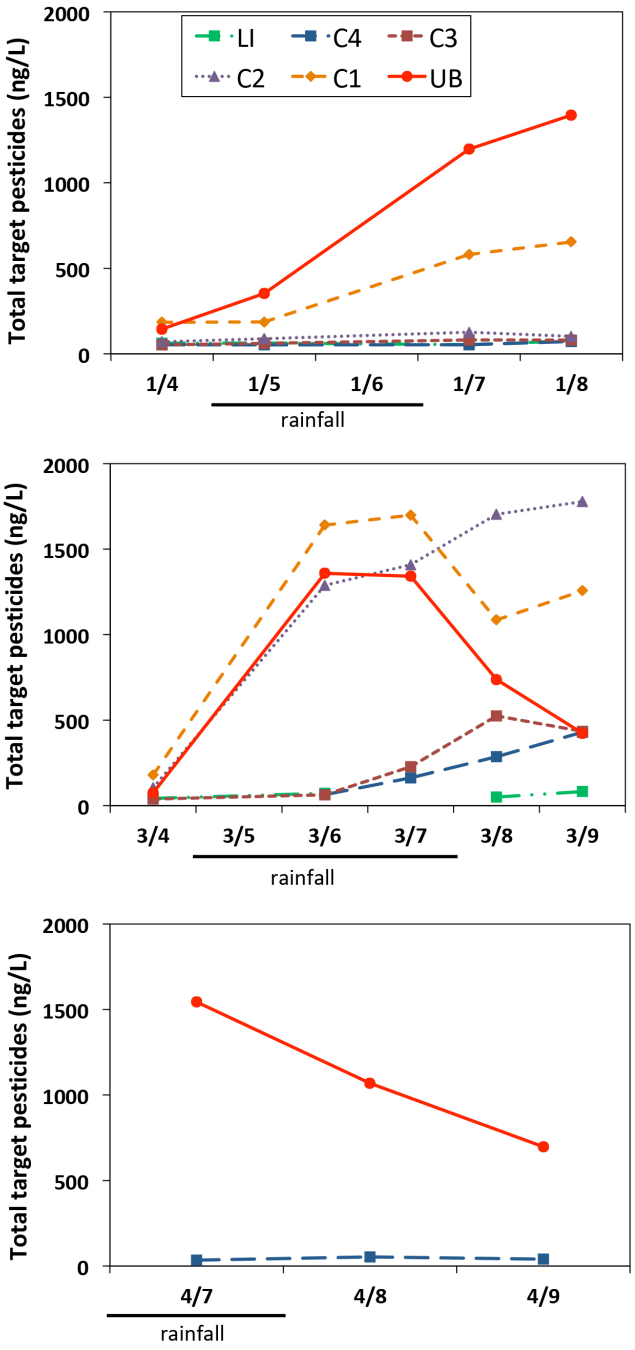

Figure 3 Temporal and spatial variation in the total pesticide concentration (sum of all 48 target compounds) during the storms in January 2016 (*top*), March 2016 (*middle*), and April 2017 (*bottom*; samples collected only at sites UB and C4)

Storm-Driven Input Patterns for Specific Compounds

Cache Slough receives chemical inputs from both continuous sources (e.g., wastewater treatment plant discharge of chemicals used indoors) and episodic sources (e.g., stormwater runoff of

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chemicals applied outdoors). We used temporal patterns in constituent concentrations across a rainstorm to draw inferences about whether the primary source was approximately continuous or rain-driven. An example concentration profile over storms for a compound with an approximately continuous source was the artificial sweetener sucralose, an indicator of wastewater treatment discharge (Oppenheimer et al. 2011). For compounds in this category, the concentration decreased during the initial portion of the rainstorm because stormwater runoff that contained no sucralose diluted the nearly constant input load from the wastewater treatment plant (Figure 4, *left panels*). Concentrations of compounds within this source category increased again after the rain. A number of pharmaceuticals exhibited similar temporal profiles, including carbamazepine, which is used to treat seizures and nerve pain.

In contrast to pharmaceuticals and food additives, compounds with primarily outdoor uses featured clear rain-driven concentration patterns. The herbicide diuron provides a good example of such compounds (Figure 4, *right panels*). This category of compounds increased distinctly in concentration during the initial portion of the rainstorm, caused by wash-off from surfaces treated around residences, highway and powerline rights-of-way, and agricultural lands (Huang et al. 2009). Numerous other target pesticides exhibited similar, rain-driven patterns indicative

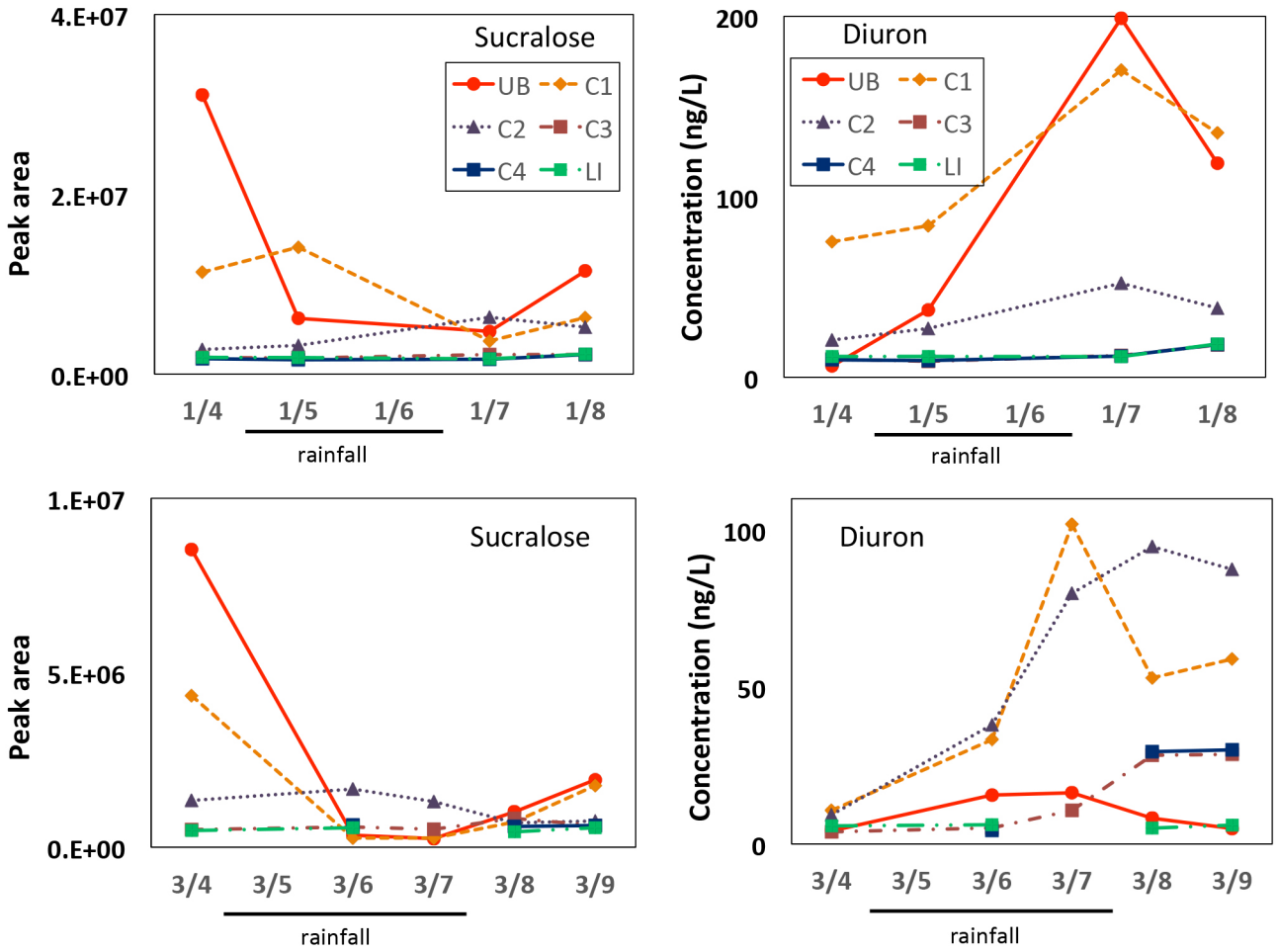

Figure 4 Typical concentration profiles for a compound with a primarily municipal wastewater input source (sucralose, *left panels*) and one with a stormwater input source (diuron, *right panels*) during rainstorms in January (*top*) and March (*bottom*). Sucralose concentration indicated by ion abundance from the mass spectrometer, with arbitrary units, since it was not a target compound.

of outdoor uses, including the fungicide boscalid and the herbicide 2,4-D.

Like the total pesticide profiles shown in Figure 3, the fiproles showed clear increases in concentration at site UB during the initial stages of the rainstorms (Figure 5). Because the first April sample was taken after rain had fallen for a day, concentrations declined across that storm. In all cases, the fiproles decreased at site UB by the end of the storm. In January 2016 samples, fipronil generally had the highest concentrations among the fiproles, followed by fipronil amide, fipronil-sulfone, fipronil-desulfinyl amide, and fipronil-desulfinyl. Some of the byproducts, particularly fipronil amide, exceeded the concentration of the parent compound in the subsequent rains. The concentrations of fipronil-sulfide, a byproduct formed primarily under reducing conditions, were low in all storms — as would be expected for these generally aerobic surface waters.

Pyrethroid insecticides were not detected as frequently as fipronil and its degradation products, but their high toxicity (many of the group <5 ng L^{-1} 96-h LC50 for *H. azteca*; Weston and Jackson 2009) makes these compounds of

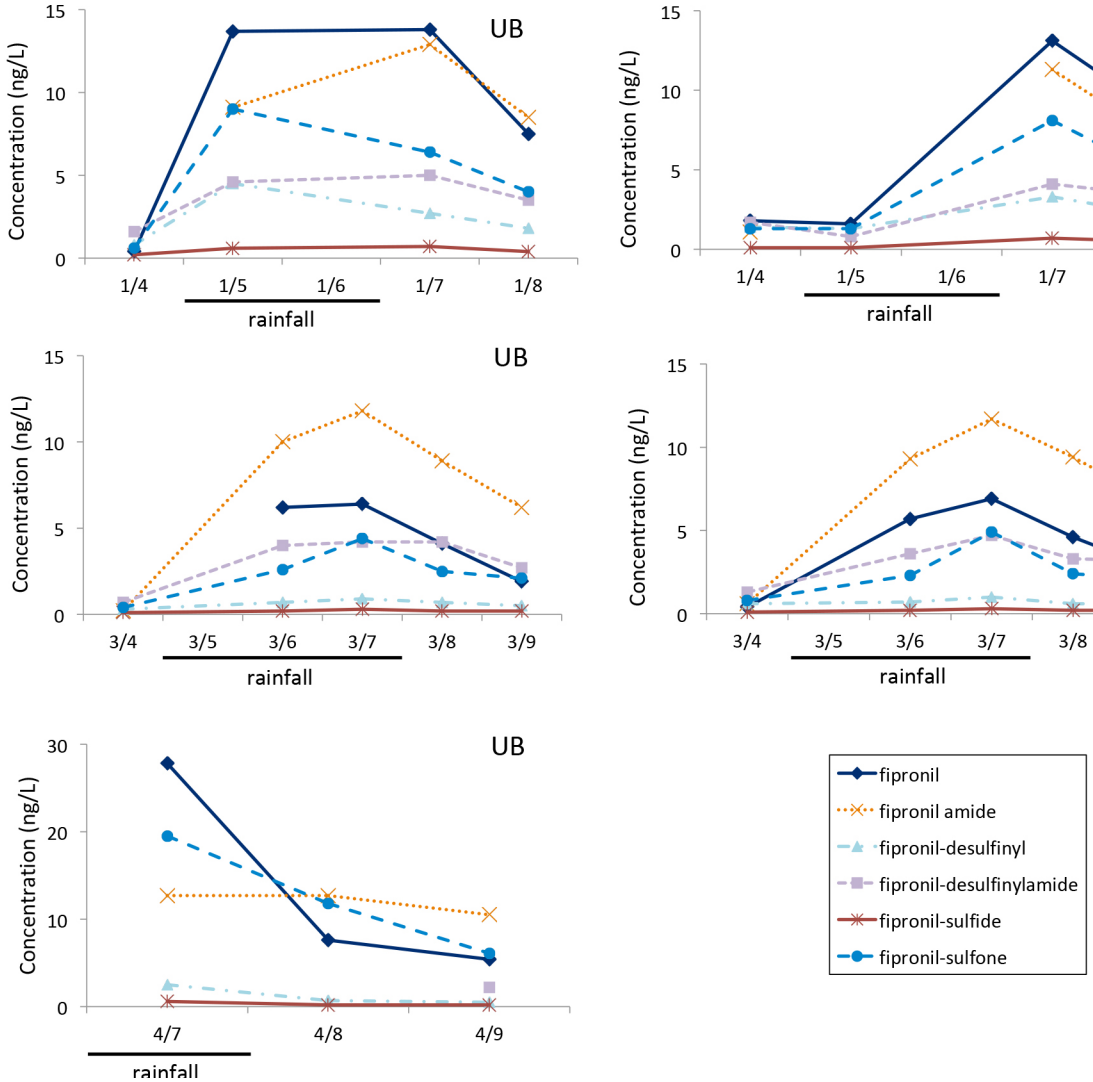

Figure 5 Concentrations of fipronil and five fipronil degradation products during three rain events in Ulatis Creek (site UB; *left panels*) and upper Cache Slough (site C1; *right panels*)

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concern nonetheless. The pyrethroid compounds routinely quantifiable were bifenthrin, cyfluthrin, cyhalothrin, and cypermethrin. We quantify "total pyrethroids," as in Figure 6 and in the text, as the sum of these four compounds, both because of their frequent occurrence and their similar toxicity (so as to aid interpretation of toxicity testing results below). Other than these four commonly detected pyrethroids, there were only two detections each of deltamethrin (1 and 12 ng L^{-1}) and esfenvalerate (1.9 and 2.8 ng L^{-1}; complete pyrethroid data in Appendix C).

The pyrethroids exhibited the same spatial and temporal patterns as previously discussed for the target pesticides in general; that is, very low or immeasurable concentrations before rainfall, a rapid increase upon entry of runoff into the aquatic system, and highest concentrations in Ulatis Creek (site UB) and the upper end of Cache Slough nearest the discharge point of the creek (sites C1 and C2). Concentrations at sites C3 and C4 were typically below quantification by the time the water reached these more seaward locations, but cypermethrin was detected at C3 in March (1.5 ng L^{-1}) and cyhalothrin at C4 in April (1.1 ng L^{-1}). Only one sample from LI contained measurable concentrations of pyrethroids (0.4 ng L^{-1} esfenvalerate and 0.1 ng L^{-1} cyhalothrin), and they were found before the onset of rains in January.

Although pyrethroid concentrations of 5-10 ng L^{-1} in upper Cache Slough appeared typical in these data, there were a few events of remarkably higher levels. On January 5, cyfluthrin reached a concentration of 29.1 ng L^{-1} at UB. Three days later, cypermethrin reached 32.8 ng L^{-1} at this same point. The April storm was noteworthy in that cyhalothrin reached 149.4 ng L^{-1} at UB on April 7 — one of the highest concentrations we have ever observed in hundreds of storm-related pyrethroid samples throughout northern California (e.g., Weston and Lydy 2010; Weston and Lydy 2012; Weston et al. 2014; Weston et al. 2018). Concentrations gradually declined over the next 2 days (to 69.9 ng L^{-1}, then 15.5 ng L^{-1}). Although this study was not designed to determine initial pyrethroid source by sampling individual discharges to Ulatis Creek, during the

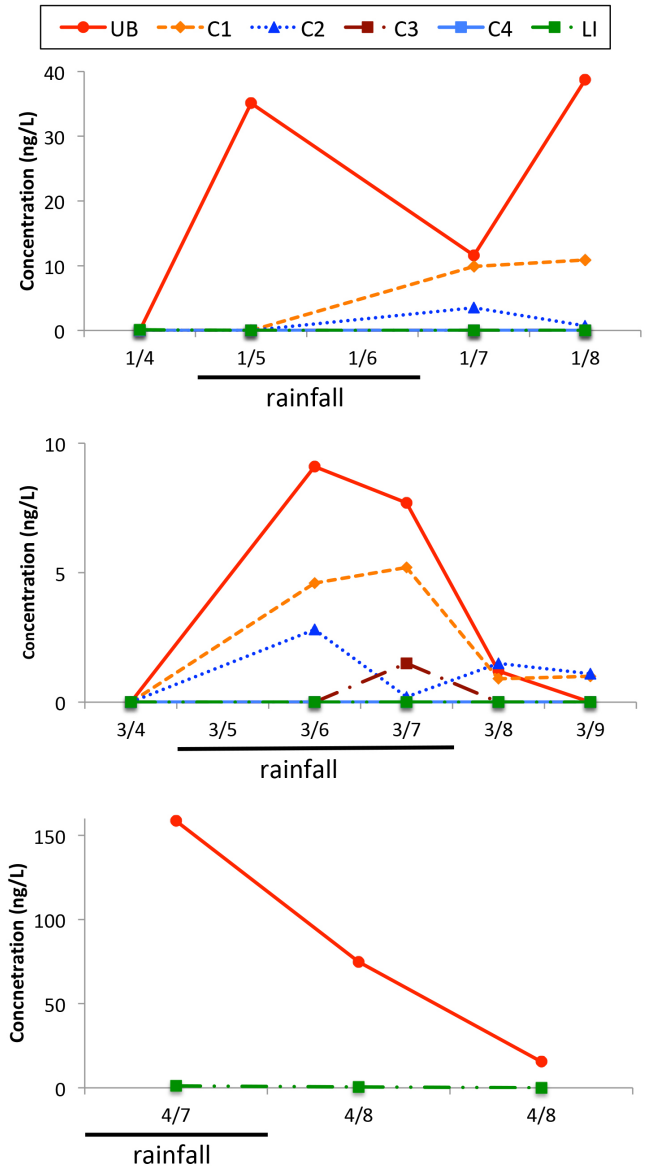

Figure 6 Temporal and spatial variation in the total pyrethroid concentration (sum of bifenthrin, cyfluthrin, cyhalothrin, cypermethrin) during the rainstorms in January 2016 (*top*), March 2016 (*middle*), and April 2017 (*bottom*; samples collected only at sites UB and C4). Note differing scales on the *y-axes*.

April 7 sampling trip we observed a large drain at the intersection of Ulatis Creek and Highway 113 — 4.5 km upstream of UB — to be discharging to Ulatis Creek, and we collected a water sample of the effluent. It contained 230.7 ng L^{-1} cyhalothrin, a concentration that, depending on flow, could potentially be enough to account for

the 149.4 ng L^{-1} seen in Ulatis Creek at UB the same day.

In Situ Toxicity Testing

We tested *in situ* at C1-C4 and LI using UCB or SIU cultures of *H. azteca*, both of which lack any genetic resistance to pesticides, and populations from Mosher Slough and the American River that carry mutations providing pyrethroid resistance. Test survival and mobility at control site LI were generally good during the 4- to 5-day *in situ* exposures. Survival and normal swimming behavior were seen in 78% to 96%, 78% to 80%, and 94% to 96% of the individuals from the UCB/SIU, American River, and Mosher Slough populations, respectively (Figure 7, Appendix D).

During the January rainstorm, toxicity among the UCB animals was limited to the uppermost reach of Cache Slough, at the mouth of Ulatis Creek (C1). It was also limited to paralysis, because 100% of the UCB animals survived, but only 64% of them could swim. The percentage of swimming individuals increased to 88% at C2, and 96% at all other sites. In contrast, no effects significantly greater than the control were seen in the pyrethroid-resistant American River and Mosher Slough populations held at any of the sites, including site C1 that caused paralysis in the non-resistant UCB organisms.

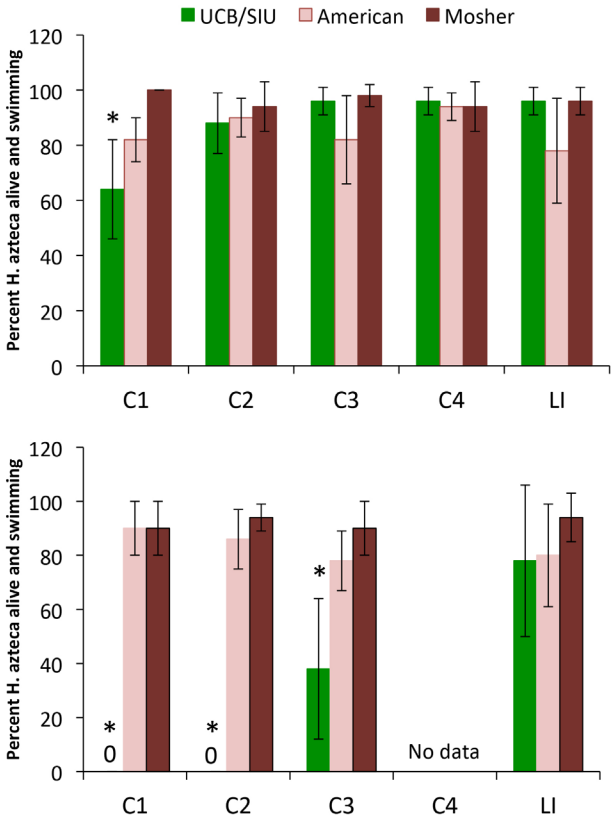

Figure 7 Results of *in situ* toxicity tests from the January storm (*upper panel*) or the March storm (*bottom panel*). Asterisks indicate statistically significant difference from results for that same population (UCB/SIU, American River, or Mosher Slough) held at the LI control site.

The March rainstorm caused widespread toxicity throughout Cache Slough to the standard laboratory-cultured group of *H. azteca*, in this case, SIU animals. None of the individuals exposed at C1 and C2 — and only 38% of the individuals at C3 — were alive and capable of swimming upon retrieval. In most instances, the animals were dead rather than paralyzed, with survival <10% at C1 and C2, and 50% at C3. Toxic effects extended over at least 8 km of Cache Slough (C1 to C3), but, lacking *in situ* deployment of *H. azteca* at C4 during this event, we don't know if toxicity continued seaward beyond C3.

Given the severe and widespread toxicity seen in the SIU animals during the March rain, it is noteworthy that the pyrethroid-resistant animals were unaffected at all sites. At C1 and C2, where every SIU individual exhibited toxicity, 86% to 90% of the American River individuals, and 90% to 94% of the Mosher Slough individuals were alive and swimming normally. Effects to the non-resistant SIU population, but no effects to either of the two pyrethroid-resistant populations, provides strong evidence that one or more pyrethroids was responsible for toxicity to the non-resistant population.

Resident *Hyalella*

Although the *in situ* toxicity tests indicated widespread toxicity in Cache Slough to the laboratory-cultured *H. azteca* strain, the slough contains dense resident populations of the species, suggesting these organisms may have acquired resistance comparable to the resistant strains we used for *in situ* testing. Figure 8 (upper panel) illustrates a 96-h cyfluthrin test with standard laboratory-reared *Hyalella* producing an LC50

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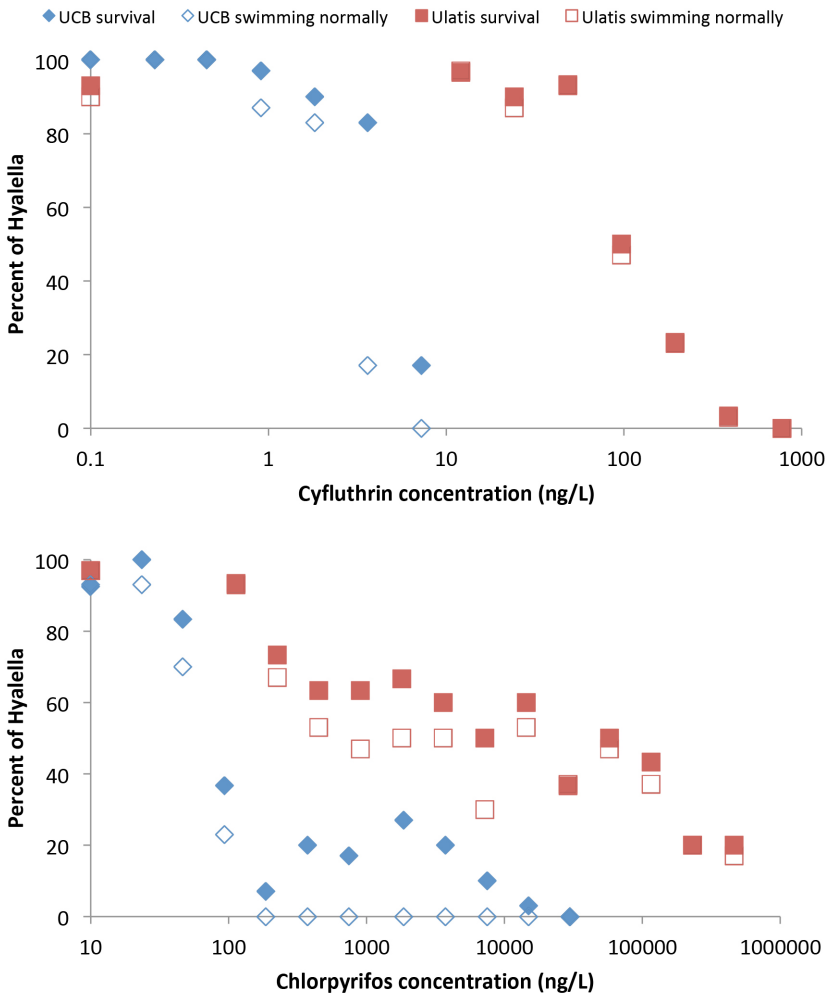

Figure 8 Concentration:response curves for cyfluthrin and chlorpyrifos exposures using both the standard U.S. laboratory-cultured *H. azteca* from the UCB laboratory and the resident *H. azteca* population from Ulatis Creek. Data are shown for both the mortality and paralysis endpoints, though at many concentrations the results were identical and the symbols are superimposed.

of 4.8 ng L^{-1} (95% confidence interval 3.9-6.2; Weston et al. 2013). The EC50, which incorporates not only death but paralysis as well, was 2.3 ng L^{-1} (1.9-2.7). The Ulatis Creek animals from site UB, for comparison, had an LC50 of 110 ng L^{-1} (91-133) and an EC50 of 112 ng L^{-1} (93-135). That is, they were 20- to 50-fold less sensitive to the pyrethroid cyfluthrin. At cyfluthrin concentrations causing near total paralysis or death in the UCB animals, the Ulatis Creek residents showed no adverse effects.

Similar resistance was observed to chlorpyrifos (Figure 8, *lower panel*). All the UCB animals were paralyzed, and about 80% dead, after 96-h exposures to 100-200 ng L^{-1} chlorpyrifos. Though a minority were able to survive higher concentrations, they were all paralyzed. In comparison, only a small number of the Ulatis Creek population began to show effects at 100 ng L^{-1}, and much of the population could tolerate concentrations orders of magnitude higher. At the highest concentration tested of 462,000 ng L^{-1}, about 20% of the Ulatis animals were still alive and swimming normally. Chlorpyrifos 96-h LC50s were 172 ng L^{-1} (128-231) and 17,758 ng L^{-1} (9,224-34,184) for UCB and Ulatis Creek animals, respectively. EC50s were 66 ng L^{-1} (57-77) and 5,509 ng L^{-1} (2,940-10,323), respectively. The data suggest that the Ulatis population contained both resistant and non-resistant individuals, but the chlorpyrifos

tolerance of the Ulatis Creek resident population when tested as a whole was approximately 100-fold greater than that of the laboratory animals.

Genetic sequencing of the resident populations indicated the presence of both several distinct species within the *H. azteca* complex, and multiple mutations associated with pesticide resistance. The dominant clade in the Cache Slough complex was species C, represented by 34 out of the 57 individuals sequenced (Table 1, Appendix E). At most sites, there was also a small proportion of individuals representing species that fell into the "B/F" clade, after Major et al. (2018), though at site RV on the Sacramento River, the wild population entirely comprised species in this clade. The Ulatis Creek site (UB) was unique in containing only species D, known from many other locations in California (Major et al. 2018; Poynton et al. 2018). Although it is possible that the *Hyalella* in Ulatis Creek truly are a different species group (D) than those further seaward in Cache Slough (C), the difference is more likely attributable to the fact that the UB site was sampled 4 months before the others. Temporal shifts in dominance at a given site have been documented (Weston et al. 2013; Weston et al. 2018), and we know from unpublished data of animals collected from UB at other times that species C occurs at the site.

Very few individuals within the Cache Slough complex carried the wild-type allele at the *vgsc* locus, with most bearing one or more mutations associated with resistance to pyrethroid pesticides (Table 1). Wild-type alleles were found only at upper Lindsey Slough (LU) and Rio Vista (RV), but, even at these sites, most individuals had at least one allele with a mutation. Of the 57 individuals sequenced for this study, only one animal, at site RV, was homozygous wild-type at the *vgsc* locus.

We found four different *vgsc* mutations among the individuals sequenced. Most common was substitution of isoleucine for leucine at position 925 (i.e., L925I). This mutation was fixed in the population at Ryer Island (RI) and in the lower portion of Lindsey Slough (LL), and was very common at all other sites. The Ulatis Creek population had two additional mutations not seen elsewhere in the Cache Slough complex: One was valine substituted for leucine at the 925 position (L925V), and the other was a leucine substitution for methionine at position 918 (M918L). A fourth mutation was a phenylalanine-for-leucine substitution at position 936 (I936F), found in half the individuals at Rio Vista, and less frequently in the Toe Drain.

Table 1 Species identify and genotype frequencies for the *vgsc* locus in *Hyalella* populations collected at six different sites in the Cache Slough complex. The percentage of individuals ($n = 8–10$) with the given homozygous (*on left*) or heterozygous (*on right*) genotypes is given for each site. See Appendix E for detailed genotypes for each individual. WT = wild-type.

		Homozygous		Heterozygous			
Site	Species group	WT	L925I	WT/ L925I	M918L/ L925I [a]	L925I/ L925V	L925I/ I936F [a]
UB	D [b]		60		30	10	
LL	B/F [c], C		100				
LU	C		20	80			
TD	B/F [c], C		80				20
RI	B/F [c], C		100				
RV	B/F [c]	12.5	25	12.5			50

a. For M918L/L925I and L925I/I936F heterozygous mutations, the assay used in the present study cannot distinguish which allele the mutations are on. In previous work we have not found any individuals with both resistant mutations on a single allele, and therefore, our best approximation is that they are heterozygous for those two mutations.
b. While only species D was found in the present study, sampling of the site at other times has also found species C individuals.
c. While species B and F were distinguishable in previous work (Major et al. 2018) in the current data set it could not be determined to which of these two groups the individuals belonged.

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The mode of toxic action for organophosphate pesticides differs from that of pyrethroids. These insecticides target acetylcholinesterase, and mutations at the *ace-1* locus can confer resistance. All six sampling locations in the Cache Slough complex (Table 2) contained at least some individuals with serine substituted for glycine at position 119 at the *ace-1* locus. This mutation is associated not only with organophosphate resistance, but resistance to carbamate insecticides as well, a group that similarly targets AChE (Essandoh et al. 2013). This mutation was most prevalent, with 70-90% of the individuals bearing it in Ulatis Creek (UB) and the Toe Drain (TD). It was least common at Ryer Island (RI), but still found in 20% of the individuals. The mutation was found only in the heterozygous state.

Table 2 Genotype frequencies for the *ace* locus in *Hyalella* populations collected at six different sites in the Cache Slough complex. The percentage of individuals ($n = 8-10$) with either a homozygous wild-type or heterozygous genotype at position 119 is shown. See Appendix E for detailed genotypes for each individual. WT = wild-type.

Site	Homozygous WT	Heterozygous WT/G119S
UB	10	90
LL	56	44
LU	70	30
TD	30	70
RI	80	20
RV	56	44

DISCUSSION

The Contaminant Environment of Cache Slough

From the broadest perspective, two general conclusions can be drawn from this work. First, the occurrence of toxicity closely parallels the TSS and target pesticide concentrations. That is, wherever the turbid runoff was seen, subsequent analyses indicated elevated pesticide concentrations and increased toxicity. For all three measures: (1) effects were more pronounced at the uppermost end of Cache Slough, and diminished with distance seaward, (2) effects extended farther seaward after the March storm than they did after the January storm, and (3) Liberty Island waters were largely unaffected by the physical, chemical, and biological storm-related events that occurred in adjacent Cache Slough.

Second, our results show that, like most inhabitants of water bodies at the urban/agricultural interface, the aquatic organisms in Cache Slough are continuously exposed to a wide variety of compounds derived from multiple human activities. The potential toxicological effects of pesticides are often the focus of investigative attention, understandably because these compounds are inherently designed to cause mortality or at least an adverse, sublethal effect. Furthermore, efforts often focus on single pesticides, as if they existed in isolation or, at best, there may be very limited information available on how two different classes of pesticides interact. However, the present study illustrates that such a perspective is overly simplistic. Current science provides no guidance whatsoever on the potential toxicity of simultaneous exposure to, for example, three pyrethroid pesticides, fipronil, multiple fipronil degradation products, several fungicides, an artificial sweetener, an anti-seizure medication, and a flame retardant. However, that is precisely the environment that exists in Cache Slough, and undoubtedly in most other water bodies influenced by our current urban, industrial, and agricultural landscapes. The pharmaceuticals and personal care products, in particular, have been brought into widespread use with no *a priori* expectation that they would even reach aquatic systems; thus, we know almost nothing about their toxicity to aquatic organisms.

Given the current state of science, we are limited to considering single compounds or related compounds within a single group, but even at this level of analysis there is cause for concern about two pesticide groups found in the present study: the fiproles and the pyrethroids.

Fiproles within Cache Slough

Fipronil provides an example of a compound for which concentration profiles were helpful in source identification. The vast majority of the fipronil-containing products registered in

California are for application to pets or for indoor uses, though a small number are registered for structural pest control, largely to control ants and termites. There are no registered fipronil-containing products for production agriculture in California. Washing pets (and hands, after petting animals treated with fipronil) results in sustained discharge of fipronil to municipal wastewater treatment plants (Teerlink et al. 2017; Sadaria et al. 2017). However, the concentration pattern for fipronil observed in Cache Slough, with increased concentrations after rain began, suggests that — at least during the runoff events monitored in this study — outdoor wash-off was a more significant source than baseline discharge from wastewater treatment plants.

The fiproles were among the most ubiquitous compounds of all those measured, with both the parent fipronil and three of its degradation products (the amide, sulfone, and desulfinyl) found in every water sample collected. One of the species more sensitive to the fiproles is the chironomid, *Chironomus dilutus* (Weston and Lydy 2014), an especially significant fact considering chironomids serve as the primary diet item in the Cache Slough complex for out-migrating juvenile Chinook Salmon (Sommer et al. 2001). Although concentrations capable of causing mortality to *C. dilutus* (Weston and Lydy 2014) are well above those observed in the present study, environmentally relevant concentrations can affect their movements. Reported 96-h *C. dilutus* EC50s for inhibited movement (inability to perform a distinctive thrashing movement upon disturbance) have been reported as 30-35 ng L^{-1} (Weston and Lydy 2014). The peak concentration fipronil found (27.8 ng L^{-1}, site UB in April) was very near that EC50. Several of the fipronil degradation products are even more toxic than the parent compound (Schlenk et al. 2001), and the *C. dilutus* EC50 for fipronil-sulfone is 7.5-7.9 ng L-1 (Weston and Lydy 2014). Four out of 57 samples in the present study had sulfone that exceeded these values (all at UB or C1), with the peak concentration of 19.5 ng L^{-1} (site UB in April). We should note that although the EC50 provides a benchmark against which to compare measured values, it represents a concentration that causes effects to half the exposed population, so it should not be construed as an environmentally protective threshold. That four samples exceeded the EC50 indicates that observed concentrations of fipronil-sulfone could affect the sensitive species of the benthic community, at least within Ulatis Creek.

The fiproles are commonly found in urban waterways of California, often exceeding concentrations of concern (Budd et al. 2015). The concentrations seen in the Cache Slough area are actually lower than commonly found elsewhere, such as the median fipronil of 33 ng L^{-1} (Budd et al. 2015) reported in northern California water bodies. Because of potential toxicity, in 2017, the California Department of Pesticide Regulation accepted a new California-specific label for fipronil-containing products with restrictions on how professional pesticide applicators may use the pesticide around structures, including a complete prohibition on use during the rainy season. There has not yet been sufficient time to establish if these label changes have reduced surface water concentrations.

Causes of *In Situ H. azteca* Toxicity

The *in situ* deployments of the standard laboratory-cultured strain *H. azteca* demonstrated toxicity limited to the extreme upper end of Cache Slough after the January storm, but extending at least 8 km down the slough after the March storm. From 2011 to 2016, we have sampled Cache Slough after seven storms (Weston et al. 2014; this study), and we have observed toxicity after five of them (Figure 9). The earlier work involved collected water being transported to the laboratory for toxicity testing, whereas this study used *in situ H. azteca* testing, but the results were similar. Winter storm runoff that enters Cache Slough via Ulatis Creek almost always carries substances that induce paralysis or death in sensitive organisms. The geographic extent of this effect varies, depending on factors such as the intensity of the storm and pesticide use patterns, but penetration of toxicity to varying distances into the Slough, up to at least 8 km, is apparently common. In observations to date, Liberty Island has been unaffected, and the toxicity has been reduced below measurable levels by the time

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water reaches the point where the Deep Water Ship Channel joins Cache Slough.

The fiproles were unlikely to have played in role in the toxicity observed, or to represent a threat to resident *H. azteca* of Cache Slough. The 96-h LC50 for *H. azteca* has been reported as 426-748 ng L^{-1} for fipronil-sulfone, and over 1,000 ng L^{-1} for fipronil and fipronil-sulfide (Weston and Lydy 2014). Even the concentrations that impair swimming in *H. azteca* are at least 10 times greater than any observed, with EC50s of approximately 200 to 700 ng L^{-1}, depending on the specific fiprole. Similarly, although chlorpyrifos has been previously observed in the study area at concentrations as high as 80 ng L^{-1} as a result of agricultural discharges, and likely caused *H. azteca* toxicity on that occasion (Weston et al. 2014), during this study it never exceeded 12 ng L^{-1}, a concentration well below those causing measurable effects (Figure 8), and was not considered a contaminant of concern during the sampling period.

There is, however, abundant evidence that pyrethroid insecticides were responsible for the observed toxicity. As previously shown in Figure 6, total pyrethroid concentrations of 1-10 ng L^{-1} were routinely found in Cache Slough after rains, with concentrations one to two orders of magnitude greater than that occasionally seen in Ulatis Creek. The observed concentrations exceeded benchmarks for toxic effects in *H. azteca*. For example, for the four compounds we summed and referred to as "total pyrethroids," their *H. azteca* 96-h EC50s have been reported

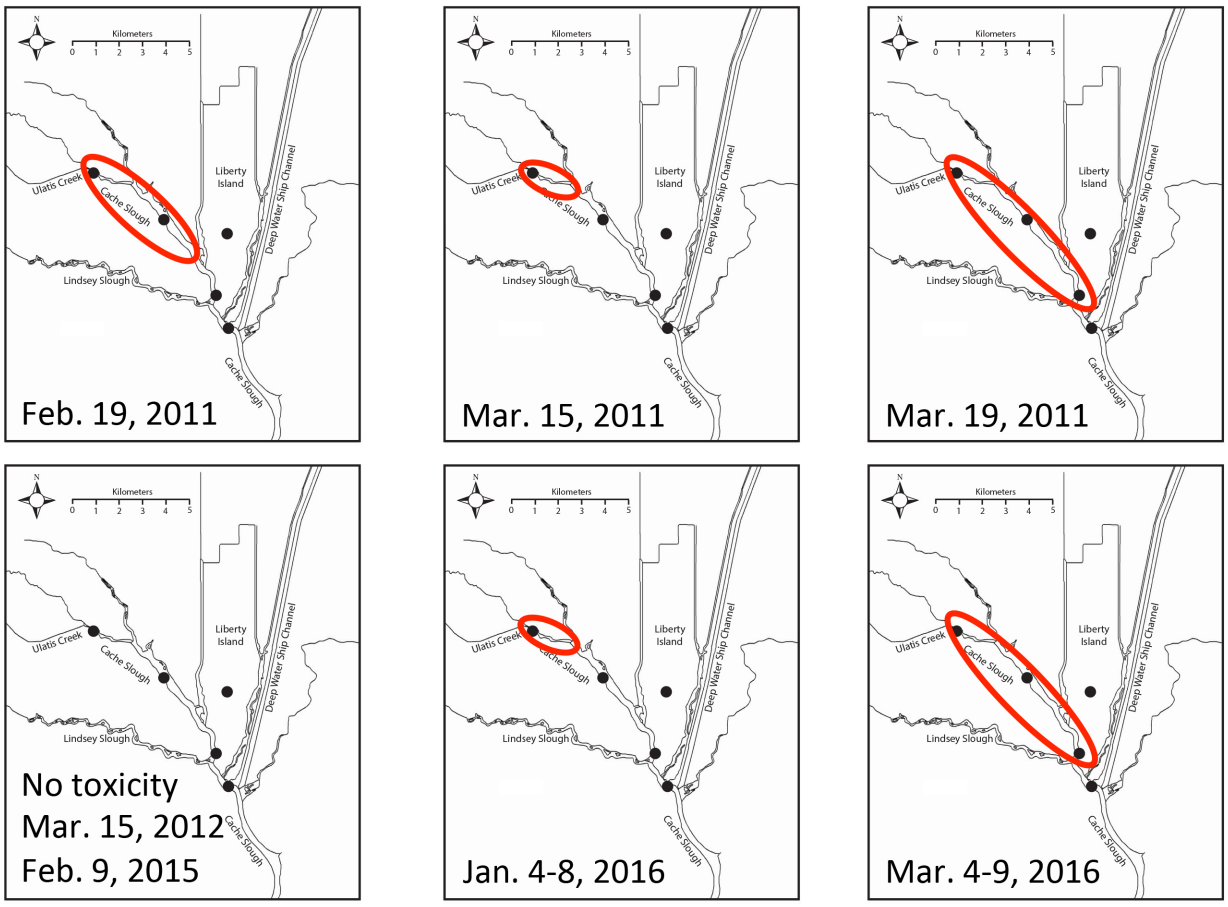

Figure 9 Spatial extent of toxicity in Cache Slough after seven storms as measured by testing with the standard laboratory strain of *H. azteca*. The *red circles* enclose the sites with toxicity, with their boundaries drawn approximately midway between toxic and non-toxic locations. Data from Weston et al. (2014) and present study.

to all fall within the range of 1.3-3.5 ng L^{-1}, with 96-h LC50s of 1.7-8.2 ng L^{-1} (Weston and Jackson 2009; Maund et al. 1998). Using an unpublished data set with different testing methodology, California regulators reported *H. azteca* LC50s to fall within 0.3-0.56 ng L^{-1} (CVRWQCB 2017). The same source reported the 5th percentile of each compound's species sensitivity distribution to all fall within 0.7-1.0 ng L^{-1}. Thus, regardless of which benchmark one chooses to use, the routinely observed 1-10 ng L^{-1} in Cache Slough exceeds it — potentially by as much as an order of magnitude. Although we recognize suspended solids in the water can reduce pyrethroid bioavailability and confound direct comparisons between actual measured pyrethroid concentrations and lab-derived toxicity benchmarks (Yang et al. 2006), quantitative adjustment for the role of TSS cannot be done reliably, especially considering its large variation over the duration of the *in situ* deployment. Furthermore, although suspended sediment could make pyrethroids less toxic than these numerical benchmarks would suggest, the opposite effect would result from the low winter temperatures of Cache Slough (11-13 °C) that approximately triple the toxicity of pyrethroids to *H. azteca* relative to the warmer temperatures at which the benchmarks were derived (Weston et al. 2009). So as a first approximation, pyrethroid concentrations appear more than adequate to explain the mortality and paralysis observed.

The *in situ* tests with multiple strains of *H. azteca* that varied in their sensitivity provide additional and compelling evidence of pyrethroid-related toxicity. At every site where toxicity (and at times near total mortality) to the standard U.S. laboratory strain of *H. azteca* was observed (C1 in January; C1, C2, and C3 in March), the strains that bore mutations making them resistant to pyrethroid toxicity were entirely unaffected. Furthermore, two different pyrethroid-resistant strains were used, and both showed identical results. The value of this innovative approach in establishing causality lies in its ability to eliminate confounding variables. Had we simply deployed the laboratory strain *in situ* and seen toxicity, it would be difficult to rule out potential contributions to toxicity by TSS, food availability, flow, or any other of a host of factors that concurrently vary during a large runoff event. Yet when multiple strains of *H. azteca* are used — some resistant to pyrethroids and others not — and significant differences in survival are seen, the potential that these differences can be attributed to factors other than pyrethroids is considerably diminished.

Sources of Pyrethroids to Cache Slough

Three potential major contributors of pyrethroids to Ulatis Creek are: (1) urban runoff that carries residues from pesticide applications to structures and landscaping, (2) municipal wastewater discharged from the Vacaville Easterly Wastewater Treatment Plant, and (3) agricultural runoff discharged from multiple drains along Ulatis Creek and its tributaries. This study was not designed to establish the relative importance of these various sources, but some conclusions are possible.

Deltamethrin was only occasionally detected, but it certainly originated from urban runoff or municipal wastewater, because the compound is not used agriculturally in Solano County, in which most of the watershed lies (CDPR 2017). Similarly, esfenvalerate almost certainly originated agriculturally, because agriculture in Solano County accounts for about 90% of its total county-wide usage (CDPR 2017).

Bifenthrin, cyfluthrin, cyhalothrin, and cypermethrin all have substantial use in both agricultural and urban environments in the county (CDPR 2017). All have been detected in either Vacaville stormwater runoff or in the creeks at the downstream boundary of the city (Weston and Lydy 2010; Weston et al. 2014), so urban sources are undoubtedly a partial contributor, though additional inputs to Ulatis Creek from the wastewater treatment plant or agricultural drains are also possible (Weston and Lydy 2010). However, the dramatically elevated concentrations of cyhalothrin observed in the April sampling period were highly likely to originate from agricultural sources, probably from application to alfalfa fields (CDPR 2017). We observed 230.7 ng L^{-1} cyhalothrin in the discharge from the Highway 113 agricultural drain to Ulatis Creek on April 7, 2017, concurrently with finding

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149.4 ng L^{-1} at site UB, 4.5 km downstream of the drain. This finding is similar to a previously reported 1,235 ng L^{-1} cyhalothrin being released from the same drain on March 18, 2011, and the concurrent appearance of 27 ng L^{-1} at UB (Weston et al. 2014). The same drain released a remarkably high concentration of 453 ng L^{-1} chlorpyrifos on March 13, 2012, corresponding to the finding of 28 ng L^{-1} chlorpyrifos at site UB (Weston et al. 2014). Although this drain can at times carry urban runoff from Dixon, California, supplementing its usual agriculturally-derived flow, urban runoff in the region typically contains <5 ng L^{-1} cyhalothrin or chlorpyrifos (Weston and Lydy 2010; Weston et al. 2014; Weston et al. 2018), indicating that the observed concentrations >100 ng L^{-1} were likely agricultural. However, we cannot rule out the possibility that other agricultural inputs, in addition to the Highway 113 drain, are further contributors to Ulatis Creek, and eventually Cache Slough.

Pesticide Resistance in Resident Hyalella

Hyalella azteca is recognized to be a species complex with many members. Coincidentally, however, the *Hyalella* species most commonly found in the Cache Slough complex is actually the same species as is widely used for toxicity testing in laboratories throughout the U.S., both being species C (Weston et al. 2013). Thus, one would expect toxicity testing with the laboratory strain to be an ideal choice when trying to assess risk to the resident animals. Yet there is at least one critical difference between the laboratory strain and the residents: Our data show that the resident animals carry any of four mutations that make them resistant to pyrethroid toxicity. The diversity of pyrethroid resistance mutations found in the Cache Slough complex is striking, but all likely work in the same manner. The target site for the pyrethroid molecule is the voltage-gated sodium-channel protein, and, once bound to it, the pyrethroid disrupts sodium flux across the nerve cell membrane, causing tremors, paralysis, and, ultimately, death (Davies et al. 2007). The mutations found all consist of the substitution of only a single amino acid, but all likely serve to alter the configuration of the protein, and thereby inhibit the pyrethroid's binding to its target. As a result, we found the LC50 of Ulatis Creek individuals to be at least 20 times higher than that of the standard laboratory strain. These same mutations, and similarly elevated LC50 values, have been found in *H. azteca* throughout California wherever there has been a history of pyrethroid exposure (Weston et al. 2013; Major et al. 2018).

Only one individual, out of 57 individuals sequenced, was homozygous for the wild-type gene, thus lacking any pyrethroid resistance. The wild-type allele, usually heterozygous with a mutant allele, was found only at the extreme upper end of Lindsey Slough (LU) and at Rio Vista (RV). These two sites would *a priori* be expected to be the least exposed to pyrethroids. Site LU is outside of the irrigated agricultural lands that surround Cache Slough, and instead is surrounded by rangeland where little pesticide use would be expected. Site RV lies in the Sacramento River, thus providing opportunity for considerable dilution of waters coming from the Cache Slough complex. The sites where only mutant alleles were found (UB, LL, RI, TD), are all either in or within a few kilometers of areas found in the present study to be affected by pyrethroids, or they are in waterways not sampled during this study but likely to be exposed to pyrethroids because of their agricultural character (e.g., another location in the Toe Drain, upstream of site TD, contained 3.3 ng L^{-1}L bifenthrin and 1.6 ng L^{-1} cyhalothrin in an earlier study [Weston et al. 2014]). These data indicate that although individuals carrying the wild-type allele are present in some areas of the greater North Delta, they are excluded from inhabiting much of the Cache Slough complex.

Not only are *H. azteca* in the area resistant to pyrethroids, but they also demonstrate resistance to the organophosphate, chlorpyrifos. The chlorpyrifos LC50 of the Ulatis Creek population was 100 times greater than that of the standard laboratory strain. Again, the resistance seems to be a consequence of an amino acid substitution that alters the configuration of the pesticide's target site, though, in the case of organophosphates, it is a mutation of the AChE gene locus. The mutation is well documented in many taxa (Fournier 2005), and is recognized to provide resistance not only to organophosphate pesticides but to carbamate pesticides as well,

since both groups share the same mode of toxic action. The mutant genotype was found in every population studied throughout the Cache Slough complex, though always as heterozygous with the wild-type allele. Unlike the pyrethroid mutations, for which the wild-type genotype is very nearly absent, we found homozygous wild-type individuals that lacked organophosphate resistance to comprise 10-80% of the individuals, depending on location. All other individuals carried at least a single copy of the wild-type allele. This finding suggests that exposure to organophosphate and/or carbamate pesticides in the Cache Slough area has exerted a selective pressure for mutation that confers resistance, but retention of at least one copy of the wild-type allele may be necessary for proper AChE functioning, resulting in the death of any homozygous mutant individuals. This constraint would lead to a substantial lost reproductive capacity (i.e., 25% of the offspring from a pairing of two heterozygous individuals may be non-viable), suggesting a high fitness cost of the mutation. Several studies in mosquitoes support a high fitness cost of the G119S mutation, reducing the function of the AChE enzyme, and resulting in reproductive and developmental impairment and increased mortality in juveniles (Bourguet et al. 1997, 2004; Berticat et al. 2008; Djogbénou et al. 2010).

There is reason to believe alteration of a species' genome, particularly when it has occurred over an area as large as much of California — as is the case with pyrethroids and *H. azteca* (Major et al. 2018) — is not an environmentally neutral outcome. Since the discovery of pyrethroid resistance in California *Hyalella* is relatively new (Weston et al. 2013), and the discovery of its chlorpyrifos resistance newer still (Major et al. forthcoming), the ramifications of these widespread genetic changes are not yet clear. But theoretical reasons and experimental data suggest the dominance of a mutant genotype that allows population-level survival in the short-term may not be without adverse long-term consequences. In fact, previous investigations have generally viewed adaptation to pollution as itself a significant indicator of ecological impairment (Klerks 2002) and deserving of greater consideration in ecological risk assessment (Klerks and Weis 1987; Medina et al. 2007).

Costs of Resistance

It would generally be assumed that, given enough time, evolution will lead to a genotype that is optimally suited to the organism's environment. If the introduction of a new stressor — in this context, the use of pyrethroids and chlorpyrifos — alters the genotype in response, that change may come at the cost of the organism's becoming less adapted to other environmental factors. Similarly, if the response to the new stressor requires alteration of a metabolically critical enzyme — in this context, the voltage-gated sodium channel or AChE — the altered enzyme may not serve its original metabolic purpose as efficiently. Numerous examples are in the literature of pesticide resistance being achieved only at a significant adaptive cost. *Daphnia* cultured with periodic exposure to the pesticide carbaryl evolved resistance to the pesticide, but at the cost of greater susceptibility to parasites (Jansen et al. 2011), and similar susceptibility to parasites has been seen in pesticide-resistant mosquito strains (Berticat et al. 2002). Mosquitoes homozygous for the same G119S mutation as seen in Cache Slough *Hyalella* were more likely to die during pupation than a non-resistant strain (Djogbénou et al. 2010). Aphids with resistance to pyrethroids through a mechanism similar to that seen in Cache Slough *Hyalella* showed a reduced ability to respond to an alarm pheromone, thus increasing their susceptibility to parasites and predators (Foster et al. 1999). *Hyalella azteca* with the same L925I pyrethroid resistance mutations widely found throughout Cache Slough are less tolerant of heat stress than non-resistant strains, and possibly more susceptible to other toxicants (Heim et al. 2018). Many more such examples in the literature suggest the mutations in Cache Slough that allow survival upon pesticide exposure may come at the cost of greater susceptibility to other abiotic stressors, parasites, or predators.

Loss of Critical Genetic Variability

Resistant populations of *H. azteca* present throughout Cache Slough have survived as a result of evolutionary rescue (Bell and Gonzalez

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2009), where mutations that provided resistance to insecticides allowed the populations to persist after storm-related pesticide pulses. Although evolutionary rescue allows populations to continue, adapted populations may have experienced genetic bottlenecks that resulted in reduced genetic diversity (Bickham et al. 2000). The loss of genetic diversity may persist long after the population appears to have recovered, with several other hidden effects (Bickham et al. 2000; Morgan et al. 2007).

Genetic diversity is a critical component of overall biodiversity. Reductions in genetic diversity, especially as a result of contaminant-induced selection (Medina et al. 2007; Ribeiro and Lopes 2013), can cascade into reductions in species-level biodiversity through several avenues. Spielman et al. (2004) demonstrated that a reduction in genetic diversity in *Drosophila* through inbreeding induced the loss of polymorphic disease resistance alleles, causing increased susceptibility to infection (Spielman et al. 2004). Decreases in genetic diversity may also be associated with reduced phenotypic plasticity, leaving populations more vulnerable to changing environmental conditions (Fasola et al. 2015). Finally, genetic diversity loss through pollution-induced evolutionary rescue may "erode evolutionary potential," making populations more vulnerable to novel environmental challenges (Laroche et al. 2002). For example, when clonal lines of *Daphnia longispina* were selected for tolerance to metals, they were rarely co-tolerant to a pesticide (Lopes et al. 2009). Thus, a population composed of individuals selected for tolerance to one stressor will likely lack the genetic diversity needed for evolutionary rescue if exposure to a second stressor occurs soon after (Ribeiro and Lopes 2013).

Increased Trophic Transfer of Contaminants to Predators

The evolution of resistance allows survival in pesticide-contaminated environments where there would otherwise be mortality. Muggelberg et al. (2017) have demonstrated that *H. azteca* with the same L925I mutation as seen in Cache Slough can bioaccumulate pyrethroids at exposure concentrations that would kill a non-resistant strain, and these body residues can be transferred to a predator upon ingestion. Thus, resistant organisms can acquire elevated body residues of the pesticide without ill effects, thus creating a route of trophic transfer of these body residues that, in the absence of resistance, would not exist. Cache Slough contains very dense populations of *H. azteca* in extensive mats of aquatic plants, and in such environments the species can be a major prey item for fish species such as Prickly Sculpin, bluegill, Tule Perch, Largemouth Bass, and Chinook Salmon (Toft et al. 2003). Widespread pesticide resistance in *H. azteca* could increase the potential of indirect pyrethroid and chlorpyrifos effects on the fish of Cache Slough.

Potential Impacts to Other Taxa

Demographic factors such as large population sizes, short generation time, and high fecundity provide *H. azteca* with high evolutionary potential. Species without these characteristics are unlikely to adapt quickly enough to avoid high toxicity (Bell 2013), and the severe worldwide decline of aquatic insect species may be direct evidence that this is occurring (Sánchez-Bayo and Wyckhuys 2019). For example, the copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi* are important prey for Delta Smelt in Cache Slough (Nobriga 2002). Their 96-h LC50s for bifenthrin and cyhalothrin fall within the range of 16-20 ng L^{-1} (Weston et al. 2014), approximately twice the total pyrethroid concentrations seen in Cache Slough. However, if their pyrethroid sensitivity is temperature-dependent (as it is in most species), and if they respond to temperature similarly as *H. azteca* does, with a tripling of sensitivity at the 11-13 °C of Cache Slough in winter, then their pyrethroid LC50s would fall to within the range of concentrations commonly found in upper Cache Slough, and copepod toxicity is possible. Similarly, exposures of fish species, such as the Inland Silversides (*Menidia beryllina*), to bifenthrin concentrations as low as 0.5 ng L^{-1} (15% of the maximum seen in Cache Slough) resulted in a reduction in reproductive output and offspring viability, biased sex ratios, and developmental deformities across multiple generations (Brander et al. 2013; White et al. 2017; DeCourten and Brander 2017). Concentrations of bifenthrin found in this

study have also been shown to alter predator avoidance behavior via olfactory mechanisms in Inland Silversides (Frank et al. 2019), and neurodevelopment in both Inland Silversides and zebrafish (*Danio rerio*) (Frank et al. 2018, 2019). In short, although resident *H. azteca* may no longer be at risk of direct pesticide toxicity because of past and ongoing selective pressures, other species in the system cannot be assumed to possess comparable resistance.

Regulatory Implications of Pesticide Resistance

Cache Slough is an example of a habitat where the results of a toxicity test widely used for regulatory decisions suggest that a contaminant input presents a risk to resident biota, yet because that input has continued for decades, the resident population of the very same species as used in the testing have evolved resistance, and are likely now unaffected by that input. Given the wide occurrence of pyrethroid resistance in *H. azteca* throughout California (Major et al. 2018), there are undoubtedly many similar situations elsewhere. Pyrethroid manufacturers and registrants have argued that regulatory limits should be relaxed when the resident animals have attained resistance, as indicated by the following excerpts from their filings with California regulatory agencies:

> *"Moreover, field populations of Hyalella have been reported to be the most dominant taxa in California water bodies such as Pleasant Grove Creek (a 303(d) listed water body based on pyrethroids) (Hall et al. 2014b), and native Hyalella have been reported to be much more tolerant of pyrethroids such as bifenthrin and cypermethrin than laboratory reared Hyalella (Clark et al.* [2015]*). Results from the field studies described above would certainly question the adoption of water quality objectives that are based solely on impacts to laboratory reared species for assessing the possible impacts of pyrethroids to resident aquatic taxa found in the environment."* (Dunham 2014, unreferenced, see "Notes")

> *"Third, other available evidence suggests that the UCD [water quality] criteria, and as updated in 2015, are overly conservative because the criteria derived are based on toxicity tests using sensitive laboratory Hyalella azteca, which do not necessarily reflect the native populations in California's water bodies."* (Dunham 2017, unreferenced, see "Notes")

Such arguments obscure the fact that it is not "native *Hyalella*" that are much more tolerant of pyrethroids. It is native *Hyalella* from environments in which long-term releases of pyrethroids have exerted a selective pressure for a resistant genotype (i.e., they have been inadvertently selectively bred). Multiple studies have shown that in areas with little or no pyrethroid use, the native *Hyalella* have a pyrethroid sensitivity comparable to the laboratory-reared populations (Weston et al. 2013; Clark et al. 2015; Weston et al. 2015; Major et al. 2018). Furthermore, this argument only serves to reward environmentally irresponsible behavior by essentially claiming that if, through chronic exposure, a discharger can eliminate wild-type individuals and foster the spread of mutations conferring resistance, then the pesticide tolerance of the resistant population should guide derivation of water quality objectives.

Additionally, the pyrethroid registrants argue that if no degradation is evident in benthic community assessments (i.e., the *Hyalella* population remains), then mitigation measures may be unnecessary:

> *"This [pyrethroid sensitivity in laboratory reared Hyalella] has significant implications as the results of standard, regulatory toxicity tests are used by regulatory agencies to characterize the condition of storm water and effluent discharges, ambient surface waters, and sediments. Results from these standard studies can trigger mitigative action, such as determination of compliance by responsible parties (e.g., dischargers) and 303(d) listing. It is recommended that follow-up confirmatory analysis of hypothetically affected in situ communities be evaluated prior to enactment of regulatory decisions that have significant consequences that might not be warranted. By way of example,*

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> *California's sediment quality objectives program...relies on a "triad" of data types, including the results of toxicity tests as well as chemistry data and benthic community data, to make a determination of whether or not a site is impacted. Such complementary data would strengthen the weight-of-evidence of impairment determinations made by regulators."* (Clark et al. 2015)

Benthic community assessments are, by their very nature, blind to a population's replacement of a pollution-sensitive genotype with an insensitive one. The argument implicitly assumes that if a genetic mutation arises which allows continued survival in the presence of pesticide, thus producing no apparent change in benthic community metrics, then no harm has occurred. However, as discussed above, there are many reasons why this assumption may very well be wrong (e.g., costs of resistance, loss of genetic variability, increased trophic transfer of contaminants). Although the topic has received limited study, in no small part because the genetic knowledge and tools needed to address the question have only recently become available and are now advancing rapidly, the available data and theoretical evolutionary considerations suggest it would be a mistake to assume no harm exists.

CONCLUSIONS

The aquatic biota of the Cache Slough complex live within two alternating chemical environments. In dry periods, the system contains a wide variety of substances that originate from wastewater treatment, including pharmaceuticals and personal care products. During rainstorms, these materials decline in concentration, but a variety of insecticides, herbicides, and fungicides enter the system via runoff from the surrounding lands. The toxicological effects of the former environment, if any, are unknown. Some of the toxicological effects of the latter were clearly evident, and have been documented repeatedly in storms over many years. Fipronil — and at least one of its degradates — attain concentrations essentially at effects benchmarks for *C. dilutus*. Pyrethroid insecticides attain concentrations in Cache Slough that are well above those expected to be toxic to sensitive aquatic species; in fact, when the common toxicity testing species, *H. azteca*, is placed in Cache Slough waters, toxicity, and at times near-complete mortality, is seen over lengthy stretches of the waterway. Pyrethroids are likely the cause, because strains of *H. azteca* bearing a mutation that provides resistance to these compounds show no ill effects. Our results suggest that multiple species within the system, beyond *H. azteca*, could be at risk from pesticide toxicity after storms.

Yet, remarkably, given the toxicity testing results, the Cache Slough complex contains dense assemblages of resident *H. azteca*. Genetic sequencing reveals that this population has, as a consequence of a long history of exposure, attained resistance to pyrethroids by any of four different mutations, and also possesses a fifth mutation that provides resistance to organophosphate pesticides and quite likely carbamate pesticides as well. Thus, in Cache Slough, and probably many other areas not yet studied, we have a population that has evolved tolerance to three of the major insecticide classes used in both agricultural and urban environments. Regulatory agencies, and society in general, have not yet grappled with the ramifications of the emergence of pollutant resistance. Should we relax water quality objectives or discharge restrictions developed to protect individuals with the wild-type genotype, if they are no longer there? If non-resistant individuals are eliminated, and replaced by individuals with a resistant genotype, thereby allowing community-level functioning to continue, is that change inconsequential? We offered a variety of reasons above to argue that neither of these views is wise. In the past, society has considered the question of whether "dilution is the solution to pollution," and has decided that, in general, dilution is not a permissible way to attain water quality objectives. We are now faced with the question of whether "evolution is the solution to pollution." As the application of genetic tools becomes increasingly common in ecotoxicology, it is likely to be a question society will need to address sooner than later.

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