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GENOTOXICITY IN NATIVE FISH ASSOCIATED WITH AGRICULTURAL RUNOFF EVENTS

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Abstract—The primary objective of the present study was to test whether agricultural chemical runoff was associated with in-stream genotoxicity in native fish. Using Sacramento sucker (*Catostomus occidentalis*), we combined field-caging experiments in an agriculturally dominated watershed with controlled laboratory exposures to field-collected water samples, and we coupled genotoxicity biomarker measurements in fish with bacterial mutagenicity analysis of water samples. We selected DNA strand breakage as a genotoxicity biomarker and Ames *Salmonella* mutagenicity tests as a second, supporting indicator of genotoxicity. Data from experiments conducted during rainfall runoff events following winter application of pesticides in 2000 and 2001 indicated that DNA strand breaks were significantly elevated in fish exposed to San Joaquin River (CA, USA) water (38.8, 28.4, and 53.6% DNA strand breakage in year 2000 field, year 2000 lab, and year 2001 field exposures, respectively) compared with a nearby reference site (15.4, 8.7, and 12.6% DNA strand breakage in year 2000 field, year 2000 lab, and year 2001 field exposures, respectively). Time-course measurements in field experiments supported a linkage between induction of DNA strand breakage and the timing of agricultural runoff. San Joaquin River water also caused significant reversion mutation in two Ames *Salmonella* tester strains. *Salmonella* mutagenicity corroborated in-stream effects, further strengthening a causal relationship between runoff events and genotoxicity. Potentially responsible agents are discussed in the context of timing of runoff events in the field, concordance between laboratory and field exposures, pesticide application patterns in the drainage, and analytical chemistry data.

Keywords—Genotoxicity Pesticides Field study Native fish DNA strand breaks

INTRODUCTION

Agriculture is the primary land use in California (USA), and pesticides are significant contaminants in major rivers of the state. In 2000, more than 14 million acres of land were dedicated to agriculture, and 188 million pounds of pesticide active ingredient were applied (California Department of Pesticide Regulation Pesticide Use Database, http://www.cdpr.ca.gov/dprdatabase.htm). Of this, 126 million pounds were utilized in the Central Valley. Pesticides are applied throughout the year, but heavy use occurs during the winter months. Significantly, the winter months in the Central Valley are the wettest of the year, and the surface runoff that follows storm events provides the major transport mechanism by which pesticides enter watersheds [1,2]. Acute toxicity to standard test organisms, such as *Ceriodaphnia dubia* and *Pimephales promelas*, have been attributed to specific chemicals in these mixtures [1,3]. Toxicity tests have provided good preliminary data, but other approaches may be necessary to evaluate the effects on native species. Accordingly, biomarker measurements are often the most informative approach for evaluating ecotoxicological effects on nonstandard organisms in the field.

In contrast to several physiologic endpoints, genotoxic responses may reflect ecotoxicological effects of greater importance, because they often have been more convincingly correlated with impairment of development, growth, and reproduction [4–12]. In addition, genotoxic endpoints tend to reflect exposure to a broad range of chemical stressors and may be more appropriate markers for initial screening of complex agricultural chemical mixtures than the chemical-specific measures. Because of their potential ecological importance and broad applications in screening, genotoxic responses to agricultural runoff may be of particular interest to environmental managers and regulators, especially if these responses are demonstrated in multiple test systems and in both the laboratory and the field.

Many potential genotoxicants are applied to agricultural commodities in the Central Valley. Organophosphate insecticides often have been implicated as toxic agents in field runoff events in the Central Valley [1,2], and some are alkylating agents and, thus, potential genotoxicants [13,14]. Other known genotoxicants, such as captan, carbaryl, malathion, methyl bromide, trifluralin, and ziram, also are applied at high rates in the Central Valley (>100,000 pounds of active ingredient in 2000; California Department of Pesticide Regulation Pesticide Use Database). Furthermore, application of the pyrethroid insecticides is increasing, and some evidence indicates that these chemicals are genotoxic in some test systems, including fish [15]. To our knowledge, the genotoxic potential of complex agricultural runoff mixtures has not been tested.

The overall goal of the present study was to combine laboratory and field studies, along with bioindicators and analytical chemistry, to examine whether agricultural runoff events are associated with genotoxicity. Experiments were conducted in the agriculturally dominated landscape of the San Joaquin River watershed, where storm runoff is primarily agricultural runoff. Exposure designs included field caging of fish and controlled laboratory exposures to field-collected water. The DNA strand breakage was applied as a sensitive gen-

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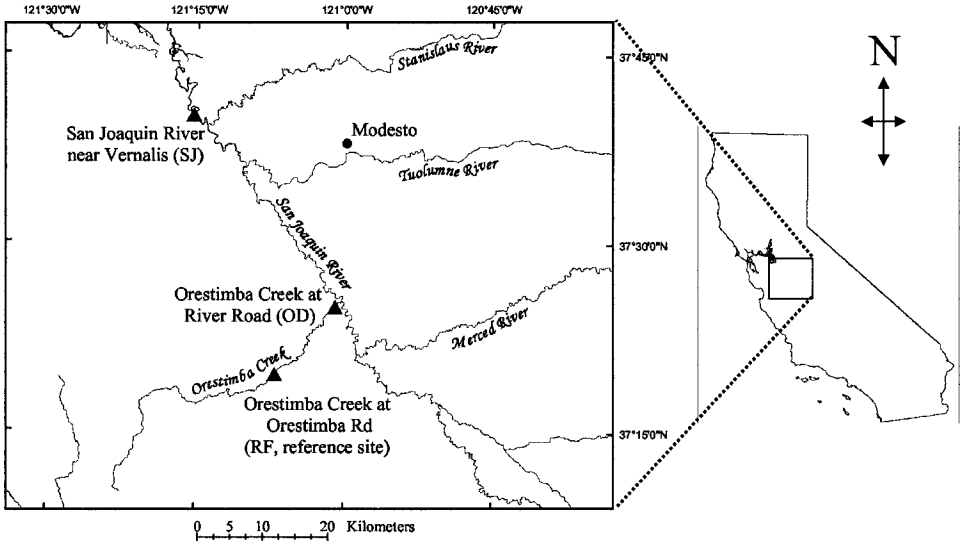

Fig. 1. California (USA) map highlighting the area in which field-caging experiments were conducted. Triangles indicate the three field-caging sites.

otoxicity biomarker that responds to a wide spectrum of chemicals. Mutagenicity of water samples was tested using the Ames *Salmonella* reversion mutation assay to complement DNA strand breakage in fish as a second indicator of runoff genotoxicity. Pesticide concentrations were monitored in the field both as a surrogate for the general timing of agricultural chemical runoff events and to identify potential inducers of genotoxic responses. Treatment comparisons included within-site time-course comparisons as well as comparisons between exposed and reference sites. Examination of genotoxicity associated with agricultural runoff in the field and laboratory may serve as a first step in identifying ecologically relevant damages that may be of importance to managers and regulators.

MATERIALS AND METHODS

Animals and maintenance

Sacramento suckers (*Catostomus occidentalis*) were selected for use in caging and laboratory studies, because they are a widely distributed species native to the Sacramento–San Joaquin watershed [16]. All fish used within each experiment were caught from a single field site and on the same date. Considering that *C. occidentalis* home ranges are considerably larger than the 100-m stretch of river in which they were caught [16], they were most likely from a single population. Suckers were 30 to 50 mm in length (fork length) and were captured from the upper Putah Creek (Napa County, CA, USA) or Russian River (Mendocino County, CA, USA) watersheds above any agricultural inputs and, presumably, had little or no recent history of pesticide exposure. Fish were subsequently maintained for a minimum of two weeks at the University of California, Davis, Bodega Marine Laboratory (BML), in aerated, freshwater flow-through tanks before use in experiments. Water temperatures ranged from 15 to 17°C, and the fish were fed (No. 3 Crumbles; Rangen, Buhl, ID, USA) ad libitum daily.

Characterization of storm events

Streamflow for the San Joaquin River near Vernalis, Orestimba Creek at Orestimba Road, and Orestimba Creek at River Road (CA, USA) were recorded at U.S. Geological Survey gauges located at Vernalis, Newman, and River Road, respectively (U.S. Geological Survey, Surface-Water Data for the Nation, http://waterdata.usgs.gov/ca/nwis). Rainfall was recorded at Modesto (California Irrigation Management Information System gauge). Personal communication with local agricultural commissioners indicated the timing of major pesticide applications. Field-caging experiments were timed to coincide with the first major rainstorm event (≥0.5 inches of rain within a 24-h period and sufficient to cause a rise in the hydrograph) following application of winter-season pesticides to orchards.

Field caging

Field-caging sites (Fig. 1) included the mainstem San Joaquin River near Vernalis (SJ) downstream of all tributaries (downstream of agriculture), Orestimba Creek at River Road (OD) just above the confluence with the San Joaquin (downstream of agriculture), and Orestimba Creek at Orestimba Road upstream of all agriculture (reference site [RF]). The San Joaquin River drains a very large landscape area (19,002 km^2), whereas the Orestimba Creek watershed is significantly smaller (603 km^2).

During year 2000 and 2001 field-caging experiments, multiple cages (eight replicate fish per cage) were deployed at each site, and cages were subsequently retrieved at different time points to evaluate changes in biomarker responses during the rise and fall of pesticide concentrations. Water temperature and dissolved oxygen concentrations were monitored daily.

In 2000, rain from a large storm started on February 12, and all cages were deployed on February 13 before hydrographs started to rise (Fig. 2). Timing of pesticide pulses was predicted based on the stage of the hydrograph and observed sediment loading, and these predictions were used to select time points for cage recovery. Two cages were deployed at OD. The first cage was retrieved on February 14, 12 h after predicted peak pesticide concentrations, and the second cage was retrieved on February 19, 6 d after predicted peak pesticide concentrations. Three cages were deployed at SJ. The first cage was retrieved on February 14, immediately following predicted peak pesticide concentrations. The second cage was retrieved

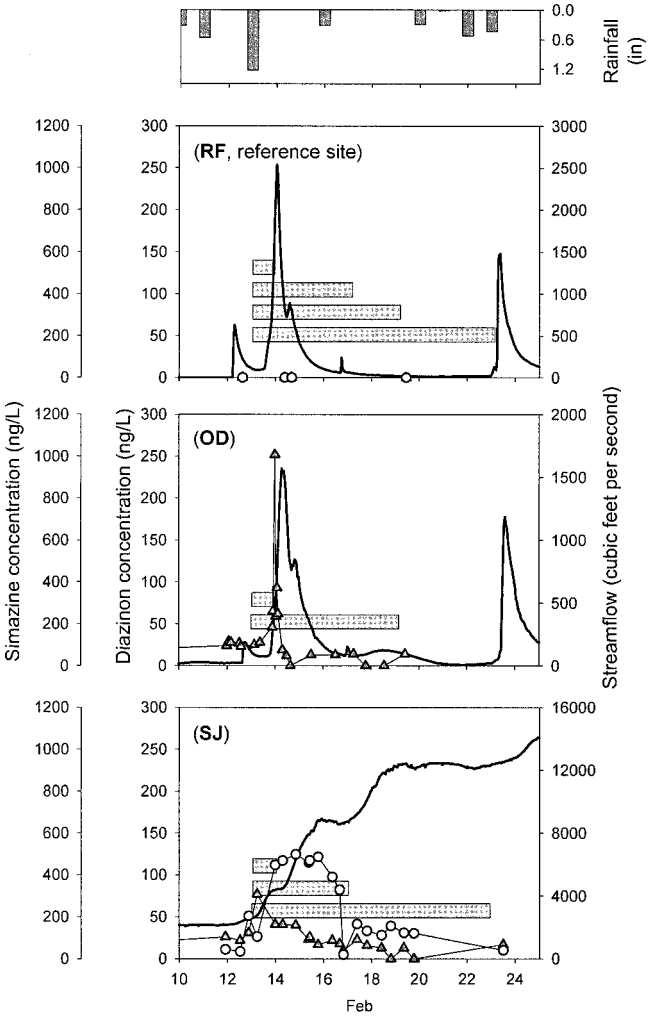

Fig. 2. The 2000 winter storm events following pesticide application and timing of field-caging experiments at the reference field site (RF), Orestimba Creek downstream (OD), and San Joaquin River near Vernalis (SJ; all CA, USA). Vertical bars at the top represent rainfall at Modesto (CA, USA). Bold lines represent streamflow. Thin lines with closed triangles and open circles indicate concentrations of diazinon and simazine, respectively. Broad horizontal bars indicate the duration of caging times for individual cages.

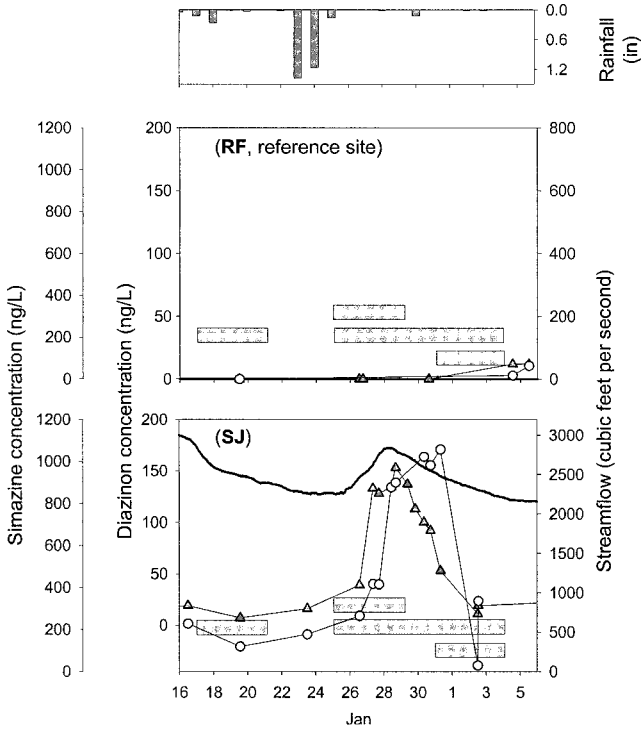

Fig. 3. The 2001 winter storm events following pesticide application and timing of field-caging experiments at the reference field site (RF) and San Joaquin River near Vernalis (SJ; all CA, USA). Vertical bars at the top represent rainfall at Modesto (CA, USA). Bold lines represent streamflow. Thin lines with closed triangles and open circles indicate concentrations of diazinon and simazine, respectively. Broad horizontal bars indicate duration of caging times for individual cages.

on February 17, 4 d after predicted peak pesticide concentrations, and the third cage was retrieved on February 23, 10 d after predicted peak pesticide concentrations. Four cages were deployed at RF, and retrievals were paired with retrieval of both SJ or OD cages.

During 2001, early cage deployments were included to obtain baseline, prerunoff biomarker measurements. One set of cages was deployed on January 17 and retrieved 4 d later (January 21) at SJ and RF before any runoff events (Fig. 3). Additional cages were deployed to obtain typical mid- and post-storm measurements. Rain from a large storm began on January 24, and cages were deployed on January 25 before hydrographs started to rise. Two cages were initially deployed at the San Joaquin site. The first cage was retrieved on January 29, immediately following predicted peak pesticide concentrations, and the second cage was retrieved on February 4, 6 d following predicted peak pesticide concentrations. A third cage was deployed on January 31 (at both SJ and RF), after the predicted peak pesticide concentrations had passed, and was retrieved on February 4. The purpose of the late deployment was to compare biomarker responses to responses of fish that were exposed to the main pesticide pulse (but retrieved on the same day) to distinguish acclimation from recovery responses. At RF, cage deployments and retrievals were timed to coincide with caging at SJ. For year 2000 and 2001 experiments, pesticide concentrations were measured at many time points throughout the storm events (Figs. 2 and 3).

Laboratory exposure to field-collected water

In 2000, composite water samples were collected concurrently with the field-caging experiment and returned to the BML for controlled laboratory exposures. These field-water samples were collected when pesticide concentrations were predicted to be highest. Water samples were obtained by pumping directly into 10-gallon, stainless-steel milk cans (or soda kegs) using a Masterflex peristaltic pump (Cole-Parmer Instrument, Vernon Hills, IL, USA) equipped with a single stainless-steel and Teflon® inlet hose suspended in midchannel. The SJ composite was composed of river water collected from February 14 to 16. The OD composite was collected at late night and early morning on February 13 and 14, respectively. The RF sample was collected on February 14. Exposures were initiated within 48 h of composite collections. Sacramento suckers were exposed to field-collected water in the laboratory for 6 d, with water changes every 48 h, and water quality (dissolved oxygen, pH, ammonia, and temperature) was monitored daily. Collected water samples to be used for water changes were maintained at 15°C in a temperature-controlled room. Exposure temperature was maintained at 15 ± 1°C to match temperatures observed in the field, and light exposure was maintained on a natural cycle. Pesticide concentrations

were measured for each composite (see the following section for analytical methods). Fish from both field-caging and laboratory-exposure experiments were killed and weighed, and tissues were excised and archived for subsequent biomarker analysis.

Water, chemistry, sampling, and analysis

Water samples for pesticide analysis and laboratory exposures were collected concurrently with field experiments but under varying schedules depending on the hydrologic characteristics of the individual sites [17]. At SJ and OD, samples were collected from a bridge as midchannel surface grabs or by pumping. At RF, all samples were collected as midchannel grabs or from the shore. All water samples were collected in close proximity to the fish exposure cages at each site and at a depth of 0.5 m beneath the water surface. Water was obtained either by pumping directly into 1-L, amber glass bottles using a Masterflex peristaltic pump (Cole-Parmer) equipped with a single stainless-steel and Teflon inlet hose suspended in midchannel or as single midchannel grabs using a weighted, 3-L Teflon bottle sampler from a bridge and then poured directly into 1-L, amber glass bottles.

Collected water samples were preserved on ice and, within 24 h, were filtered through baked, 0.7-μm glass-fiber filters. Samples were then extracted using C8 solid-phase extraction cartridges. A surrogate compound, terbuthylazine, was added to each sample before extraction to provide quantitative data regarding extraction efficiency. The cartridges were then dried using a syringe to repeatedly force air through each cartridge, frozen, and delivered to the U.S. Geological Survey organic chemistry laboratory in Sacramento, where they were stored frozen for two to six months. Once removed from storage, each cartridge was eluted with 9 ml of ethyl acetate, and internal standards were added. The cartridge was then analyzed using a Varian Saturn gas chromatograph mass spectrometer (Varian, Inc., Palo Alto, CA, USA). Samples collected in the year 2000 were analyzed for 26 individual pesticides, whereas 31 pesticides were analyzed for the year 2001 (Table 1).

Four types of quality-control data were collected: Field and laboratory equipment blanks, replicate samples, matrix spikes, and surrogate recovery. Equipment blanks were analyzed every 20 to 30 samples (totaling two in 2000 and three in 2001); none of the pesticides was detected in the blanks. Replicate samples constituted 33% of the samples analyzed and were within 25% agreement for each of the pesticides detected. As part of the method validation, matrix spike samples accounted for 10% of the samples. Recovery of the surrogate, terbuthylazine, was recorded to assess the efficiency of each extraction, because this compound represents the class of pesticides that is most sensitive to extraction conditions. The average percentage recovery and standard deviation for terbuthylazine was calculated for each year. Sample data were excluded if the recovery of terbuthylazine was outside the control limit of the annual mean ± two standard deviations (95% ± 20%) [17].

Reagents

All chemicals were purchased from Sigma Chemical (St. Louis, MO, USA) unless otherwise noted.

DNA strand break (comet) assay

Methods were based on those described by Singh et al. [18] with modifications. Blood (1–2 μl) was collected by heart puncture, preserved in buffer (100 μl), immediately frozen in liquid nitrogen, and then transferred to −70°C for storage. Storage time of all frozen blood samples did not exceed six weeks. Blood preservation buffer consisted of Hanks balanced salt solution (Ca^{2+}- and Mg^{2+}-free) with 20 mM ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate, and 10% dimethyl sulfoxide (DMSO). The following procedure was conducted under yellow light: Blood samples were thawed on ice, and 7 to 10 μl of cell suspension (~4,500 cells/μl) were mixed in 250 μl of melted 0.5% low-melting-point agarose, placed on a coated slide, and covered with a glass coverslip. Slides were immersed in cold, freshly made lysis buffer (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, pH lysis buffer to 10.0). Then, 1% sodium lauroylsarcosinate, 1% Triton X-100, and 10% DMSO were added for a minimum of 1 h. Slides were then transferred to the electrophoresis apparatus (kept at 4°C) and immersed in alkaline electrophoresis running buffer for 20 min to allow for unwinding of DNA. Twenty-four samples could be electrophoresed at once, and slides were randomly assigned to electrophoresis runs. Slides were electrophoresed for 20 min at 25 V with current adjusted to 0.5 A by raising or lowering the volume of electrophoresis buffer. To visualize DNA strand breaks, slides were stained with a 100-μl volume containing 2 μg/ml of ethidium bromide, and images were captured at ×40 magnification using an Olympus fluorescent microscope (Model BH2-RFCA; Olympus America, Melville, NY, USA). Fifty cells per slide were randomly captured, representing 50 replicate measures per individual. The DNA strand breakage was quantified as the amount of fluorescence in the comet tail divided by the amount of fluorescence in the comet head multiplied by 100 (%DNA in comet tail) using a macro within Scion Image software (Scion Image for Windows 2000; Scion, Frederick, MD, USA).

Table 1. Maximum concentrations (ng/L) of pesticides measured during field-caging experiments and laboraory experiments in 2000 and 2001[a]

	2000			2001	
	SJ	OD	RF	SJ	RF
Atrazine	13.0	11.0	ND	19.3	ND
Butylate	ND	16.0	ND	ND	ND
Carbaryl	ND	ND	ND	31.6	ND
Chlorpyrifos	18.0	11.0	ND	18.2	ND
Dacthal	ND	17.0	ND	10.4	ND
Diazinon	77.0	252.0	ND	154.0	12.0
Diethatyl-ethyl	ND	ND	ND	32.0	ND
Eptam	ND	19.0	ND	ND	ND
Ethalfluralin	ND	21.0	ND	ND	ND
Hexazinone	—	—	—	106.0	17.3
Methidathion	51.0	95.0	ND	33.0	ND
Metolachlor	35.0	226.0	ND	25.6	ND
Molinate	ND	ND	ND	ND	9.4
Napropamide	46.0	ND	ND	90.7	ND
Oxyfluorfen	—	—	—	57.7	23.2
Pendimethalin	43.2	41.0	ND	72.7	ND
Piperonyl butoxide	—	—	—	28.2	16.2
Simazine	487.0	981.0	ND	731.0	26.1
Trifluralin	21.0	41.4	ND	35.4	19.1

[a] All study locations are in California (USA); reference field site (RF), Orestimba Creek downstream (OD), and San Joaquin River near Vernalis (SJ). Pesticides not detected were alachlor, azinphos-methyl, carbofuran, cyanazine, cycloate, fonofos, malathion, methyl parathion, pebulate, phosmet, sulfotep, and thiobencarb. See Orlando et al. [17] for concentrations of pesticides in all field samples. ND = not detected; — = not analyzed.

Ames bacterial mutagenicity assay

The Ames assay was used to test for mutagenicity of composite water samples collected from the field in 2001. Composite collection, transport, and storage procedures were the same as those described for year 2000 laboratory exposures. The SJ composite was comprised of river water collected on January 27, 28, and 29, and the RF sample was collected on January 26.

The plate incorporation assay was conducted according to the method described by Maron and Ames [19] using *Salmonella typhimurium* tester strains TA98 and TA100. Tester strains were supplied by Bruce N. Ames (University of California, Berkeley, CA, USA). Negative controls were sterile water and DMSO solvent control. Positive controls were 2-aminoanthracene (0.1 μg/plate) for TA98 and sodium azide (NaN_3; 10.0 μg/plate) for TA100. Samples tested were organic extracts of BML well water as a laboratory control, RF composite, and SJ composite. The SJ extracts were tested undiluted as well as diluted to 50%, 25%, and 1% of the original extract concentration. The organic fraction of samples was recovered by liquid–liquid extraction and then concentrated 500-fold. Organics from unfiltered composite water samples and the laboratory control were extracted by mixing a 2.0-L water sample with 100 ml of high-performance liquid chromatography–grade hexane in a separatory funnel. The solvent phase was drawn off, and each water sample was extracted three times. The solvent phase was then evaporated using a rotary evaporator, and diluents were resuspended in 2.0 ml of DMSO in an ultrasonic bath for 20 min. Samples were tested in duplicate, and with and without rat liver S9 metabolic activation (S9 obtained from Moltox, Boone, NC, USA).

Statistical methods

Nested analysis of variance (ANOVA) was applied to DNA strand break data to characterize within-individual variation. Field-caging experiments in the year 2000 consisted of a single deployment of multiple cages, and mean DNA strand break differences among cages were tested using a nested two-way ANOVA design with retrieval time point and site as factors. Data were tested for normality and homogeneity of variances, and the T-method was applied following ANOVA to test for significant differences among means. For laboratory exposures in 2000, nested one-way ANOVA was used to test for differences in mean DNA strand breakage in fish exposed to field-collected water samples.

Field-caging experiments in 2001 consisted of three separate deployments. Therefore, ANOVA could not be applied to test for mean differences among all time points. Alternatively, nested one-way ANOVA was used to test for significant difference of mean biomarker responses among pairs of cages (SJ vs RF) for the January 17, 2001, and January 31, 2001, deployments at SJ and RF. For the January 25, 2001, deployment, mean DNA strand break differences among cages were tested using nested two-way ANOVA with time point and site as factors.

Mutagenicity of treatments was considered to be positive if the mean number of colonies growing was greater than double (i.e., twofold) the mean number of colonies growing in the vehicle (DMSO) control [20].

RESULTS

Storm runoff events

The Orestimba Creek sites were characterized by very steep and rapid changes in the hydrograph during year 2000 runoff events, whereas the San Joaquin site was characterized by comparatively broad changes in the hydrograph (Fig. 2). The 2001 storm was not as severe as the storm in 2000, and changes in hydrographs were much less pronounced (Fig. 3).

Pesticide concentrations

The two pesticides with high applications that were detected most consistently in water samples were diazinon and simazine, so these were selected to illustrate the timing of runoff events. Detected pesticide concentrations varied among sites (Table 1), and concentration profiles of different pesticides within sites varied with time. At SJ in 2000, diazinon and simazine profiles overlapped, but peak timing and duration varied (Fig. 2). Peak diazinon concentrations of 77.0 ng/L were reached on February 13, with the pulse lasting approximately 2 d. Simazine concentrations peaked at 499 ng/L at 1.5 d later, and high concentrations were detected for 4 d. All deployed cages were exposed to both pulses. At OD in 2000, no simazine was detected during cage deployments (Fig. 2). Diazinon concentrations peaked at 252 ng/L (greater than threefold higher than at SJ) following the deployment of cages, and the pulse was of very short duration (<24 h). Similar to differences detected in the field, diazinon in composite water samples used for laboratory exposures was highest in the OD composite (152 ng/L) compared to the SJ composite (29 ng/L). In contrast, simazine concentrations were highest in the SJ composite (565 ng/L) compared to the OD composite (69 ng/L). No pesticides were detected in RF water.

Pesticide concentrations were generally higher in 2001 than in 2000. In 2001, diazinon and simazine profiles at SJ again overlapped, but they differed in degree and peak timing (Fig. 3). The duration of the pulses were similar, but diazinon peaked on January 28 at 153 ng/L on January 28 and simazine 3 d later at 1,057 ng/L. Cages deployed on January 25 and January 31 were all exposed to the simazine pulse, whereas only the cages deployed on January 25 were exposed to the diazinon pulse. Diazinon and simazine concentrations in the SJ composite samples used in the Ames assay were similar to peak concentrations observed during field experiments (diazinon, 149 ng/L; simazine, 1008 ng/L). A detailed report of detected concentrations for all pesticides analyzed is found in Orlando et al. [17].

DNA strand breaks

The DNA strand breakage was variable among sites but significantly elevated in both 2000 and 2001 in fish caged at SJ. In 2000, DNA strand breaks were not induced in fish caged at OD (Fig. 4A) but were induced at all time points in fish caged at SJ (Fig. 4B). For the three pairs of cages deployed at SJ and RF on February 13, DNA strand breakage was significantly higher in SJ-caged fish compared to fish caged at the RF ($p < 0.001$) on February 14 (44.6% and 16.3% DNA in comet tail for SJ and RF, respectively), February 17 (33.5% and 13.6% DNA in comet tail for SJ and RF, respectively), and February 23 (38.2% and 16.7% DNA in comet tail for SJ and RF, respectively), whereas strand breakage was not significantly induced in OD-caged fish compared to fish caged at the RF site ($p = 0.142$) (Fig. 4A).

In support of field data, DNA strand breaks were elevated in fish exposed for 6 d in the laboratory to SJ-collected water (year 2000) compared to fish exposed to RF water (Fig. 5). Strand breaks were significantly ($p = 0.007$) induced in SJ-exposed fish (28.5% DNA in comet tail) compared to fish

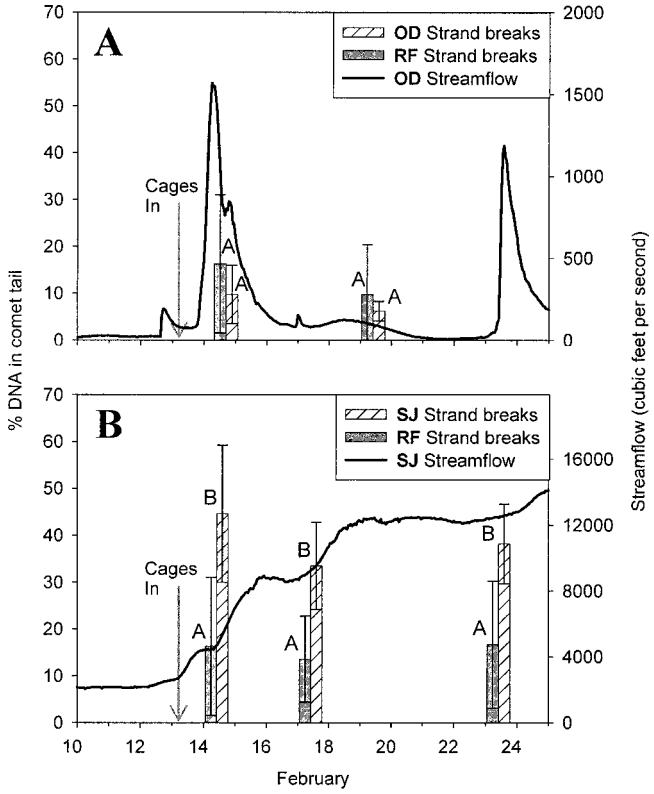

Fig. 4. The DNA strand breaks from the year 2000 field caging at the reference field site (RF), Orestimba Creek downstream (OD), and San Joaquin River near Vernalis (SJ; all CA, USA). Line graphs streamflow. Hatched bars represent mean DNA strand breaks (%DNA in comet tail ± standard deviation) in fish caged at (**A**) OD and (**B**) SJ. Solid bars represent DNA strand breaks in fish caged at RF. All cages were deployed on February 13. Bars with the same uppercase letter have no significant ($p < 0.05$) differences among mean DNA strand breaks.

exposed to RF water (8.7% DNA in comet tail). However, DNA strand breaks in OD-exposed fish were not significantly higher than in RF-exposed fish.

In 2001, DNA strand breaks were again elevated in fish caged at SJ, and more extensive experimental design indicated

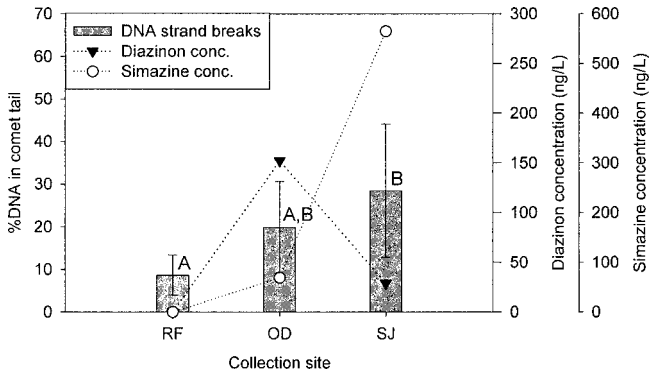

Fig. 5. The DNA strand breaks from year 2000 laboratory exposures to field-collected water at the reference field site (RF), Orestimba Creek downstream (OD), and San Joaquin River near Vernalis (SJ; all CA, USA). Lines with closed triangles and open circles indicate concentrations of diazinon and simazine, respectively. Bars represent mean DNA strand breaks (%DNA in comet tail ± standard deviation) in fish exposed to water collected from field sites. Bars with the same uppercase letter have no significant ($p < 0.05$) differences among mean DNA strand breaks.

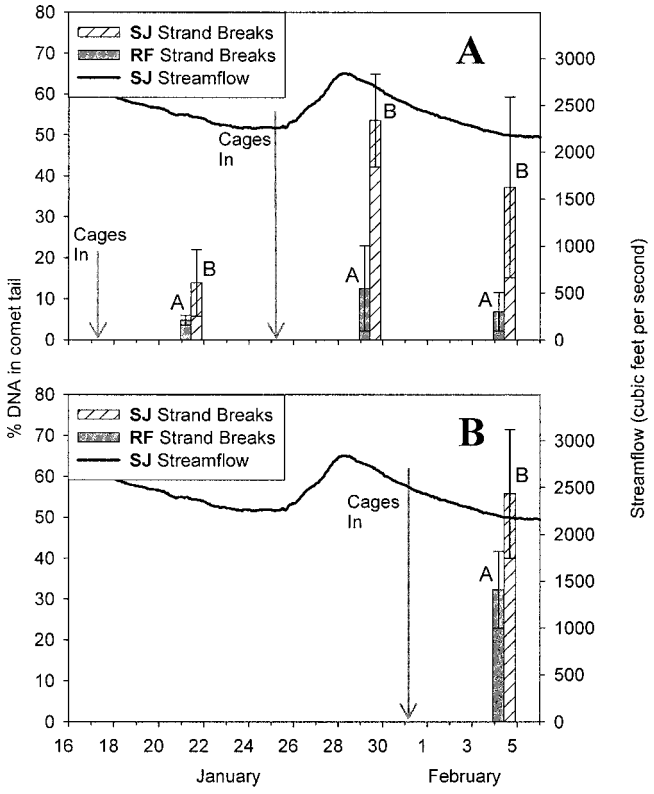

Fig. 6. The DNA strand breaks from the year 2001 field caging at San Joaquin River near Vernalis (SJ) and the field reference site (RF; both CA, USA). Lines represents streamflow. One deployment/retrieval (January 17–21) was prerunoff (**A**). The second deployment/retrieval (January 25 to January 29 or to February 4) captured pesticide runoff (**A**). The third deployment/retrieval (January 31 to February 4) was post–diazinon runoff (**B**). Hatched bars represent mean DNA strand breaks (% DNA in comet tail ± standard deviation) in fish caged at SJ, and solid bars represent DNA strand breaks in fish caged at RF. Bars with the same uppercase letter have no significant ($p < 0.05$) differences among mean DNA strand breaks as determined by nested one-way analysis of variance (for the first and third deployments) or by the T-method following nested two-way analysis of variance (for the second deployment).

that the timing of strand break induction coincided with the timing of the runoff (Figs. 3 and 6). For the pair of cages deployed on January 17 and retrieved before runoff occurred (Fig. 6A), DNA strand breakage was low. Considering the magnitude of responses in the rest of the data set, the 13.9% DNA in comet tail of SJ-caged fish likely is within the region of background strand breakage. Throughout this early deployment, the dropping hydrograph represented reservoir release rather than runoff from a previous storm, and application of winter-season pesticides occurred during the dry week immediately preceding the January 25 deployment [21]. For the two pairs of cages deployed on January 25 at the onset of rain (Fig. 6A), DNA strand breakage was significantly higher in SJ-caged fish compared to fish caged at the RF site ($p < 0.001$) in both the 4-d exposure (53.6% and 12.6% DNA in comet tail for SJ and RF, respectively) and the 10-d exposure (37.3% and 6.9% DNA in comet tail for SJ and RF, respectively). From these data alone, it is unclear whether the elevated DNA strand breaks in SJ fish retrieved on February 4 reflect a continued exposure to stressors remaining after the diazinon pulse (which had passed 4 d earlier) (Fig. 3) or simply a lack of recovery from exposure to stressors associated with the diazinon pulse. The final deployment of cages discriminated be-

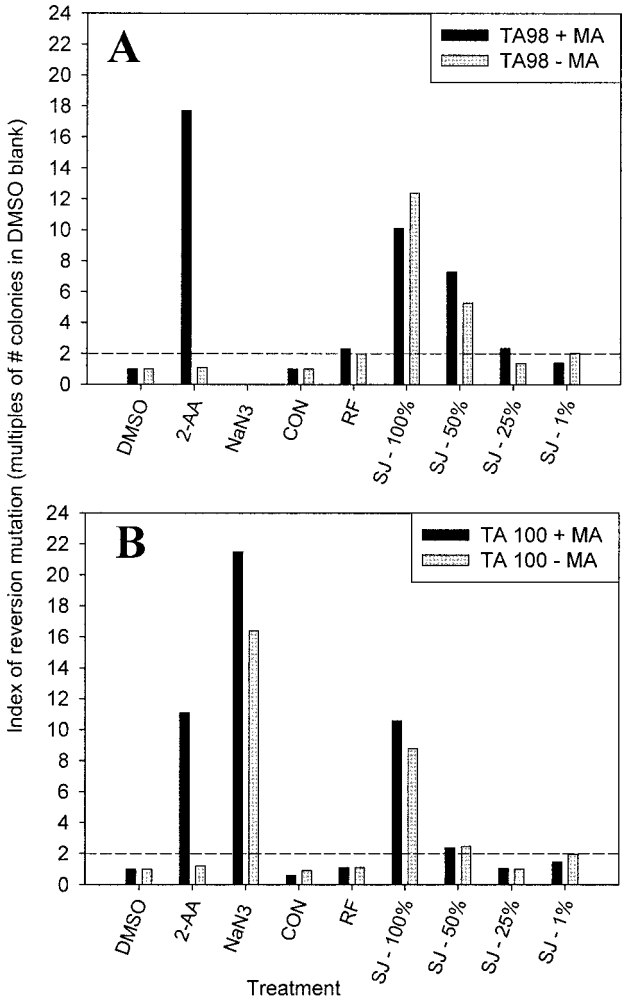

Fig. 7. Mutagenicity of year 2001 field-collected water samples using the *Salmonella typhimurium* tester strains (**A**) TA98 and (**B**) TA100. Dark bars and gray bars indicate reversion mutation of treatments with and without S9 metabolic activation (MA), respectively. Dashed line indicates the twofold threshold for considering mutagenic response as being significantly elevated above the dimethyl sulfoxide (DMSO) control. Treatments are DMSO control (i.e., DMSO), positive controls 2-aminoanthracene (2-AA) and sodium azide (NaN_3), laboratory control water (CON), field reference water (RF), and water from the San Joaquin River near Vernalis both undiluted (SJ-100%) and diluted to 50%, 25%, and 1% of the original concentration.

tween these two alternatives (Fig. 6B). This pair of cages, deployed on January 31 after the diazinon pulse (Fig. 3), indicated that DNA strand breakage was significantly higher ($p = 0.008$) in SJ-caged fish (55.8% DNA in comet tail) compared to fish caged at the RF site (32.3% DNA in comet tail). Among-individual variance (detected using nested ANOVA) was often significant ($p < 0.05$) but was insufficient to mask added variance because of treatments.

Mutagenicity

Among all samples tested, San Joaquin River water was most mutagenic to both TA98 and TA100, with and without metabolic activation (Fig. 7). Total mean revertants per plate in DMSO-negative controls were 24.5 and 20.0 in TA98 with and without S9, respectively, and 105.0 and 99.0 in TA100 with and without S9, respectively. Mutagenicity (Fig. 7) is expressed relative to the mean number of colonies growing in the DMSO control (e.g., if 20 colonies were growing in the DMSO controls, the number of colonies growing in all treatments was divided by 20). Total mean revertants per plate in sterile water–negative controls were essentially the same as in DMSO controls (A. Whitehead, University of California, Davis, unpublished data). San Joaquin River water collected during the 2001 dormant season was mutagenic both with and without S9 metabolic activation in both tester strain TA98 (10.1 × DMSO with S9, 12.4 × DMSO without S9) (Fig. 7A) and TA100 (10.6 × DMSO with S9, 8.8 × DMSO without S9) (Fig. 7B), and mutagenicity decreased on dilution. Laboratory control water (BML well water) and field control water (from RF composite) did not elicit mutagenic responses using either TA98 or TA100, with or without metabolic activation.

DISCUSSION

Experiments were specifically timed to coincide with runoff following pesticide applications to orchards in the study area. Winter storm events increased water flows, transported agricultural chemicals to watersheds, and were coincident with the induction of DNA strand breaks in caged fish over two consecutive field seasons. In 2000 (the first field season), laboratory exposures to field-collected water were included concurrently with field experiments, and DNA strand break data supported the field results. In 2001, Ames *Salmonella* reversion mutation assays on field-collected water were included, again concurrently with field experiments, and mutagenicity data corroborated genotoxicity findings in field-caged fish.

Sources of runoff during storms

Analytical chemistry was used primarily to characterize the timing of chemical runoff events. In addition, because toxicity was observed concurrently with these events, chemistry data were further used as evidence to implicate or rule out causative agents. Although urban runoff contributes pesticides during rainfall–runoff events, organophosphate loads were more than three orders of magnitude greater at SJ than in Modesto storm drains during the 2001 runoff event [21]. Agricultural development accounted for 24% of land use in the San Joaquin River watershed, whereas urban development accounted for only 1.7%. Furthermore, the timing of experiments specifically followed notification of winter-season pesticide applications to orchards within the watershed, and locations of caging experiments were selected to emphasize agricultural inputs. Additional study, however, would be needed to eliminate the alternative hypothesis that the relatively sparse urban inputs in this region are responsible for the genotoxicity observed.

Two widely applied pesticides, diazinon and simazine, were monitored among others as surrogates for the general timing of agricultural chemical runoff events and tended to predict successfully the onset of DNA strand breakage. These chemicals are widely applied at high rates. Their analysis in environmental media is routine, and their detection limits are low. Although some organophosphates and triazines may be genotoxic, diazinon and simazine measurements were mainly included for these reasons. Our assumption was that the timing of runoff of other chemicals, some potentially genotoxic, would coincide with runoff of diazinon and simazine. Indeed, the validity of this assumption was suggested by the genotoxicity data.

Diazinon and simazine runoff profiles differed, and they probably represent runoff from different geographical sources within the San Joaquin watershed. In 2000 at SJ, diazinon and simazine runoff profiles overlapped, but simazine concentra-

tions peaked 1.5 d after diazinon. The hydrograph continued to rise until February 18, representing releases from upper-watershed reservoirs in the Sierra Nevada. Similarly, in 2001 at SJ, runoff profiles overlapped, but simazine concentrations did not peak until 3 d after diazinon. The longer lag time between the two peaks is consistent with the lower flows in 2001. Geographical Information System mapping of pesticide application shows that even though simazine and diazinon applications overlap geographically, simazine is also applied higher in the watershed. This results in broader profiles of simazine that reach the downstream SJ caging site later than diazinon. One may interpret both the diazinon and simazine profiles as representing the pulse of pesticides applied in the lower watershed and, in addition, the simazine profile as representing the pulse of pesticides applied in the upper watershed.

Genotoxicity associated with runoff

Many aspects of our data support the hypothesis that genotoxicity was associated with agricultural chemical runoff events in the field. An alternate hypothesis is that urban stormwater runoff contributed to observed genotoxicity. Although pesticide loads in 2001 were more than three orders of magnitude higher at SJ than in Modesto storm drains [21], the data presented in the present study do not rule out this possibility. More extensive geographical and time-course testing is necessary to definitively examine this alternate hypothesis.

Experiments were specifically timed to immediately follow winter-season pesticide applications to orchards within the watershed. At SJ in 2000, DNA strand breakage was induced in fish at all time points following the onset of runoff, and all deployed cages were exposed to both diazinon and simazine pulses. More extensive field exposures in 2001 again induced DNA strand breakage in SJ-exposed fish. Importantly, strand breaks were induced in fish exposed during runoff but not in fish exposed before the runoff. Fish from all three cages deployed during the runoff event were exposed to chemicals associated with either the diazinon pulse or the simazine pulse, and these fish had high DNA strand breakage compared to RF-exposed fish.

Very little is known about the genotoxic potential of agricultural runoff, and few studies have tested for associations between genotoxicity in resident organisms in situ and exposure to these complex mixtures. Others have observed elevated DNA strand breakage in resident tadpoles or frogs from ponds and ditches of heavily agriculturalized landscapes relative to those from nonagricultural areas [22,23]. However, exposure to agricultural chemicals was not assessed, and it was unclear whether agricultural stressors were responsible. In the present study, the pulsed nature of winter-season runoff events following agricultural pesticide applications in the Central Valley provided a unique opportunity to test more clearly the associations between agricultural runoff and the timing of in situ genotoxic responses.

Bioindicators of greatest potential utility in ecotoxicological studies are those that have been validated in both the laboratory and the field and linked to higher-order effects. The DNA strand break assays have been confirmed as being sensitive indicators of exposure to a variety of chemical genotoxicants in several model organisms, including fish [24]. Field studies have detected DNA strand breakage in response to exposure to polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and heavy metals (see, e.g., [11,25–27]) and to contamination by radionuclides (see, e.g., [10,28]). To our knowledge, the present study is the first to have coupled genetic biomarkers with other measures of exposure to test for genotoxic effects of agricultural runoff in the field. Linkages between genotoxic endpoints (including DNA strand breakage) and fitness parameters, such as reproduction, growth, and developmental abnormalities, have been demonstrated in various laboratory [4,5,12] and field [6–11] evaluations. However, a thorough characterization of the genetic structure of *C. occidentalis* populations in the Sacramento and San Joaquin basins did not reveal evidence of population-level effects relative to long-term historical pesticide exposure [29]. This may imply that fitness effects have been of insufficient intensity to be detectable at the population genetic level or, alternatively, that chemicals relatively new to the watershed are responsible for the observed genotoxicity and too few generations have passed for the effects to propagate to the population genetic level.

San Joaquin River water induced reversion mutation in two *Salmonella* tester strains both with and without S9 metabolic activation. These data from 2001 corroborated the 2001 field biomarker data in fish, further strengthening a causal relationship between genotoxicity and exposure to San Joaquin River water during runoff. However, the relationship between DNA strand breakage in fish and in vitro mutagenicity is empirical, and one cannot presume that the same genotoxins are responsible for both effects.

Whereas Ames testing is considered to have high power for predicting carcinogenic potential [30,31], few studies have examined the capacity of in vitro reversion mutation for predicting in vivo genotoxic effects in aquatic organisms. In a thorough field study, Theodorakis et al. [32] observed correlations between *Salmonella* reversion mutation, DNA strand breakage, and community disturbance levels. The DNA damage in fish and the mutagenicity of sediment decreased following a downstream gradient of complex contamination, and both were related to community-level disturbances. Another study found that fractions of Rhine River water were mutagenic in *Salmonella* tester strains compared to reference groundwater, which is in agreement with increased metaphase chromosomal aberrations in the gills of exposed fish [33]. Mutagenicity data from the present study tend to support observed in-stream genotoxicity in native fish, but further investigation to characterize responsible agents is warranted. Bacterial mutagenicity tests have been systematically applied to characterize the mutagenic potential of industrial wastes [34] but not to assess complex agricultural runoff mixtures. Data from the present study indicate that this approach might be a sensible way to determine the chemical fractions responsible for observed genotoxicity.

Possible causes of observed genotoxicity

Although the elucidation of responsible agents often is speculative in the absence of chemistry data specifically supporting a cause–effect relationship, we consider the possible influence of some likely candidates to direct future studies. Modifying variables that could contribute to genotoxic responses in field studies may include physical stress and exposure to ultraviolet radiation. Because controlled laboratory exposures and bacterial assays support the field data, oxidative damage induced by physical stress during turbulent flows was unlikely to be the responsible agent. Indeed, turbulent flows at the reference caging site were not associated with genotoxicity. High turbidity of river water associated with particulate

runoff likely reduced severely the ultraviolet penetration to the river bottom, where the cages were situated. Furthermore, strand breaks were not induced in fish exposed before the runoff event, at which time ultraviolet penetration likely was higher. Thus, chemical stressors associated with runoff, rather than physical or radiation stressors, mostly likely were the responsible agents.

Even though genotoxicity appeared to be associated with the timing of agricultural runoff, it is unlikely to be attributable to diazinon exposure. First, diazinon concentrations in 2000 and 2001 were much lower at SJ and OD than expected based on the concentrations detected in previous years [1,2], which reflects the shift toward replacement by the pyrethroid insecticides. Such concentrations were many orders of magnitude lower than those demonstrated to be genotoxic in laboratory studies [13] and were too low to induce inhibition of acetylcholinesterase enzyme, a sensitive biomarker of exposure to organophosphate and carbamate insecticides, in field- and laboratory-exposed fish [35]. Second, in 2000, diazinon concentrations were higher in OD water in the field and laboratory than in SJ water; however, DNA strand breakage was much higher in SJ exposures than in OD exposures. Third, continued DNA strand breakage in fish retrieved from late-deployed cages in 2001 supported the hypothesis that elevated strand breaks were caused by exposure to stressors associated with the simazine pulse after the diazinon pulse had passed. Diazinon appears to have been an adequate surrogate for exposure to genotoxic agents associated with early runoff but not for those associated with late runoff.

Stressors associated with the simazine pulse are more consistent with the field and laboratory genotoxicity data. In 2000, all caged fish at SJ had elevated DNA strand breakage and had been exposed to the simazine pulse. In 2000, simazine was not detected at OD during caging, and DNA strand breaks were not elevated. In year 2000 laboratory exposures, simazine concentrations were much higher in SJ water than in OD or RF water, and DNA strand breakage was induced only in SJ-exposed fish. In 2001, strand breaks at SJ were elevated in all fish caged during the runoff event. Those deployed at the onset of runoff were exposed to both diazinon and simazine pulses, whereas those deployed later during runoff were exposed to the tail end of the simazine pulse but not to the diazinon pulse. Although it seems to be consistent that simazine may be responsible for observed DNA strand breakage, the literature provides little indication that simazine is genotoxic (EXTOXNET, August 2003, http://extoxnet.orst.edu). Other chemicals co-occurring with simazine in these complex runoff mixtures are more likely to be responsible for the observed genotoxicity.

Those agricultural chemicals that may warrant further investigation are genotoxins (i.e., chemicals with at least two positive genotoxicity results, as summarized in [36]) applied at high rates in the Central Valley (>100,000 pounds of active ingredient per year in 2000; California Department of Pesticide Regulation Pesticide Use Database), such as captan, methyl bromide, and ziram (these chemicals were not analytes in the present study). Carbaryl, malathion, and trifluralin are also genotoxins applied at high rates in the Central Valley, but these were not detected at elevated concentrations in the present study [17]. In addition, the pyrethroid insecticides are starting to replace the organophosphates for applications, including those to Central Valley orchards in the winter. These chemicals are highly toxic in aquatic organisms, and some evidence indicates they are genotoxic in some test systems, including fish [15]. Unfortunately, pyrethroids are notoriously difficult to detect in environmental samples, even at acutely toxic concentrations [3]. Other classes of known genotoxicants, such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls, may be considered as analytes in future studies.

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