Aquatic Pesticide Monitoring Program

Aquatic Pesticide Monitoring Program
Phase 3 (2004)
Monitoring Project Report

Edited by:
Geoffrey S. Siemering

Contributing Authors:
Geoffrey Siemering, SFEI   Daniel Schlenk, UC-Riverside
Jennifer Hayworth, SFEI   Lingtian Xie, UC-Riverside
Aroon Melwani, SFEI       Mary Ann Irwin, UC-Riverside
Dave Crane, CDFG-WPCL     Kevin Berry, Univ. of Louisiana
Abdou Mekebri, CDFG-WPCL  Benjamin K. Greenfield, SFEI
Gloria Blondina, CDFG-WPCL Kelly Thrippleton, UC-Riverside

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Gloria Blondina, CDFG-WPCL

San Francisco Estuary Institute
7770 Pardee Lane, 2nd Floor
Oakland, CA 94621

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1 INTRODUCTION

Funding for Phase 3 (2004) of the APMP was possible because of judicious financial planning throughout the project and the guidance of both the APMP steering committee and the APMP Technical Review Committee that kept the project focused on the data needs of all stakeholders involved.

This report will focus solely on the results derived from Aquatic Pesticide Monitoring Program studies conducted during 2004. Data from 2003 and 2002 will be referenced only where appropriate to deepen the discussion and understanding of the 2004 data. For a more thorough review of the entire APMP please refer to the Phase 2 (2003) monitoring report available at http://www.sfei.org/sfeireports.htm. The Phase 2 (2003) results summary is included below (from Siemering 2004).

1.1 Phase 2 (2003) Results Summary

Use of the data gathered during the 2002 and 2003 pesticide application seasons of the APMP should be limited to screening purposes only, to identify where further risk characterization or research may be needed. The APMP is not of sufficient spatial or temporal scope to directly inform regulatory change. Due to the limited time and budget of the project, no definitive conclusions can be drawn from the data accumulated to date. The APMP generated chemical characterization, toxicity, and bioassessment data. The chemical characterization and toxicity data can be used for screening purposes. In complex field situations, bioassessments require multiple years of data before even preliminary conclusions can be drawn from them.

Specific conclusion concerning individual pesticides:

2,4-D

Only one application of 2,4-D (in the 2,4-D dimethylamine salt formulation) with added surfactant was monitored. At this single application, no toxicity was observed nor did risk
quotients indicate the need for further information. Vitellogenin induction experiments indicated that 2,4-D may possibly cause endocrine disruptor at application rates in the laboratory.

The vitellogenin induction finding indicates the need for further study particularly under normal field conditions. This is a special study and not a routine monitoring recommendation.

**Acrolein**

Due to acrolein’s rapid volatilization, work focused on development of a field sampling method that would allow for accurate determination of the pesticide in water. Current standard environmental sampling methods are inadequate for sampling of acrolein treated water. Due to acrolein’s rapid volatilization, it is currently not possible to conduct standard water toxicity tests. Since acrolein exhibited extremely low Lowest Observable Effect Concentration (LOEC) values, the detectable presence of acrolein suggested very high mortality to EPA water and sediment toxicity test species. APMP could not locate toxicological data on acrolein’s principle breakdown product 3-hydroxypropanal.

Further refinement of the sampling methodology begun in 2003 is warranted including investigation of 3-hydroxypropanal. It is recognized that residue values for this pesticide may be difficult to determine. Therefore, development of diagnostic response tests (i.e. phytomonitoring, sentinel bivalves and fish, etc.) should be explored.

**Copper Sulfate**

Copper sulfate applications were monitored in two reservoirs. In one reservoir treatment area dissolved copper sulfate toxicity (in the form of mortality) was observed 24 hours after application in juvenile trout. Lethal (mortality) and sublethal (reproduction) toxicity was observed in Ceriodaphnia (water flea) up to one week after application. Peak concentration risk quotients showed acute and chronic U.S. EPA Office Pesticide Programs Levels-of-Concern (LOC) exceedances. At 24 hours after application the risk quotients showed acute and chronic LOC exceedances. At one-week post application the risk quotients only showed acute LOC exceedances.
In the reservoir treated with granular copper sulfate, significant mortality was observed in Ceriodaphnia and juvenile trout water toxicity tests immediately after application within the treatment area. Follow up water sampling was not conducted and the reservoir received only one application in 2003. Mortality and growth inhibition was also observed in a number of the sediment samples. Sediment copper concentrations exceeded National Oceanographic and Atmospheric Administration (NOAA) Effect Ratio Low and Medium values. However, the limited toxicity observed in the sediments indicated that the majority of the copper was not bioavailable.

These findings indicate the need for further risk characterization associated with copper sulfate applications.

Chelated Copper

Chelated copper pesticides were monitored during applications in two irrigation canal systems. One system used a product of mixed copper ethanolamines and the other the same product of mixed copper ethanolamines in an emulsified formulation. Chelated copper formulations are likely to have distinct behavior between copper sulfate and each other in aquatic environments based on the chelating agent and other adjuvants.

In both systems, the water samples were similarly toxic both before and after application. Therefore, no definitive conclusions could be drawn on the toxicity of mixed copper ethanolamines. Risk quotients showed some Levels-of-Concern exceedances depending on species sensitivity. It should be noted that copper carbonate is the active ingredient in other chelated copper products and no monitoring of copper carbonate based pesticides was conducted.

Based on the lack of definitive data, further risk characterization associated with chelated copper applications is warranted.

Glyphosate

Glyphosate was monitored at several locations. No toxicity was found to be associated with glyphosate applications. Risk quotients for Selenastrum indicated that immediately after application (when glyphosate concentrations are highest) Levels-of-Concern are exceeded.
Glyphosate is often applied with a surfactant, which may have much higher toxicity than the active ingredient.

Based on risk quotient calculations and toxicity data, no further risk characterization associated with glyphosate applications alone is necessary. Risk characterizations may be warranted to further investigate a surfactant used in conjunction with the glyphosate.

Diquat Dibromide

Diquat dibromide was monitored at two locations, one small pond and a Delta slough. At both sites, 100% mortality was observed in the acute and chronic Ceriodaphnia toxicity tests one hour after application. Twenty-four hours after application to the Delta slough, no toxicity was observed in the treatment area. Additional samples were not gathered from the pond site. Risk quotients similarly exceeded Levels-of-Concern at all sampling periods in the Delta slough (including pre-application) and at one hour after application in the pond. Diquat may be applied with a surfactant which may have much higher toxicity than the active ingredient. Diquat sediment concentrations were not considered as diquat is irreversibly adsorbed to sediments and thereafter not bioavailable.

Toxicity test and risk quotient results indicate the need for further risk characterization.

Fluridone

Fluridone (applied in pellet or liquid form) was not found to be definitively toxic in three EPA species for water or sediment amphipod toxicity tests. The peak concentration risk quotient for Stonewort did exceed an acute Level-of-Concern (LOC). Risk quotients for other species did not exceed LOCs. Fluridone caused sublethal toxicity (decreased shoot and root length) to Typha. This would indicate a potential for impacts on non-target plants.

Further risk characterization of impacts on non-target plants is warranted. There is also cause for concern over development of genetic resistance to fluridone, which is emerging in plant populations in Florida.
Methoprene

Monitoring for methoprene is challenging because it is commonly applied to environments that do not lend themselves to traditional water and sediment sampling and testing methods (i.e. extremely shallow water and highly anoxic sediments). In-situ and laboratory toxicity tests were completed, but the results were inconclusive. From the one site monitored for methoprene, water and porewater risk quotients indicated no need for further risk characterization. Methoprene was persistent in marsh sediments for several weeks up to the ppm level. Little methoprene sediment toxicity data could be located in the literature.

Future work is warranted to further characterize the risk of methoprene in sediments. Additional studies may also be warranted due to the common simultaneous application of methoprene and Bacillus Thuringiensis israelensis (BTi).

Triclopyr

Triclopyr (in the triclopyr, triethylamine salt formulation) was monitored at one location. Due to sampling error, the toxicity tests were rendered inconclusive and therefore no conclusions can be drawn as to the toxicity of triclopyr. Triclopyr peak concentration risk quotients showed no Levels-of-Concern exceedances. Triclopyr is often applied with a surfactant, which may have much higher toxicity than the active ingredient.

Limited further risk characterization is necessary to conduct toxicity testing. Risk characterizations may be warranted to further investigate a surfactant used with triclopyr.

Nonionic surfactants

The most commonly used surfactants at APMP monitoring sites were Target Prospreader Activator and R-11. Both are nonylphenoletxoxylate surfactants. Peak concentration risk quotients indicated Levels-of-Concern exceedances for a wide range of animal species including Delta Smelt and Sacramento Splittail. Vitellogenin induction experiments in Rainbow trout indicated that these nonylphenol surfactants could be an endocrine disruptor at application rates. There is a wide range of surfactants available, each one having a different toxicological profile. There is limited data available on surfactants.
1.2 Current Aquatic Pesticide Regulatory Status

In 2004, the emergency NPDES permit promulgated in 2001 expired. At the April 2004 meeting of the State Water Resources Control Board (SWRCB), two new aquatic pesticide NPDES permits, one for weed control and the other for mosquito control, were adopted by the Board. At this meeting the Board also requested that the SWRCB staff develop an option for joint or regional monitoring by stakeholder groups and an unannounced compliance monitoring program. No timeline was given for the development of these additions to the current permits.

The joint or regional monitoring option would allow individual stakeholder groups to leverage the funds they are currently required to expend for monitoring to provide better scientific data to address the technical concerns raised by the APMP data. The general concept of the joint and regional monitoring efforts would be to allow applicator groups to fund more extensive monitoring at certain application areas in lieu of more widely spread compliance monitoring. The more extensive studies would allow for the determination of longer term or more subtle effects than are detectable under most compliance monitoring scenarios. This data would ultimately inform the revision of the NPDES permits. This form of monitoring is straightforward to organize with the primary difficulty arising from the establishment of a funding mechanism and acceptable contribution levels by applicators. The scientific objectives for this monitoring could be taken directly from the Directions for Future Monitoring discussed at the end of this report.

The unannounced compliance monitoring would help to alleviate the environmental advocacy community concerns that pesticides are being applied at off-label rates in excess of the legal limits. This type monitoring would require the retention of a single organization charged with randomly selecting applications to monitor and organizing the sampling and analytical efforts. The sampling would take place shortly after an application to determine if the amount of pesticide was within the allowable limit. The primary difficulty in organizing this type of monitoring is establishing a list of scheduled applications. Many applicators currently apply on a schedule (irrigation districts in particular) and many others could establish one. However, many applications are conducted with little to no lead time (on an as-needed basis) based on field observations. Other applications are scheduled, but are then altered due to weather (e.g. Delta sites) or tidal conditions.
1.3 Scientific Objectives

The APMP studies conducted in 2004 were special projects that were required based on results of the 2002 and 2003 monitoring. With no additional funding identified past the current contract amount, the decision was made to curtail the widespread triad-approach monitoring utilized in 2002 and 2003. From the 2002 and 2003 results the following six special studies were planned:

1) Conduct longer-term bioassessment analysis of Bon Tempe and Lake Lagunitas drinking water reservoirs of the Marin Municipal Water District. In addition to benthic bioassessment data, phytoplankton and zooplankton studies were conducted. Morphological deformations of benthic macroinvertebrates were analyzed as well as copper bioaccumulation.

2) The analysis of in-field derivitized acrolein treated water samples was further refined.

3) Diagnostic tests were used to evaluate the impacts of acrolein inside and outside of a treatment area.

4) Endocrine disrupting effects of tank-mixed pesticide/nonylphenol based surfactant combinations applied at label application rates were determined.

5) Benthic macroinvertebrate communities present in an acrolein treated canal were surveyed.

6) The potential long-term non-target plant toxicity of pelleted fluridone was investigated.

All of the above mentioned studies with the exception of the bioassessment work are presented herein. The bioassessment report is presented under separate cover (Hayworth and Melwani, 2005).

1.4 References


2 IN-FIELD DERIVITIZATION OF ACROLEIN TREATED WATER SAMPLES

2.1 Introduction

Acrolein (2-propenal, CAS# 107-02-8), which is the active ingredient in the aquatic herbicide Magnacide® H (Baker Petrolite Corp.), was monitored in irrigation canal systems within the Merced Irrigation District. This monitoring was done in support of the San Francisco Estuary Institute’s Aquatic Pesticide Monitoring Program (APMP). The goal of the APMP was to provide the California State Water Resources Control Board with scientific information upon which California’s aquatic pesticide National Pollutant Discharge Elimination System (NPDES) permits could be based. States in the western U.S. are currently required to issue NPDES permits as a result of the U.S. Ninth Circuit Court Talent Decision.

Acrolein is used routinely in agricultural canals of the Western U.S. primarily to control submerged aquatic weeds (Nordone et al., 1996), although its major use is as a biocide for aquatic flora and fauna (Ghilarducci and Tjeerdema, 1995). Acrolein dissipates rapidly in irrigation canals due to processes such as degradation (hydrolysis DT$_{50}$ = 1.5 d at pH 7.0), volatilization (Henry’s $K_H$ = 7.9-19.5 kPa m$^3$ mol$^{-1}$), adsorption, and dilution. Dissipation half-lives have been reported at 10.2 and 7.3 h in weedy and non-weedy canals, respectively (Nordone et al., 1996). The rapid dissipation generally results in low-levels of residual acrolein in treated irrigation canals following its application at the label recommended concentrations.

Several methods have been developed to quantify acrolein in aquatic media that include a laboratory or field derivatization step to stabilize acrolein for instrumental detection. For instance, Ogawa and Fritz (1985) trapped acrolein from water samples using a hydrophobic Zeolite ZSM-5 column, eluted the column with acetonitrile, derivatized the eluted acrolein with 2,4-dinitrophenylhydrazine (DNPH), and then they analyzed the acrolein-DNP adduct using HPLC-UV. Nordone et al. (1996) derivatized acrolein in irrigation canal water samples with pentafluorophenylhydrazine (PFPH), liquid-liquid extracted the derivatized samples, and then analyzed the acrolein-PFPH adduct using GC-ECD. In the latter method, derivatization in the field of acrolein to its corresponding pentafluorophenylhydrazone prevented degradation after the
samples were removed from the canal (Nordone et al., 1996). This suggests that laboratory measurements of acrolein containing aquatic samples are likely to misrepresent the actual site concentrations if acrolein is not stabilized in the field at the time of sample collection.

Our initial field sampling results indicated that there were serious limitations with the sampling methods used to collect water samples for acrolein analysis at trace levels. Briefly, the laboratory results showed that acrolein was detectable in water samples at 2 h post application of Magnacide® H (range 4500-4600 µg/L, mean 4550 µg/L); however, it was not detectable in water samples collected at 72 h post application. The non-detection of acrolein in the 72 h post application sample was unexpected given that at the time of sample collection, a Baker test kit (a field colorimeter designed specifically for detecting acrolein) indicated the presence of acrolein in the canal. Based on these results, we determined that it was necessary to stabilize acrolein-containing water samples immediately following their collection and prior to transport to the laboratory for analysis. Therefore, this prompted us to develop an in situ chemical stabilization method to improve recovery of acrolein in surface water samples. The methodology used for this study was a modified EPA Method 8315 where DNPH is used to derivatize acrolein and its metabolites at the time of sample collection. At the laboratory, the sample is solvent extracted, evaporated, and reconstituted for analysis by LC-MS. The method is simple enough to be performed in the field by the pesticide applicator.

2.2 Materials and Methods

2.2.1 Method Validation – Field Work

Field samples were obtained from the LeGrand and Planada Canals 24 h after an acrolein application at the LeGrand Canal headgates. Acrolein was injected at three application points to insure a uniform concentration over the length of both the Planada and LeGrand Canals. Treatment occurred at each application point for 2 h. The pre-application water sample was collected just above the LeGrand headgates. Site 1 water samples (n=3) were collected from the LeGrand Canal three miles downstream of the LeGrand headgates, while Site 2 samples (n=3) were collected from the Planada Canal, 6 miles downstream of the LeGrand headgates (Figure 1).
Samples were collected by submerging a wide mouth Nalgene® collection bottle to 1 m water depth. Once retrieved, the sample was carefully decanted to minimize aeration into a 500 mL wide mouth amber glass bottle, leaving sufficient space for the addition of DNPH reagent. After the addition of DNPH (45 mL of 1.0 g/L DNPH solution), the bottle was filled completely to eliminate any headspace. The collection bottle was rinsed thoroughly with deionized water between sites. In previous field sampling events, samples were collected by pumping water through a peristaltic pump into collection bottles with the pump tubing held just above the bottle mouth to prevent possible sample contamination. This is the typical method for collecting water samples for pesticide analysis; however, based on the chemistry results we concluded that the use of a peristaltic pump could have contributed to the non-detectable concentrations observed for acrolein since pumping increases the level of aeration of water samples.

At each location four samples were collected. Three samples were treated with DNPH and one was left untreated. A group of pre application control samples were also collected upstream of the LeGrand Canal headgates. The temperature of the water samples ranged from 19.7 to 22.7°C with a pH range of 6.6-6.8. All of the samples were kept cold in ice chests (4°C) and delivered to the laboratory at the end of the collection day where they were extracted immediately upon arrival.

2.2.2 Sample Extraction and Analysis

Samples were liquid-liquid extracted two times using a hexane:dichloromethane (70:30) solvent solution. After which, the organic phase was dried with sodium sulfate, concentrated by rotary evaporation, reconstituted in methanol, and the final extract volumes were adjusted to 5 mL. The extracts were then analyzed for acrolein and its derivatives using an Agilent LC-MSD 1100 Series equipped with an Agilent Zorbax C-18 column (150 mm x 4.6 mm i.d., 5 µm, @ 38°C). Gradient conditions were optimized to achieve separation of the derivitized acrolein and its hydrolysis product (3-HPA). Mobile phase A was 99:1 water-methanol and mobile phase B was 100% acetonitrile. The gradient program was 90%A:10%B held for 1 min, then linearly increased to 80%B over 30 min and then returned to initial conditions (90%A:10%B) over 2 min. Injection volume was 20 µL and the mobile phase flow rate was 1.0 mL/min. The MS was configured with Atmospheric Pressure Chemical Ionization (APCI) in negative mode. To enhance sensitivity, sample extracts were analyzed and quantified using Selected Ion Monitoring (SIM) mode set for

m/z = 235 (acrolein-DNPH) and m/z = 253 (3-HPA-DNPH) and confirmed using full scan mode (extracted ion technique) (see Figures 2 and 3). For quantification, a standard curve was made from acrolein, which was derivatized at the same time as the samples. The reporting limit was 0.02 ppb (µg/L). Since the acrolein metabolite 3-HPA is not commercially available, 3-HPA concentrations were estimated by using a degraded acrolein standard where the quantified loss of acrolein is used as the concentration of the 3-HPA peak. Recoveries of laboratory control standards and matrix spikes ranged from 80-85%.

2.3 Results and Discussion

The results from this study demonstrated that water samples treated with DNPH immediately after field sampling resulted in higher acrolein recoveries compared to untreated samples (Table 1). Acrolein concentrations in DNPH treated water samples were 2-4 times higher than non-treatment controls. By derivatizing acrolein with DNPH at the sampling site, it is stabilized, allowing a few hours for the water sample to be transported to the laboratory for immediate processing and analysis.

Acrolein stability: The temperature of the field samples at the time of collection was 19.7-22.7ºC and the pH was 6.6-6.7. The sample collection method and holding time both had a significant effect on the stability of acrolein. Dip sampling with a wide mouth bottle minimized the level of aeration of the water sample. Acrolein mean concentrations were approximately 2-4 times higher than the reporting limit (RL=0.02 ppb) in DNPH treated field samples but was not detected or was below the reporting limit (<0.02 ppb) in untreated samples collected from the same sampling site (Table 1). Delaying the addition of DNPH to the water samples for as little as a few hours resulted in decreased acrolein recovery.

DNPH stability and addition to acrolein: The DNPH solution should be prepared, kept cold (4ºC) and used within 24-48 h. The solution must be added to the water at the time of sample collection and the samples must be kept cold (4ºC) in order for the acrolein-DNPH adduct to remain stable. After addition of DNPH, extraction should occur as soon as possible (samples for this study were extracted within 8 h). Once extracted with organic solvent, the acrolein-DNPH adduct is very stable. Acrolein-DNPH adduct standards analyzed over a one year period gave
consistent results. Addition of 45 mL (1 g/L DNPH) can derivatize one liter of water containing 1 mg/L concentration of acrolein with 100% efficiency (correlation factor = 0.9999).

3-HPA: One major metabolite of acrolein is 3-hydroxypropanal (3-HPA). This hydrolysis product is not available from any major U.S. supplier and must be made in the lab. Concentrations of 3-HPA were detected in both the DNPH treated field samples and the untreated field samples (see Table 1).

The field derivatization method used here coupled with LC/MS instrumental analysis has allowed for the detection of trace levels of acrolein and 3-HPA in surface water samples. One advantage of using LC/MS is that it allows acrolein detection without giving false negatives or positives, as is a common occurrence with other instruments (i.e., spectrometers) due to the selectivity of mass spectra detection.

**Abbreviations Used**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>APMP</td>
<td>Aquatic Pesticide Monitoring Program</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DNPH</td>
<td>2,4-Dinitrophenylhydrazine</td>
</tr>
<tr>
<td>HPA</td>
<td>3-Hydroxypropanal</td>
</tr>
<tr>
<td>NPDES</td>
<td>National Pollutant Discharge Elimination System</td>
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**Literature Cited**

Analysis of DNPH-aldehydes using LC-MS. *LC-MS Application Data Sheet, No. 031*. Shimadzu, Japan.


**Figure Captions**

Figure 1. Representative schematic showing acrolein application sites and surface water sampling sites in the LeGrand and Planada Canals of the Merced Irrigation District.

Figure 2. LC/MS chromatograms of DNPH derivatized water samples: A) DNPH derivatized 3-HPA (RT = 13.597 min, m/z = 253.4) and acrolein (RT = 20.658 min, m/z = 235), and B) DNPH derivatized 3-HPA (RT = 13.626 min, m/z = 253.4) and acrolein (RT = 20.642 min, m/z = 235) from full scan (m/z = 60-300) analysis.

Figure 3. Mass spectra of extracted ions from full scan analysis: A) DNPH derivatized 3-HPA (m/z = 253), and B) DNPH derivatized acrolein (m/z = 236).
Figure 1
Figure 2
Figure 3
Table 1. Acrolein and 3-HPA Field Study Results.

<table>
<thead>
<tr>
<th>Sampling Site</th>
<th>Acrolein ppb (SD)</th>
<th>3-HPA ppb (SD)</th>
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</thead>
<tbody>
<tr>
<td><strong>Pre-application Site</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(LeGrand Canal above headgates)</td>
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<td></td>
</tr>
<tr>
<td>Sample without DNPH</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Samples treated with DNPH (n=2)</td>
<td>&lt;0.02</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Site 1 LeGrand Canal</strong></td>
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<tr>
<td>Sample without DNPH</td>
<td>&lt;0.02</td>
<td>14</td>
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<tr>
<td>Samples treated with DNPH (n=3)</td>
<td>0.05 (0.003)</td>
<td>46 (4)</td>
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<tr>
<td><strong>Site 2 Planada Canal</strong></td>
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<tr>
<td>Sample without DNPH</td>
<td>&lt;0.02</td>
<td>42</td>
</tr>
<tr>
<td>Samples treated with DNPH (n=3)</td>
<td>0.08 (0.01)</td>
<td>413 (15)</td>
</tr>
</tbody>
</table>

Abbreviations:
3-HPA = 3-hydroxypropanal
DNPH = 2,4-dinitrophenylhydrazine
ND = not detected
SD = standard deviation
3 DIAGNOSTIC TESTS AS INDICATORS OF ACROLEIN ECOSYSTEM IMPACTS

3.1 Introduction

As a counterpoint to the development of a field sampling method for the accurate analysis of water samples treated with acrolein, the APMP TRG felt that testing of simple low-cost diagnostic tests for acrolein impacts would be useful. Diagnostic tests using algal growth on artificial substrates as metrics may be able to show impacts of acrolein outside of application zones at a fraction of the cost of chemical analysis. Under the premise that acrolein in water at any concentration is considered toxic, if the diagnostic tests indicate impacts, then water quality could be considered impaired.

3.2 Approach

Acrolein use is restricted to treatment of areas that can be completely isolated from natural waterbodies. In California, acrolein is currently used only to treat irrigation source water canals managed by irrigation districts. These canals terminate at farm fields. However, along the length of many canals there are outfalls blocked by canal gates that do lead to natural or other uncontrolled waterways. These canal gate structures vary in construction and effectiveness. More sophisticated gate structures have very little leakage, whereas the gates consisting primarily of wooden slats exhibit greater leakage. Although the leakage of acrolein treated water through these gate structures is technically in violation of the label requirements, it would not be financially feasible to upgrade every canal gate to a structure that allowed for absolutely no leakage.

Since leakage does occur, it is important to determine potential acrolein impacts outside the treatment area. To accomplish this, artificial algal substrates were deployed and algal response (sloughing and bleaching) analyzed.

3.3 Methods

Two types of artificial substrates were deployed. One substrate type was 8” diameter clay flower pots. At each test station, the pots were suspended by rope upside down approximately 12”
below the water surface. Prior to submersion, ¾” by ¾” grids on the pot tops (eight sections) and sides (nine sections) were marked with indelible marker to allow for quantification of algal growth.

The second substrate type was four glass slides within an enclosure. The enclosures allowed for sufficient water flow to encourage growth, but limited the mechanical removal of algae by turbulent flow conditions. The slide containing enclosures were also attached to ropes, weighted, and submerged 12” below the water surface.

Substrates were deployed between 10-14 days prior to two different treatments occurring to allow for algal colonization. During the first treatment, to test the methods, only the pots were deployed. These were recovered eight hours after cessation of treatment, inspected, and percent algal coverage, percent density, and color (percent bleaching) estimated and recorded according to the scales shown in Tables 1, 2 and 3.

For the second treatment, algal pots were deployed at all three transects and slide enclosures were deployed at two transects (LC-1 and LC-2). For the second event, algal metrics were recorded one hour prior to the application and 30 hours post application. As acrolein is acutely toxic, all algal impacts should have occurred within a 30 hour timeframe. Algal measures at the three locations were compared to determine impacts. Basic water quality measures, including DO, pH, and temperature, were collected at all sampling times.

### Table 1. Algal Cover Ratings

<table>
<thead>
<tr>
<th>Value</th>
<th>% Cover</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0-24%</td>
</tr>
<tr>
<td>2</td>
<td>25-49%</td>
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<td>3</td>
<td>50-74%</td>
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<tr>
<td>4</td>
<td>75-100%</td>
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### Table 2. Algal Density Ratings

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<th>Value</th>
<th>Density Characteristics</th>
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<tr>
<td>0</td>
<td>Substrate rough with no visual evidence of microalgae</td>
</tr>
<tr>
<td>0.5</td>
<td>Substrate slimy, but no visual accumulation of microalgae is evident</td>
</tr>
<tr>
<td>1</td>
<td>A thin layer of microalgae is visually evident</td>
</tr>
<tr>
<td>2</td>
<td>Accumulation of microalgal layer from 0.5-1 mm thick is evident</td>
</tr>
<tr>
<td>3</td>
<td>Accumulation of microalgae layer from 1 mm to 5 mm thick is evident</td>
</tr>
<tr>
<td>4</td>
<td>Accumulation of microalgal layer from 5 mm to 2 cm thick is evident</td>
</tr>
<tr>
<td>5</td>
<td>Accumulation of microalgal layer greater than 2 cm thick is evident</td>
</tr>
</tbody>
</table>
### Table 3. Algal Color Ratings

<table>
<thead>
<tr>
<th>Value</th>
<th>Color Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No visual evidence of microalgae</td>
</tr>
<tr>
<td>0.5</td>
<td>Substrate slimy, faint green apparent</td>
</tr>
<tr>
<td>1</td>
<td>Bright, solid green with no other colors (white/grey) present</td>
</tr>
<tr>
<td>2</td>
<td>Substrate predominantly green with few spots of other colors (white/grey) present</td>
</tr>
<tr>
<td>3</td>
<td>Substrate predominantly green with many spots of other colors (white/grey) present</td>
</tr>
<tr>
<td>4</td>
<td>Substrate predominantly white/grey with few spots of green</td>
</tr>
<tr>
<td>5</td>
<td>All white/grey</td>
</tr>
</tbody>
</table>

### 3.3.1 Site Description

All tests were carried out in the Merced Irrigation District Livingston Canal at the point where it is diverted by a canal gate into the Hammett lateral canal (Figure 1). If the canal gate was open, the Livingston Canal could continue several hundred yards further before entering a spillway that flows in the Merced River. There are significant flows through the spillway during the high flow winter months only and at no time does acrolein-containing water enter the Merced River. The canal gate remains largely closed during the entire irrigation season, but water does leak through the control structure at all times and, outside of treatment periods, the gate may be partially opened for water control purposes. This site is approximately nine miles downstream of the nearest acrolein injection point.

At this location, substrates were deployed along three transects. One transect was located outside of the canal gate just downstream of the turbulent area where the water flows through the structure. In this area the substrates were submerged with some treated water leaks, but otherwise the area is untreated. Another transect was immediately inside the canal gate where the substrates were submerged in treated water. This transect is in a low flow back-water area. The final transect of three pots was 20 feet further downstream where the Livingston Canal diverts into the Hammett lateral canal. This transect is in a high flow area.
3.4 Results and Discussion

On 7/16/04 only clay pots were deployed along the three transects. This date was 10 days prior to the application of acrolein to allow algal colonization of the pots. Acrolein treatment occurred on 7/26/04 in the early morning and the pots were removed and analyzed that afternoon. At the transect that was outside of the treatment area (LC-1), the majority of the pots were broken due to turbulence from increased water flow. Despite being broken, it was still possible to collect data from most of the pot tops and often from the side quadrants. Pots at all three transects were left in place to allow additional time for algal colonization.

The enclosures were deployed on 7/28/04. At this time the substrates (clay and slide) were secured to prevent movement. These substrates were observed on 8/9/04 and metrics recorded.
Acrolein treatment occurred on 8/9/04 and the substrates were observed the following day and algal characteristics recorded.

Table 4 summarizes the data from the surviving substrate grid sections. For all grid sections data gathered, the algal cover was estimated at 0-24%, with no visual evidence of microalgae. This lack of difference can likely be attributed to insufficient algal colonization and growth time. Algal colonization of clay or glass substrates generally requires three weeks.

Table 4. Clay Pot Algal Substrate Parameters

<table>
<thead>
<tr>
<th>Transect</th>
<th>Average Post-treatment Algal Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cover</td>
</tr>
<tr>
<td>LC-1</td>
<td>1.0</td>
</tr>
<tr>
<td>LC-2</td>
<td>1.0</td>
</tr>
<tr>
<td>LC-3</td>
<td>1.04</td>
</tr>
</tbody>
</table>

Data from the second treatment event are summarized in Tables 5 and 6. These data indicate that greater algal growth occurred outside of the treatment zone both prior to treatment and no change in characteristics post-treatment. The lack of algal growth in the treatment zone before application is due to the fact that Merced Irrigation District treats this canal on a bi-weekly basis which does not allow sufficient time for substantive growth. The more robust algal growth outside of the treatment area coupled with the lack of change in the characteristics both before and after treatment, indicated that there were no impacts of leakage of acrolein treated water into this canal section.

Table 5. Clay Pot Algal Substrate Parameters

<table>
<thead>
<tr>
<th>Transect</th>
<th>Average pre-treatment Algal Parameters</th>
<th>Average Post-treatment Algal Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cover</td>
<td>Density</td>
</tr>
<tr>
<td>LC-1</td>
<td>3.0</td>
<td>2.0</td>
</tr>
<tr>
<td>LC-2</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>LC-3</td>
<td>1.0</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Table 6. Slide Enclosure Algal Substrate Parameters

<table>
<thead>
<tr>
<th>Transect</th>
<th>Average pre-treatment Algal Parameters</th>
<th>Average Post-treatment Algal Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cover</td>
<td>Density</td>
</tr>
<tr>
<td>LC-1</td>
<td>3.0</td>
<td>2.0</td>
</tr>
<tr>
<td>LC-2</td>
<td>3.0</td>
<td>1.67</td>
</tr>
</tbody>
</table>

Algal growth in the slide enclosures occurred more rapidly both inside and outside the treatment zone. The more rapid growth in the enclosures was likely due to the decreased water...
flow through the slide enclosures. The exposed clay substrate surfaces were continually scoured by water movement through the canal.

Outside the treatment zone (LC-1), algal cover remained unchanged both before and after treatment, while density decreased and color increased after treatment. This slight change in characteristics may be due to the subjective nature of the sample analysis. The post-treatment characteristics observed on the clay substrates and the slides were numerically identical. This would indicate that there were no impacts of the leakage of acrolein treated water through the canal gates on the algal growth within the slide enclosures.

Inside the treatment zone, a slight degradation of algal characteristics in the slide enclosures was observed in cover and density parameters between the pre- and post-treatment analysis. The degradation was not to the level seen on the clay substrate. This may be due to more robust algal growth being present prior to treatment and also the fact that water exchange within the slide enclosures is limited. Treatment along this canal occurs at two injection points where the acrolein is injected for two hours at each point. During treatment, acrolein is injected at two locations for two hours, thereby creating a treated slug of water moving through the canal.

3.5 Conclusions

Initial results indicated that algal substrate diagnostic tests could prove useful as sentinel stations to detect impacts of acrolein treated water. Comparing algal characteristics before and after treatment of substrates inside and outside of treatment zones demonstrated if acrolein is reaching areas outside of the treatment zone. Diagnostic tests do not provide numerical values that are currently required under the California NPDES permit. However, the costs of deploying the substrates, retrieving them, and data analysis is substantially lower than that of conducting chemical analysis.
4 LONG-TERM NONTARGET PLANT TOXICITY OF PELLETED FLURIDONE

4.1 Introduction

In 2003, fluridone (liquid and pellet formulations) was monitored in Big Bear Lake and Costa Ponds. Data indicated that fluridone was not definitively toxic in three U.S. EPA species for water or sediment amphipod toxicity tests. The peak concentration risk quotient for Stonewort did exceed an acute LOC and fluridone caused sublethal toxicity (reduced shoot and root length) to Typha. These findings indicated that 2004 efforts should focus on potential non-target plant impacts.

Given the extensive long-term use of pelleted fluridone in Clear Lake, it was selected as the sampling site for 2004. Clear Lake is being treated for Hydrilla control. Clear Lake is almost 21 miles long and eight miles wide, has a surface acreage of 43,000 acres, and has approximately 100 miles of shoreline. The lake is relatively shallow with an average depth of 26 feet and in the summer temperatures range from the mid to high 30°C’s. These temperatures are ideal for Hydrilla germination and growth. Hydrilla was first detected in Clear Lake in 1994. A management and treatment plan was developed shortly after detection. To facilitate the eradication efforts, the lake’s shoreline was divided into 80 management units each with independent treatment schedules (Figure 1).

These management units are surveyed annually for Hydrilla presence. If Hydrilla is detected, treatment takes place multiple times throughout the growing season. A segment is considered Hydrilla-free if none are detected for three years. Treatment continued during the three APMP observation years.
Figure 1. Fluridone treatment zones in near shore areas of Clear Lake, California.

4.2 Approach

The segmented treatment approach used in Clear Lake enabled APMP to conduct studies in lake regions ranging from actively treated segments to segments not treated for several years. The hypothesis guiding the study approach was:

*Pelleted fluridone causes non-target plant toxicity for a significant length of time after the labeled application period and pellet degradation occurs*

Lake segments were selected that were currently being treated, that had never been treated, and where treatment had not occurred for a range of years (Table 1). The goal was to select sites that would help deduce the potential for long term toxicity of pelleted fluridone to non-target plants. A Typha toxicity test was utilized to determine potential plant impacts. Although segments of Clear Lake are known to have high mercury sediment concentrations, mercury is not considered
an acute phytotoxin and therefore should not impact the Typha test used. Two liters of sediment were collected at each station.

### Table 1. Clear Lake Site Selection

<table>
<thead>
<tr>
<th>Lake Segment</th>
<th>APMP Site ID</th>
<th>Treatment Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CL02 (zone 2)</td>
<td>Currently treated</td>
</tr>
<tr>
<td>1 (edge)</td>
<td>CL03 (zone 1)</td>
<td>Currently treated</td>
</tr>
<tr>
<td>3</td>
<td>CL08</td>
<td>No treatment for 2 years</td>
</tr>
<tr>
<td>4</td>
<td>CL9</td>
<td>No treatment for 2 years</td>
</tr>
<tr>
<td>5</td>
<td>CL10</td>
<td>No treatment for 1 year</td>
</tr>
<tr>
<td>6</td>
<td>CL11</td>
<td>No treatment for 1 year</td>
</tr>
<tr>
<td>7</td>
<td>CL04</td>
<td>Currently treated</td>
</tr>
<tr>
<td>7 (edge)</td>
<td>CL05</td>
<td>Currently treated</td>
</tr>
<tr>
<td>8</td>
<td>CL06</td>
<td>Reference</td>
</tr>
<tr>
<td>13</td>
<td>CL13</td>
<td>No treatment for 3 years</td>
</tr>
<tr>
<td>14</td>
<td>CL12</td>
<td>No treatment for 3 years</td>
</tr>
<tr>
<td>20</td>
<td>CL07</td>
<td>Reference</td>
</tr>
<tr>
<td>21</td>
<td>CL01</td>
<td>Reference</td>
</tr>
</tbody>
</table>

At the treated sites, sediment was collected prior to treatment and then four weeks after treatment occurred. At the legacy sites, two liters of sediment were collected. All sampling was done in accordance with the APMP QAPP. The sediment was transported over ice to Pacific Ecorisk laboratories and the California Department of Fish and Game Water Pollution Control Laboratory. Typha toxicity tests and chemical characterization (sediment quality parameters, fluridone, and mercury) was performed on all collected samples.

### 4.3 Results and Discussion

All samples from currently treated areas exhibited no toxicity prior to fluridone application in 2004 (Table 2). Conversely, all samples (including reference sites) exhibited some level of toxicity after application occurred. Typha toxicity appeared to have no relation to fluridone concentration in the samples. For some samples, the fluridone concentrations declined after treatment as compared to the pre-treatment samples even though samples exhibited phytotoxicity after fluridone treatment.

Similarly there was no clear pattern of toxicity due to fluridone at the legacy application sites. Both samples taken from sites treated in 2003 were toxic, whereas only two of the four
samples collected from sites treated in 2001 and 2002 were toxic. There was a large variation in fluridone concentrations.

Table 2. Clear Lake Typha Toxicity Test Summary

<table>
<thead>
<tr>
<th>Clear Lake Site ID (CDEA Lake Segment ID)</th>
<th>Date Segment Last Treated</th>
<th>Pretreatment</th>
<th>Post Treatment</th>
<th>Legacy Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL01 (21)</td>
<td>Prior to 1999</td>
<td>No (120: 1.2)</td>
<td>G, R (388: 2.03)</td>
<td></td>
</tr>
<tr>
<td>CL02 (1)</td>
<td>2004</td>
<td>No (131: 1.09)</td>
<td>G, R, S (27.4: 1.05)</td>
<td></td>
</tr>
<tr>
<td>CL03 ((1 Edge)</td>
<td>2004</td>
<td>No (59.8: 0.68)</td>
<td>R (226: 6.44)</td>
<td></td>
</tr>
<tr>
<td>CL04 (7)</td>
<td>2004</td>
<td>No (1740: 4.47)</td>
<td>G, R (1110: 5.28)</td>
<td></td>
</tr>
<tr>
<td>CL05 (7 Edge)</td>
<td>2004</td>
<td>No (375: 1.63)</td>
<td>G, R, S (460: 3.19)</td>
<td></td>
</tr>
<tr>
<td>CL06 (8)</td>
<td>Prior to 1999</td>
<td>No (30.7: 0.13)</td>
<td>R (3.19: 0.12)</td>
<td></td>
</tr>
<tr>
<td>CL07 (20)</td>
<td>Prior to 1999</td>
<td>No (24.9: 0.13)</td>
<td>R, S (27.9: 0.17)</td>
<td></td>
</tr>
<tr>
<td>CL08 (3)</td>
<td>2002</td>
<td>No (49.8: 0.472)</td>
<td>G, R (2060: 12.7)</td>
<td></td>
</tr>
<tr>
<td>CL10 (5)</td>
<td>2003</td>
<td>G, R (153: 1.82)</td>
<td>R (33.2: 1.76)</td>
<td></td>
</tr>
<tr>
<td>CL11 (6)</td>
<td>2003</td>
<td>G, R (153: 1.82)</td>
<td>R (33.2: 1.76)</td>
<td></td>
</tr>
<tr>
<td>CL12 (14)</td>
<td>2001</td>
<td>G, R (153: 1.82)</td>
<td>R (33.2: 1.76)</td>
<td></td>
</tr>
<tr>
<td>CL13 (13)</td>
<td>2001</td>
<td>G, R (153: 1.82)</td>
<td>R (33.2: 1.76)</td>
<td></td>
</tr>
</tbody>
</table>

Toxicity Indicated Legend: G=% Seed Germination, R= Mean Root Length, S= Mean Shoot Length, M= At least two of G, R, or S.

Additional analysis was performed to determine potential relationships of toxicity to sediment grain size, dissolved organic carbon and mercury concentrations. The Typha toxicity (Figures 2-5) was more closely related (p<0.05) to each of these three additional variables than to fluridone concentration (p>0.05).
Figure 2. Clear Lake Typha Toxicity. Linear Regression Relationship of Percent Fines at 4 weeks after application (p<0.05)

Figure 3. Clear Lake Typha Toxicity. Linear Relationship of Mercury at 4 weeks after application (p<0.05)
4.4 Conclusions

Given the shallow lake depth and frequent high wind conditions, it is likely that fluridone containing sediment is redistributed throughout the lake system continuously. Therefore, in a sense,
the entire lake is treated every year. This makes testing of specific sites for toxicity very difficult as fluridone concentrations are potentially highly variable.
5 EVALUATION OF ESTROGENIC ACTIVITIES OF SOME HERBICIDES AND SURFACTANTS USING RAINBOW TROUT VITELLOGENIN ASSAY

5.1 Abstract

Estrogenic potencies of four herbicides: triclopyr, 2,4-Dichlorophenoxyacetic acid (2,4-D), diquat dibromide, glyphosate, two alkylphenol ethoxylate-containing surfactants (R-11 and TPA), and the binary mixture of surfactants with the herbicides were evaluated using an in-vivo rainbow trout vitellogenin assay. Juvenile rainbow trout exposed to 2,4-D (1.64 mg/L) for 7 days had a 93 fold increase in plasma vitellogenin levels compared with untreated fish, while rainbow trout exposed to other pesticides alone did not show elevated vitellogenin (Vtg) levels compared to the control fish. When combined with surfactants, although general trends indicated enhanced estrogenicity, only 2,4 D and trichlopyr when administered in combination with surfactants caused significant induction of Vtg. Concentration-response studies demonstrated the lowest observed effect concentrations (LOECs) for 2,4-D, and triclopyr were 0.164 mg/L and 1 mg/L. In terms of measured 4-nonylphenol, the LOECs of R-11 and TPA were 20 ug/L and 9.5 ug/L respectively. Binary mixtures of TPA and 2,4-D showed a greater than additive estrogenic response at the lowest concentrations tested, but less than additive response at the highest combined concentrations. Binary mixtures of TPA with triclopyr also caused greater than additive Vtg responses in two middle-concentrations when compared to TPA or triclopyr alone. When trout were exposed to water collected from a site where triclopyr was used in combination with TPA, a concentration-dependent increase in Vtg expression was observed. Measured values of 4-NP were 3.7 ug/L and triclopyr concentrations were below detection (<5 ng/L). Estradiol Equivalents (EEQs) of the lake water were calculated from an estradiol concentration response curve, and were similar 8.5 ± 7.7 ng/L to the mean values for the combined trichlopyr and TPA treatments (9.9-12.2 ng/L) in the laboratory, suggesting the estrogenicity of the water may have been due to the treatment. These results demonstrated the binary mixture of APE-containing
Surfactants with two aquatic pesticides demonstrated greater than additive estrogenic responses in fish under laboratory conditions and in a field setting.

5.2 Introduction

Effluents from industrial, agricultural, and domestic sources contain a wide variety of natural and synthetic chemicals that alter endocrine systems in wildlife and laboratory animals (1-3). Chemicals simulating the natural estrogen, 17β-estradiol, can bind to estrogen receptors (ERs) and initiate estrogenic activities in animals (2, 4). Estrogenic chemicals include organochlorine pesticides, herbicides, polychlorinated biphenyls, alkylphenolic chemicals, and phthalates (5). Many studies have shown that environmental concentrations of many of environmental or xenoestrogens exceed laboratory-derived threshold concentrations that cause adverse effects in aquatic animals (6, 7). Environmental estrogens induce vitellogenin expression in male and juvenile fish (3, 8). In oviparous animals, vitellogenin production is normally limited to mature females. Male and juvenile fish also possess this gene, which is expressed following exposure and uptake of environmental estrogens (9). Thus, induction of vitellogenin in male and juvenile fish has been considered a reliable biomarker for exposure to environmental estrogens (3, 10).

Aquatic herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D), diquat dibromide, glyphosate, and triclopyr are widely used to selectively control broadleaf and woody plants in various waterways. 2,4-D is one of the oldest and most commonly used herbicides. Once in water, it is readily degraded to 2,4-dichlorophenol (11) which is estrogenic (12). Triclopyr has a similar chemical structure to 2,4-D and photochemically decomposes to trichloropyridinol within hours once in water (13). Trichloropyridinol is also the main metabolite of chlorpyrifos which is weakly estrogenic (14). One study has shown that diquat does not initiate responses in estrogen receptor α and β transactivation assays (15).

Alkylphenolic polyethoxylates (ApnEO, n=6-40), such as nonylphenol ethoxylates (NPE) and octylphenol ethoxylates (OPE), are often used in combination with aquatic pesticides as dispersing agents, detergents, emulsifiers, and solubilizers.
APEs are normally present in raw sewage effluent and their degraded products (alkylphenols) bind estrogen receptor (16) and cause estrogenic effects in fish (17, 18).

Few studies have addressed the estrogenic effects of combined exposure of pesticides and surfactants. The objective of this study was to investigate the estrogenic potencies of four herbicides, non-ionic surfactants, and the mixture of herbicides with surfactants using an in-vivo rainbow trout vitellogenin assay.

5.3 Materials and methods

Diquat dibromide was purchased from Syngenta (Wilmington, DE, USA). Triclopyr and glyphosate were from Dow AgroSciences (Indianapolis, IN, USA). 2,4-D was from Nufarm company (St. Joseph, MO, USA). R-11 was provided by Wilbur-Ellis (San Francisco, CA, USA). TPA was from Target Specialty Products company (Fresno, CA, USA). Estradiol was from Sigma (St. Louise, MO).

5.3.1 Measurement of chemicals

The concentrations of 2,4-D, R-11, TPA, triclopyr, glyphosate, diquat dibromide in their stock solutions were determined by Water Pollution Control Laboratory at the Department of Fish and Game (Rancho Cordova, California) (Table 1). For 2,4-D, water samples were acidified to pH<2 with sulfuric acid:water (1:1) and extracted by solid phase extraction (C18). The extracts were analyzed by LC-MS in API-ES negative mode.

Table 1. Results of chemicals analysis on the concentration of the chemicals used. Nonylphenol contents in Prospreader and R-11 were also determined.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Values in mg/L</th>
<th>Nonlyphenol (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target Prospreader Activator*</td>
<td>80000</td>
<td>19000</td>
</tr>
<tr>
<td>R-11*</td>
<td>65000</td>
<td>18000</td>
</tr>
<tr>
<td>Triclopyr</td>
<td>41000</td>
<td></td>
</tr>
<tr>
<td>2,4-D</td>
<td>60000</td>
<td></td>
</tr>
<tr>
<td>Glyphosate</td>
<td>49000</td>
<td></td>
</tr>
<tr>
<td>Diquat Dibromide</td>
<td>46000</td>
<td></td>
</tr>
</tbody>
</table>

* as nonylphenoletoxylate (up to 10 ethylene oxide units)

For the two surfactant R-11 and TPA, Water samples were extracted by solid phase extraction (C18) for nonylphenol (NP) and nonylphenoletoxylate (NPE). The extracts were analyzed by high-performance liquid chromatography (HPLC) with fluorescence detection and confirmed by LC-MS in API-ES negative and positive mode for NP and NPE respectively. For glyphosate, samples were filtered and injected directly in high-performance liquid chromatography (HPLC) with post-column derivatization. For
diquat dibromide, The pH of water samples were brought up to 10.5 ± 0.2 with 10% w/v NaOH (aq) or 10% v/v HCl (aq) prior to extraction using solid phase extraction (C₈). A Hewlett Packard 1100 HPLC equipped with a diode array detector (DAD) was used to analyze the samples (at 308 nm for diquat). For triclopyr, water samples were acidified to pH≤ 2 with sulfuric acid:water (1:1) and extracted by solid phase extraction (C₁₈). The method detection limit and percentage recovery for each method were provided in Table 2.

**Table 2. Method detection limits and percentage recoveries of the analytic methods for the chemicals tested.**

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Sample Type</th>
<th>Instrument Used</th>
<th>Method Detection Limit, ppb</th>
<th>Reporting Limit, ppb</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonylphenol</td>
<td>Water</td>
<td>HPLC</td>
<td>0.10</td>
<td>0.20</td>
<td>91.0-110</td>
</tr>
<tr>
<td>Nonylphenolethoxylate (up to 10 Ethylene oxide units)</td>
<td>Water</td>
<td>HPLC</td>
<td>0.10</td>
<td>0.20</td>
<td>91.0-105</td>
</tr>
<tr>
<td>Triclopyr</td>
<td>Water</td>
<td>LC-MS</td>
<td>0.002</td>
<td>0.005</td>
<td>98.0-100</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>Water</td>
<td>HPLC-Post Column</td>
<td>2.00</td>
<td>5.00</td>
<td>81.0-87.0</td>
</tr>
<tr>
<td>Diquat Dibromide</td>
<td>Water</td>
<td>HPLC</td>
<td>0.20</td>
<td>0.50</td>
<td>70.0-75.0</td>
</tr>
<tr>
<td>2,4-D</td>
<td>Water</td>
<td>LC-MS</td>
<td>0.01</td>
<td>0.02</td>
<td>80.0-98.0</td>
</tr>
</tbody>
</table>

5.3.2 Test species

Juvenile rainbow trout (Standard length:11.5 ± 2.2 cm) were kindly provided by the California Department of Fish and Game at the Mojave River Hatchery (Victorville, California). After being transferred to the University of California at Riverside, they were maintained in a living stream (Frigid Units, OH, USA) receiving filtered dechlorinated tap water at a flow rate of 5 L/min. The water temperature in the living stream was 14 ± 1°C. The fish were fed Purina rainbow trout chow (St. Louis, MO, USA) at approximately 1% of their body weight every day. Light cycles were at 16:8 h (light:dark). Fish were acclimated to these conditions for at least two weeks prior to exposure.
5.3.3 Range finding experimental exposure.

This pilot experiment was carried out for the selection of chemicals for concentration-response exposures. Rainbow trout were exposed to pesticides and surfactants at concentrations based on the label information provided by the pesticide manufacturers. The concentrations for pesticides: glyphosate, 2,4-D, diquat dibromide, triclopyr were 0.11, 1.64, 2.07, and 1.25 mg/L respectively. The concentrations for R-11 and Target Pro-spreadr Activator (TPA) were 1.46 and 0.8 mg/L. For exposure to mixtures, rainbow trout were exposed to the highest concentration of each pesticide combined with each surfactant at fixed ratios of 1:2 (R-11 : pesticide) or 1:4.5 (TPA : pesticide). Exposures were carried out in 20 L tanks filled with aerated water in a daily static renewal system for 7 days. In addition, rainbow trout were exposed to five concentrations of 17β estradiol to calculate estradiol equivalent values (EEQs). Each concentration of chemical (treatment or control) had three replicates with 2 fish in each tank. The test water in each individual tank was monitored daily for water chemistry after every water renewal. The hardness of the test water ranged from 142 to 162 mg/L (as CaCO₃), the free chlorine was < 0.2 mg/L. Alkalinity ranged from 148 to 180 mg/L and ammonia (as N-NH₃) was < 0.02 mg/L. Dissolved oxygen averaged 94.6% of the air saturation value and pH values ranged from 6.0 to 8.2 mg/L. Water temperature was maintained at 16 ± 1°C. Fish were fed rainbow trout chow at approximately 1% of their body weight during the exposure. The photo period was 16 h : 8 h (light : dark).

5.3.4 Dose-response exposure

A 7-day dose-response exposure was carried out for 2,4-D, triclopyr, R-11, and TPA. Mixture experiments were conducted evaluating 2,4-D with R-11 or TPA and triclopyr with TPA. Each concentration (control or treatment) had 3 replicates with 2 fish in each replicate. Water chemistry, water temperature, and photo-period were similar to that of the worst-case scenario exposure (above). Fish were exposed to 2,4-D at concentrations of 0 (control), 0.00164, 0.0164, 0.164, and 1.64 mg/L. Fish were exposed to R-11 at concentrations of 0 (control), 0.0146, 0.146, 0.73, and 1.46 mg/L. Fish were exposed to TPA at concentrations of 0, 0.008, 0.08, 0.4, 0.8 mg/L. Fish were exposed to triclopyr at concentrations of 0 (control), 1, 10, 100, and 1000 ug/L. For binary mixtures of pesticide 2,4-D with the two surfactants, a fixed ratio was used (the ratio of the two
chemicals was kept constant, while the total concentrations of the mixture was varied). For 2,4-D + R-11, the concentrations used were 0 (control), 0.00164 mg/L (2,4-D) + 0.00089 mg/L (R-11), 0.0164 mg/L (2,4-D) + 0.0089 mg/L (R-11), 0.164 mg/L (2,4-D) + 0.089 mg/L (R-11), and 1.64 mg/L (2,4-D) + 0.89 mg/L (R-11). For binary mixture of 2,4-D + TPA, the concentrations used were 0 (control), 0.00164 mg/L (2,4-D) + 0.00048 mg/L (TPA), 0.0164 mg/L (2,4-D) + 0.0048 mg/L (TPA), 0.164 mg/L (2,4-D) + 0.048 mg/L (TPA), and 1.64 mg/L (2,4-D) + 0.48 mg/L (TPA). For binary exposure of TPA + triclopyr, the concentrations used were 0, 0.013 ug/L (TPA) + 1 ug/L (Triclopyr), 0.13 ug/L + 10 ug/L (Triclopyr), 1.3 ug/L (TPA) + 100 ug/L (Triclopyr), and 13 ug/L (TPA) + 1000 ug/L (Triclopyr).

5.3.5 Plasma vitellogenin levels determination

After the exposure, the fish were euthanized in MS-222 (50 mg/L). Blood samples from rainbow trout were obtained by an incision at the caudal peduncle and collection of the blood exiting the incision. Blood was centrifuged at 10,000 rpm for 3 minutes at room temperature. After centrifugation, PMSF (Phenylmethyl sulphonyl fluoride; Stock solution 0.1M) was added to the plasma samples at a final concentration of 1mM. The plasma samples were stored at −80°C until analysis.

Plasma vitellogenin levels were determined by enzyme-linked immunosorbent assay (ELISA) as previously described using a commercially available rainbow trout ELISA kit (Biosense, Bergen, Norway) (19). Total protein concentrations of the plasma samples were determined according to the methods of Bradford using bovine serum albumin as standards (0.25 – 2 mg/ml).

Vitellogenin levels in the plasma samples were expressed as ng vitellogenin per mg of total protein. Estrogenicity of the water samples was estimated from the standard curve of estradiol exposure (the dose-response sigmoid curve had an R^2 = 0.999, results not shown). The estrogenicity of the water samples was expressed as estradiol equivalent concentrations (ng/L).

5.3.6 Field Evaluation

To evaluate the effects of combined surfactant/pesticide exposure in a field setting. Water was collected from Anderson Pond (N 40°28.070, W 12°216.372), a 10-
acre pond south of Redding California near the Sacramento River at the northern end of the Sacramento Valley. Anderson Pond is under surveillance and treatment by the California Department of Food and Agriculture for Hydrilla control. No Hydrilla has been observed in the pond since 1999 and this was the final year of required observation. Triclopyr tank mixed with TPA was applied to control emergent water primrose. The primrose was treated in order to allow sunlight to reach the pond bottom in order to provide ideal growth conditions for any Hydrilla tubers present. The pesticide mixture was applied via three-gallon hand sprayers. Each three gallon pesticide mixture consisted of 0.25oz of TPA and 19oz of Renovate (ai. triclopyr triethlyamine salt). A total of 2.5oz of TPA and 190oz of Renovate was applied to two 20 by 20 meter areas of Anderson Pond. Water was collected from the middle of one of these application areas.

Fifty-five gallons of water was collected within the treatment area one hour after application occurred. The water was collected from a hand powered Zodiac boat by submerging one-gallon cleaned stainless steel buckets into the pond just below the surface allowing them to fill. The water was transferred via metal funnel into cleaned aged plastic five gallon bottles (water cooler bottles). Three five-gallon bottles were filled before the boat returned to shore (with oars) and the water was transferred to a 55-gallon Nalgene drum. The Nalgene drum was transported that day to UC Riverside to carry out exposures with trout. Water was placed in 20L tanks and aerated and it was also diluted 50% with dechlorinated tap water as a second exposure concentration. Dechlorinated tap water served as a negative control and estradiol served as a positive control (see above). Ten trout were exposed using 5 replicates (2 fish per tank) for 7 days, euthanized and tissues removed following the guidelines above. Water samples were removed for chemical analyses of triclopyr, 4-NP and nonylphenol ethoxylates.

5.3.7 Data Analysis

All data analyses were performed using Statistical Analysis System (SAS, version 8.2, Cary, NC). Normality was evaluated using the Shapiro test and equal variance using Levene’s test. Since assumption of normality and equal variance were violated, nonparametric tests (Kruskall-Wallis) were used to test the difference in vitellogenin levels in rainbow trout among different groups.
Estrogenicity in the unit of estradiol equivalent concentrations for chemicals was estimated from the standard curve of exposure to estradiol. The estrogenicity of the mixtures was calculated based on the model of concentration addition, which assumes that mixtures act via a similar mode of action in producing an effect (20, 21).

5.4 Results

5.4.1 Single concentration exposure

Results for a “worst-case scenario” exposure were presented in Figure 1. Pesticides triclopyr, glyphosate, and diquat dibromide did not induce elevated levels of vitellogenin in juvenile rainbow trout compared with control fish (Fig.1). However, rainbow trout exposed to 1.64 mg/L of 2,4-D had a 93-fold increase in plasma vitellogenin compared to the control fish. 2,4-D + R-11 did not induce a significant increase in vitellogenin levels in juvenile rainbow trout, while 2,4-D + TPA induced an 83-fold increase in plasma vitellogenin compared with the control fish. Furthermore, trout exposed to triclopyr mixed with TPA had a 506% increase in plasma vitellogenin levels compared to the control fish. No other mixtures of the pesticides with either of the two surfactants induced elevated levels of vitellogenin levels in rainbow trout (Fig.1).

5.4.2 Dose-response curve exposure

Vitellogenin induction in rainbow trout exposed to 2,4-D, R-11, TPA, 2,4-D with R-11, and 2,4-D with TPA all showed dose-related responses (Fig.2 and Fig.3). 2,4-D, TPA (in terms of 4-nonylphenol), and R-11 (in terms of 4-nonylphenol) produced concentration-dependent increases in plasma vitellogenin (Figure 2), with lowest observed effect concentrations (LOECs) of 0.164 mg/L, 0.4 mg/L (NP: 9.5 µg/L), and 0.73 mg/L (NP: 20 µg/L) respectively (Figure 2, Table 3). The mixture of 2,4-D and TPA had a smaller LOEC of 0.0164 mg/L (2,4 D) + 0.0048 mg/L (0.114 ug/L 4-NP) (TPA) and no-observed effect concentration (NOEC) (0.00164 mg/L-2,4 D + 0.0114 ug/L 4-NP) in rainbow trout than TPA or 2,4-D used individually (Table 3). The mixture of 2,4-D and R-11 had a higher LOEC of 1.64 mg/L and 0.89 mg/L (24.6 µg/L 4-NP) and NOEC than the two components used individually (Figure 3, Table 3). Overall the estrogenicity for the mixture of TPA or R-11 with 2,4-D both showed additive dose-response curves (in both cases, $P > 0.1$ and $R^2 >0.90$ for the four parameters dose response equation).
However, compared with the estimated estrogenicity of the individual chemicals, the mixture of TPA and 2,4-D showed a higher estrogenicity than the addition of the estrogenicity of the two chemicals alone at the lowest tested concentration of 0.00048 mg/L of TPA and 0.00164 mg/L of 2,4-D ($P < 0.05$. Figure 4). The mixture of TPA and 2,4-D showed less estrogenicity than the addition of the estrogenicity of the two chemicals at the highest test concentration (0.48 mg/L of TPA and 1.64 mg/L of 2,4-D) ($P < 0.05$). The mixture of R-11 and 2,4-D showed a marginally decreased estrogenicity at the highest test concentration of the two chemicals (0.89 mg/L of R-11 and 1.64 mg/L of 2,4-D) (Figure 4). Enhanced estrogenicity of the binary exposure of 2,4-D with TPA was only observed at the lowest tested concentration (Figure 5).

Table 3. No-observed effect concentration (NOEC) and lowest-observed effect concentration (LOEC) in rainbow trout exposed to different chemicals.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>NOEC</th>
<th>LOEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPA (in term of NP)</td>
<td>0.08 mg/L (1.95 ug/L)</td>
<td>0.4 mg/L (9.5 ug/L)</td>
</tr>
<tr>
<td>R-11 (in term of NP)</td>
<td>0.146 mg/L (4 ug/L)</td>
<td>0.73 mg/L (20 ug/L)</td>
</tr>
<tr>
<td>2,4-D</td>
<td>0.0164 mg/L</td>
<td>0.164 mg/L</td>
</tr>
<tr>
<td>2,4-D + TPA (in term of NP)</td>
<td>0.00164 mg/L + 0.0048 mg/L</td>
<td>0.0164 mg/L + 0.048 mg/L</td>
</tr>
<tr>
<td>2,4-D + R-11 (in term of NP)</td>
<td>0.164 mg/L + 0.089 mg/L</td>
<td>1.64 mg/L + 0.89 mg/L</td>
</tr>
</tbody>
</table>

Note: The values in the parenthesis are the nonylphenol (NP) concentrations in the corresponded concentrations of TPA and R-11.

Triclopyr failed to induce Vtg at any tested concentration (Data not shown). However, when treated in combination with TPA, estrogenic responses were greater than responses observed with TPA alone when these data were compared with vitellogenin expression (calculated in terms of EEQs) (Figure 6). When the laboratory exposures were compared to responses from trout exposed to the pond water where trichlopyr and TPA were used in combination, EEQ values in the 100% pond water ($8.5 \pm 7.7$ ng/L) concentration were similar to the 2nd ($9.5 \pm 10.7$ ng/L) and 3rd ($12.2 \pm 11.8$ ng/L) concentrations from combined treatments in the laboratory.

5.5 Discussion

Estrogenic activities of four herbicides, two surfactants, and the mixture of 2,4-D and triclopyr with surfactants were examined in juvenile rainbow trout. Of the four herbicides tested for estrogenicity, 2,4-D was the only compound that showed estrogentic activity in a “worst case scenario” exposure (1.64 mg/L). The LOEC for 2,4-D was 0.164
mg/L in rainbow trout exposed for 7 days. One study using the yeast two-hybrid assay determined that 2,4-D was estrogenic at a concentration of approximately 0.2 g/L indicating relatively weak affinity at the ER (22). Previous studies in our laboratory have shown in-vivo assays are more sensitive and robust than the YES in-vitro assay in detecting the estrogenic activities of chemicals (19, 23-25). This is likely do the ability of the in-vivo assay to respond to multiple mechanisms of action (i.e. alterations of steroid synthesis or feed-back loops) than simple ER activation.

Mechanism(s) explaining the in-vivo estrogenicity of 2,4 D are unknown. Possibilities include the transformation, either through biotic or abiotic means to metabolites that are more potent ER ligands or possess more disruptive characteristics. In aqueous solutions, 2,4-D undergoes rapid photolysis and hydrolysis to 2,4, dichlorophenol (11). However, it is slowly metabolized within fish to amino acid conjugates with up to 90% of the parent being eliminated in the urine unchanged (36-37). Although 2,4-Dichlorophenol was not active (> 1 mM) in the in-vitro MCF-7 cell line assay for ER activity (30), it did slightly bind quail ERα, but not ERβ (35). In trout, 2,4 dichlorophenol was shown to be an ER antagonist (12). Given the lack of transformation to 2,4 dichlorophenol within fish, and its antagonistic activity at the ER, it would appear that 2,4 D itself, an amino acid conjugate or an unknown metabolite may elicit estrogenicity indirectly outside of direct ER binding. These data would be consistent with the greater than additive response observed with the APE surfactants at low concentrations.

Whereas, 2,4 D demonstrated estrogenic activity alone, triclopyr failed to induce Vtg expression alone, but in combination with APE surfactants caused a significant increase which was higher than would be predicted from the surfactant treatment alone. Unlike 2,4 D, triclopyr is slowly degraded in the environment, but undergoes biotransformation in fish to the metabolite, trichloropyridinol (39), which a the major metabolite of the slightly estrogenic organophosphate chlorpyrifos (14). Unfortunately, there have not been any reported studies showing whether trichloropyridinol is estrogenic.
For most of the pesticides examined in the current study, combination with either APE-containing surfactant enhanced estrogenic activity. This was not surprising since it has been well established that alkylphenols and even ethoxylates induce vitellogenin and activate ER, although at low potencies. The LOEC values for Vtg induction (in terms of nonylphenol) for TPA and R-11 were similar to the values (~µg/L) observed from other studies (26) and similar to concentrations of alkylphenol ethoxylate and their degraded products in various surface waters (7). Within one day of exposure to 10-150 µg/L nonylphenol, vitellogenin mRNA was detected in liver of rainbow trout, with maximum production detected after 72 hours of exposure (27). There did not seem to be a consistent difference between R-11 and TPA with regard to induction alone or in combination with the pesticides. However, the binary mixture of 2,4-D with R-11 consistently showed higher responses in rainbow trout in terms of estrogenic activities than 2,4 D and TPA. However, in contrast, it was the TPA and 2,4 D mixture which provided statistically significant effects in terms of greater than additive response of EEQs at the lowest tested concentrations. Combinations with TPA also caused a statistically significant lesser than additive response at the high concentration. It should be noted, that a similar trend was observed with the R-11 mixtures with 2,4 D, but the values were not statistically significant. The difference in responses between the mixture of 2,4-D with either surfactants may be due to the other constituents within each surfactant mixture. For example, although there were no marked differences in the content of 4-NP in the two surfactants, nonylphenolmonoethoxylates were slightly higher in the TPA (Table 1). As these values only represent two compounds out of a multitude of isomers and other “inert” ingredients within the surfactant, it is unclear whether other compounds could be influencing the responses.

A consistent U-shaped concentration-response curve was observed when 2,4 D was combined with either surfactant. Estrogenicity was higher than the predicted sum of either compound at low concentrations, with gradual movement toward additivity in mid-range concentrations, followed by less than additive (predicted) responses in the highest concentrations. Greater than additive responses were also observed for triclopyr and TPA in the laboratory and possibly the field. Reasons for this pattern are unknown, but several reports have shown that combining environmental estrogens at sub-NOEC
concentrations resulted in a dramatic enhancement of the estrogenic effect (28, 29). U-shaped dose-response curves have been documented in many biological, toxicological, and pharmacological studies (33). In one study, it was shown that certain phytoestrogens were aromatase (CYP19) inhibitors at low concentrations (< 1uM) diminishing estrogen synthesis, but ER ligands at high concentrations (> 1 uM) (34). Alternatively, signal transduction pathways may be non-ER targets as upregulation of coregulators may enhance the transcriptional activity of steroid hormone receptors even in the absence of the ligands (31). Earlier studies examining gender ratios in fish following larval treatment with 4-NP indicated masculinization at low concentrations of exposure followed by feminization at higher concentrations (38). One simple possibility for diminishing estrogenic activity at higher mixture concentrations may be the acute toxicity of the surfactants, which may inhibit overall protein synthesis and, hence, the estrogenic response in fish. The 72 hr LC 50 for 50 to 200 g rainbow trout of 4-NP was 150 to 250 ug/L (27). Although the concentrations measured in the current study were less than this, smaller fish and a longer duration (7 d) were used which have led to enhanced toxicity. Range-finding studies showed that 5 mg/L of each surfactant resulted in 30% of the mortality in rainbow trout within 48 hours. In argument against toxicity, no mortality was observed in any of the surfactant-treated fish and the Vtg response was clearly concentration-dependent when the fish were treated with only surfactants at the same concentrations as those used for the mixtures. The 96 hr LC50 for the dimethylammonium salt of 2,4-D was reported to be 100 mg/L (32). The highest concentration utilized in the current study was 1.64 mg/L and no mortality was observed in any treatment. Thus, it would appear that the diminished estrogenic responses at the highest concentrations are not likely due to acute toxicity. Clearly, numerous targets may be involved with these responses and more research dedicated to the mechanisms of estrogenic synergism and antagonism with these compounds should prove fruitful.

The environmental relevance of greater than additive responses was noted by the concentration-dependent induction of Vtg in fish treated with pond water that had recently undergone triclopyr treatment. Chemical evaluation of the water indicated no detectable triclopyr, and 4-NP concentrations that were at or below laboratory derived NOEC and LOEC values. EEQ calculations indicated estrogenicity that was similar to
EEQs derived from laboratory treatments of triclopyr and TPA mixtures, which were greater than the additive responses of the individual compounds. These data indicate the mixture of triclopyr and TPA may be responsible for the estrogenicity in this sample. However, caution should be used as water was not evaluated prior to pesticide application and other compounds, such as natural phytoestrogens may be present. Studies are currently underway to evaluate this variable.

In summary, 2,4-D, as well as the APE-containing surfactants, R-11 and TPA were estrogenic to rainbow trout at environmentally relevant concentrations. Greater than additive responses were observed in the laboratory when 2,4-D or triclopyr were combined with the surfactant TPA. Response curves differed between the pesticides with the 2,4-D + TPA mixture displaying a U-shaped dose response. The estrogenic response of triclopyr and TPA was greater than additivity at mid-range concentrations and diminished at the highest concentration. Estrogenic activity was observed in pond water treated with triclopyr and TPA that was similar to laboratory values with the combined compounds. These data suggest caution should be utilized when using NOEC and LOEC values to assess estrogenic activity for individual compounds, and that utilization of additive responses are likely inappropriate for endocrine mediated endpoints.

Acknowledgement—This research was supported by a contract from the California State Water Resources Control Board via the San Francisco Estuarine Institute (Agreement #01-130-250-0). The authors wish to thank Thi Mai (UCR) and Matt Yates (UCR) for their help in maintaining the fish during exposure and Charles Striplen (SFEI) for his assistance in field sample collection.
5.6 References
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29. Rajapakse N, Silva E, Kortenkamp A. Combining xenoestrogens at levels below individual no-observed-effect concentrations dramatically enhances steroid hormone action. Environmental Health Perspectives 110:917-921 (2002).


36. James MO, Bend JR. Taurine conjugation of 2,4-Dichlorophenoxyacetic acid and phenylacetic acid in two marine species Xenobiotica 6:393-398 (1976).


5.7 Figures

Figure 1. Plasma vitellogenin levels in juvenile rainbow trout exposed to different pesticides: Triclopyr (T), 2,4-Dichlorophenoxyacetic Acid (2,4-D), Glyphosate (G), Diquat (D); and the mixture of the pesticides with two surfactants: R-11 and TPA for 7 days.

* indicates significant difference in vitellogenin levels from control at $P < 0.05$. 

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Figure 2. Plasma vitellogenin levels in juvenile rainbow trout exposed to different concentrations of 2,4-Dichlorophenoxyacetic Acid (2,4-D), R-11, and TPA for 7 days. The number in the parenthesis in the figure label was the concentration of the chemical.

* indicates significant difference in vitellogenin levels from control at $P < 0.05$. 

![Graph showing plasma vitellogenin levels in juvenile rainbow trout exposed to different concentrations of 2,4-Dichlorophenoxyacetic Acid (2,4-D), R-11, and TPA for 7 days. The number in the parenthesis in the figure label was the concentration of the chemical. * indicates significant difference in vitellogenin levels from control at $P < 0.05$.](image-url)
Figure 3. Vitellogenin levels in juvenile rainbow trout exposed to the mixture of 2,4-D with TPA or R-11 for 7 days. The concentration of each chemical is provided in Table 4.

* indicates significant difference in vitellogenin levels from control at $P < 0.05$.

Table 4. Concentrations of TPA, R-11, and 2,4-D for the dose response experiment.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Concentrations of chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TPA (mg/L)+2,4-D (mg/L)</td>
</tr>
<tr>
<td>C 1</td>
<td>0.00048 TPA + 0.00164 2,4-D</td>
</tr>
<tr>
<td>C 2</td>
<td>0.0048 TPA + 0.0164 2,4-D</td>
</tr>
<tr>
<td>C 3</td>
<td>0.048 TPA + 0.164 2,4-D</td>
</tr>
<tr>
<td>C 4</td>
<td>0.48 TPA + 1.64 2,4-D</td>
</tr>
</tbody>
</table>
Figure 4. Estradiol equivalent concentrations (EEQs) of various concentrations of mixture of R-11 and 2,4-D.

Data are expressed as Mean and standard deviation. Black bars are the predicted values, while dashlined bars are the measured value for the mixture of R-11 and 2,4-D. Concentration of each chemical is provided in Table 5.

Table 5. Concentrations of R-11 and 2,4-D for the estimation of estrogen equivalent concentrations.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Conc.s of chemicals dose</th>
<th>Conc.s of chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TPA (mg/L) 2,4-D (mg/L)</td>
<td>TPA (mg/L)+2,4-D (mg/L)</td>
</tr>
<tr>
<td>D 1</td>
<td>0.00048 0.00164 d 1</td>
<td>0.00048 TPA + 0.00164 2,4-D</td>
</tr>
<tr>
<td>D 2</td>
<td>0.0048 0.0164 d 2</td>
<td>0.0048 TPA + 0.0164 2,4-D</td>
</tr>
<tr>
<td>D 3</td>
<td>0.048 0.164 d 3</td>
<td>0.048 TPA +0.164 2,4-D</td>
</tr>
<tr>
<td>D 4</td>
<td>0.48 1.64 d 4</td>
<td>0.48 TPA + 1.64 2,4-D</td>
</tr>
</tbody>
</table>
**Figure 5. Estradiol equivalent concentrations (EEQs) of various concentrations of TPA and 2,4-D.**

Black bars are the predicted values, while dashlined bars are the measured values for mixture of TPA and 2,4-D. Data are expressed as Mean and standard deviation. * indicates significant different between the measured values and the predicted values at $P < 0.05$. Concentration of each chemical is provided in Table 6.

![Graph showing estradiol equivalent concentrations](image)

**Table 6. Concentrations of TPA and 2,4-D for the estimation of estradiol equivalent concentrations.**

<table>
<thead>
<tr>
<th>Dose</th>
<th>Conc.s of chemicals</th>
<th>dose</th>
<th>Conc.s of chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TPA (mg/L)</td>
<td></td>
<td>TPA (mg/L) + 2,4-D (mg/L)</td>
</tr>
<tr>
<td>D 1</td>
<td>0.00048</td>
<td>d 1</td>
<td>0.00048 TPA + 0.00164 2,4-D</td>
</tr>
<tr>
<td>D 2</td>
<td>0.0048</td>
<td>d 2</td>
<td>0.0048 TPA + 0.0164 2,4-D</td>
</tr>
<tr>
<td>D 3</td>
<td>0.048</td>
<td>d 3</td>
<td>0.048 TPA + 0.164 2,4-D</td>
</tr>
<tr>
<td>D 4</td>
<td>0.48</td>
<td>d 4</td>
<td>0.48 TPA + 1.64 2,4-D</td>
</tr>
</tbody>
</table>
Figure 6. Estradiol equivalent concentrations (EEQs) of various concentrations of mixture of triclopyr and TPA and the field-collected water samples.

Data are expressed as Mean and standard deviation. Black bars are the measured values, while empty bars are the predicted value for the mixture of triclopyr and TPA, and dashlines are for the half-diluted and non-diluted field collected water samples. * indicates significant difference between the predicted value and the measured value at $P < 0.05$. Concentration of each chemical is provided in Table 7.

Table 7. Concentrations of TPA and triclopyr for the estimation of estradiol equivalent concentrations.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Conc.s of chemicals</th>
<th>Dose</th>
<th>Conc.s of chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TPA (ug/L)</td>
<td></td>
<td>TPA (ug/L)+triclopyr (ug/L)</td>
</tr>
<tr>
<td>c1</td>
<td>0.013</td>
<td>1</td>
<td>0.013TPA +1 triclopyr</td>
</tr>
<tr>
<td>c2</td>
<td>0.13</td>
<td>10</td>
<td>0.013 TPA + 10 triclopyr</td>
</tr>
<tr>
<td>c3</td>
<td>1.3</td>
<td>100</td>
<td>0.13 TPA +100 triclopyr</td>
</tr>
<tr>
<td>c4</td>
<td>13</td>
<td>1000</td>
<td>13 TPA +1000 triclopyr</td>
</tr>
</tbody>
</table>