

Evaluation of Estrogenic Activities of Aquatic Herbicides and Surfactants Using an Rainbow Trout Vitellogenin Assay

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Estrogenic potencies of four herbicides (triclopyr, 2,4-dichlorophenoxyacetic acid (2,4-D), diquat dibromide, glyphosate), two alkylphenol ethoxylate-containing surfactants (R-11 and Target Prospreader Activator (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 (Vtg) levels compared with untreated fish, while rainbow trout exposed to other pesticides alone did not show elevated vitellogenin levels compared to the control fish. When combined with surfactants, trends indicated enhanced estrogenicity for all combinations, but only 2,4-D and triclopyr caused significant induction of Vtg. Concentration-response studies demonstrated that the lowest observed effect concentrations (LOECs) for 2,4-D and triclopyr were 0.164 mg/l and 1 mg/l, respectively. In terms of measured 4-nonylphenol (4-NP), the LOECs of R-11 and TPA were 20 µg/l and 9.5 µg/l, respectively. Binary mixtures of TPA and 2,4-D showed a greater than additive estrogenic response at the lowest concentrations tested, but a 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 µg/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 triclopyr + 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 alkylphenol ethoxylate-containing surfactants with two aquatic pesticides possessed greater than additive estrogenic responses in fish under laboratory conditions and in a field setting.

Key Words: estrogenicity; surfactants; herbicides; rainbow trout; *in vivo*; vitellogenin.

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 (Heppell *et al.*, 1995; Jobling *et al.*, 1998; Sumpter and Jobling, 1995). Chemicals simulating the natural estrogen, 17β-estradiol, can bind to estrogen receptors (ERs) and initiate estrogenic activities in animals (Jobling *et al.*, 1998; Tyler *et al.*, 1998). Anthropogenic chemicals with estrogenic properties include organochlorine pesticides, herbicides, polychlorinated biphenyls, alkylphenolic chemicals, and phthalates (Allen *et al.*, 1999). Environmental estrogens induce vitellogenin (Vtg) expression in male and juvenile fish (Sumpter and Jobling, 1995; Thorpe *et al.*, 2000). 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 (Wahli *et al.*, 1981). Thus, induction of vitellogenin in male and juvenile fish has been considered a reliable biomarker for exposure to environmental estrogens (Hemmer *et al.*, 2002; Sumpter and Jobling, 1995).

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 it is in water, it is readily degraded to 2,4-dichlorophenol (Crosby and Tutass, 1966), which is an estrogen receptor (ER) ligand (Jobling *et al.*, 1995). Triclopyr has a similar chemical structure to 2,4-D and photochemically decomposes to trichloropyridinol within hours once it is in water (Petty *et al.*, 2003). Trichloropyridinol is also the main metabolite of chlorpyrifos, which also has weak ER activation (Andersen *et al.*, 2002). One study has

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shown that diquat does not initiate responses in estrogen receptor α and β transactivation assays (Kojima *et al.*, 2004).

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. Alkylphenol ethoxylates (APEs) are normally present in raw sewage effluent, and their degraded products (alkylphenols) bind estrogen receptor (Routledge and Sumpter, 1996) and cause estrogenic effects in fish (Jobling and Sumpter, 1993; Routledge *et al.*, 1998).

Few studies have addressed the estrogenic effects of combined exposure of pesticides and surfactants. Four herbicides were selected for evaluation based upon evidence of prior usage with surfactants and, in some cases, the potential for estrogenic activity. The objective of this study was to investigate the estrogenic potencies of four herbicides, nonionic surfactants, and the mixture of herbicides with surfactants using an *in vivo* rainbow trout vitellogenin assay.

MATERIALS AND METHODS

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

Measurement of chemicals. Measurements of 2,4-D, 4-nonylphenol (NP), nonylphenoethoxylates (NPE), triclopyr, glyphosate, and diquat were determined by the Department of Fish and Game Water Pollution Control Laboratory (Rancho Cordova, CA). For 2,4-D, water samples were acidified to $\text{pH} \leq 2$ with sulfuric acid:water (1:1) and extracted by solid phase extraction (C_{18}). The extracts were analyzed by LC-MS in API-ES negative mode. For the two surfactants, R-11 and TPA, water samples were extracted by solid phase extraction (C_{18}) for NP and 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 (Huggett *et al.*, 2003). For glyphosate, samples were filtered and

injected directly in high-performance liquid chromatography (HPLC) with post-column derivatization. For diquat, the pH of water samples was 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 (C_{18}). 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 $\text{pH} \leq 2$ with sulfuric acid:water (1:1) and extracted by solid phase extraction (C_{18}). The method detection limit and percentage recovery for each method were provided in Table 1.

Test species. Juvenile rainbow trout (standard length: 11.5 ± 2.2 cm) were provided by the California Department of Fish and Game Mojave River Hatchery (Victorville, California). After being transferred to the University of California at Riverside (UCR), they were maintained in a living stream (Frigid Units, Toledo, OH) receiving filtered dechlorinated tap water at a flow rate of 5 l/min. The water temperature in the living stream was $14 \pm 1^\circ\text{C}$. The fish were fed Purina rainbow trout chow (St. Louis, MO) 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 2 weeks prior to exposure. No mortality was observed during the entire treatment process.

Range finding experimental exposure. Rainbow trout were exposed to pesticides and surfactants at concentrations based upon the recommendations provided by the pesticide manufacturers. The measured concentrations for pesticides, glyphosate, 2,4-D, diquat dibromide, and triclopyr, were 0.11, 1.64, 2.07, and 1.25 mg/l respectively. The nominal concentrations for R-11 and 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 in ethanol to calculate estradiol equivalent values (EEQs) as previously described (Huggett *et al.*, 2003; Thompson *et al.*, 2000; Xie *et al.*, 2005). Each concentration of chemical (treatment or control) had three replicates with two 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 CaCO_3); the free chlorine was <0.2 mg/l. Alkalinity ranged from 148 to 180 mg/l, and ammonia (as N- NH_3) 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 $16 \pm 1^\circ\text{C}$. 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).

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 three replicates with two fish in each replicate. Water

TABLE 1
Method Detection Limits and Percentage Recoveries of the Analytic Methods for the Chemicals Tested

Analysis	Sample type	Instrument used	Method detection limit, ppb	Reporting limit, ppb	% Recovery	Reference
Nonylphenol	water	HPLC	0.10	0.20	91.0–110	Björn <i>et al.</i> , 1997
Nonylphenoethoxylate (up to 10 ethylene oxide units)	water	HPLC	0.10	0.20	91.0–105	Björn <i>et al.</i> , 1997
Triclopyr	water	LC-MS	0.002	0.005	98.0–100	EPA Method 3535A EPA Method 615
Glyphosate	water	HPLC-post column	2.00	5.00	81.0–87.0	EPA Method 547
Diquat dibromide	water	HPLC	0.20	0.50	70.0–75.0	EPA Method 549
2,4-D	water	LC-MS	0.01	0.02	80.0–98.0	EPA Method 3535A EPA Method 615

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 (control), 0.008, 0.08, 0.4, 0.8 mg/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 µg/l (TPA) + 1 µg/l (triclopyr), 0.13 µg/l + 10 µg/l (triclopyr), 1.3 µg/l (TPA) + 100 µg/l (triclopyr), and 13 µg/l (TPA) + 1000 µg/l (triclopyr).

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 min at room temperature. After centrifugation, PMSF (Phenylmethyl sulphonyl fluoride; stock solution 0.1 M) was added to the plasma samples at a final concentration of 1 mM. 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) (Xie *et al.*, 2005). 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.

Field evaluation. To evaluate the effects of combined surfactant/pesticide exposure in a field setting, water was collected from Anderson Pond (N $40^{\circ}28.070$, W $12^{\circ}216.372$), 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 treated by the California Department of Food and Agriculture for Hydrilla control. No Hydrilla has been observed in the pond since 1999, and 2004 was the final year of required observation. Triclopyr 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 3-gallon hand sprayers. Each 3-gallon pesticide mixture consisted of 0.25 oz of TPA and 19 oz of Renovate (ai. triclopyr triethylamine salt). A total of 2.5 oz of TPA and 190 oz 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 after application.

Fifty-five gallons of water was collected within the treatment area 1 h after application occurred in July of 2004. Water was also collected in February of 2005 as a negative control. The water was collected from a hand-powered Zodiac boat by submerging 1-gallon cleaned stainless steel buckets into the pond just below the surface and allowing them to fill. The water was transferred via a metal funnel into cleaned, aged plastic 5-gallon bottles (water cooler bottles). Three 5-gallon bottles were filled before the boat returned to shore, and the water was transferred to a 55-gallon Nalgene drum. The Nalgene drum was transported that day to UCR to carry out exposures with trout. Water was placed in 20-l 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 five replicates (two fish per tank) for 7 days, and euthanized, and tissues removed following the guidelines above. Water samples were removed for chemical analyses of triclopyr, 4-NP and nonylphenol ethoxylates after exposures were concluded.

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 the assumption of normality and equal variance were violated, nonparametric tests (Kruskal-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 17 β estradiol (E2). 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 (Altenburger *et al.*, 2003; Loewe and Mulschnek, 1926).

RESULTS

Single Concentration Exposure

Results for a "worst-case scenario" exposure are 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 R-11 had a 5-fold 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).

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 (Fig. 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 (Fig. 2, Table 2). The mixture of

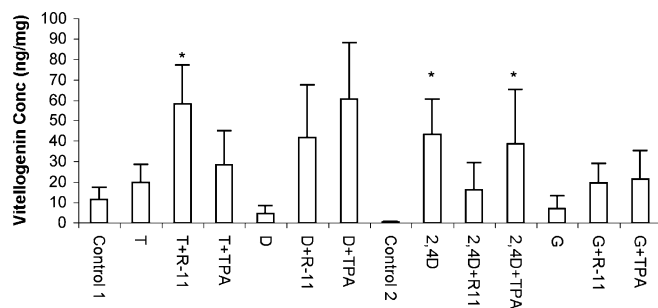


FIG. 1. Plasma vitellogenin levels in juvenile rainbow trout exposed to different pesticides: triclopyr (T), 2,4-dichlorophenoxyacetic acid (2,4-D), glyphosate (G), diquat (D); and the mixture of the pesticides with two surfactants: R-11 and TPA for 7 days. * Indicates significant difference in vitellogenin levels from control at $p < 0.05$.

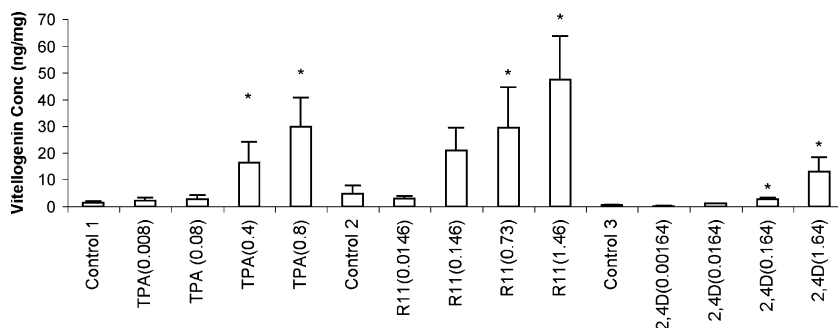


FIG. 2. Plasma vitellogenin levels in juvenile rainbow trout exposed to different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), R-11, and TPA for 7 days. The number in the parenthesis in the figure label was the concentration of the chemical. * Indicates significant difference in vitellogenin levels from control at $p < 0.05$.

2,4-D and TPA had a smaller LOEC of 0.0164 mg/l (2,4-D) + 0.0048 mg/l (0.114 μ g/l 4-NP) (TPA) and no-observed effect concentration (NOEC) (0.00164 mg/l 2,4-D + 0.0114 μ g/l 4-NP) in rainbow trout than TPA or 2,4-D used individually (Table 2). 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 μ g/l 4-NP) and NOEC than the two components used individually (Fig. 3, Table 2). Overall, the estrogenicity for the mixtures 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 ($p < 0.05$, Fig. 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) (Fig. 4). Enhanced estrogenicity of the binary exposure of 2,4-D with TPA was only observed at the lowest tested concentration (Fig. 5).

Triclopyr failed to induce Vtg at any tested concentration (data not shown). However, when triclopyr was treated in

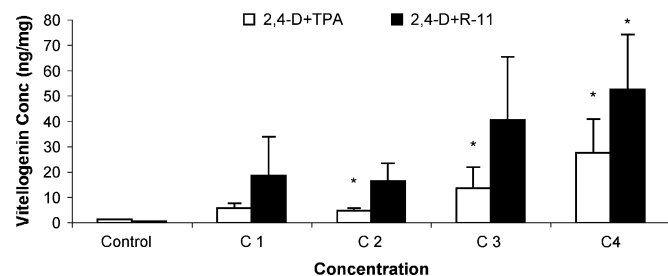


FIG. 3. Plasma vitellogenin levels (ng/mg) in juvenile rainbow trout exposed to the mixture of 2,4-D with TPA or R-11 for 7 days. The concentration of each chemical is provided in Table 3. * Indicates significant difference in vitellogenin levels from control at $p < 0.05$.

combination with TPA, Vtg expression was greater than responses observed with TPA alone at the lowest combined concentration (calculated in terms of EEQs) (Fig. 5). When the laboratory exposures were compared to responses from trout exposed to the pond water where triclopyr and TPA were used in combination, EEQ values in the 100% pond water (8.5 ± 7.7 ng E2/l) concentration were similar to the second (9.5 ± 10.7 ng E2/l) and third (12.2 ± 11.8 ng E2/l) concentrations from combined treatments in the laboratory. Nonylphenol was measured in Anderson pond after triclopyr/TPA treatment at 3.72 ± 0.056 μ g/L. Nonylphenolethoxylate concentrations were 21.3 ± 0.28 μ g/L after treatment. Triclopyr was not detected in Anderson Pond at either sampling event. None of the three analytes nor a Vtg response was observed in water collected in February 2005.

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

TABLE 2
Measured No-Observed Effect Concentration (NOEC)
and Lowest-Observed Effect Concentration (LOEC) in
Rainbow Trout Exposed to Different Chemicals

Chemicals	NOEC	LOEC
TPA (Nonylphenol)	0.08 mg/l (1.95 μ g/l)	0.4 mg/l (9.5 μ g/l)
R-11 (Nonylphenol)	0.146 mg/l (4 μ g/l)	0.73 mg/l (20 μ g/l)
2,4-D	0.0164 mg/l	0.164 mg/l
2,4-D + TPA	0.00164 mg/l + 0.0048 mg/l	0.0164 mg/l + 0.048 mg/l
(Nonylphenol)	0.00164 mg/l + 0.0114 mg/l	0.0164 mg/l + 0.114 μ g/l
2,4-D + R-11	0.164 mg/l + 0.089 mg/l	1.64 mg/l + 0.89 mg/l
(Nonylphenol)	0.164 mg/l + 2.46 μ g/l	1.64 mg/l + 24.6 μ g/l

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

TABLE 3
Concentrations of TPA, R-11, and 2,4-D Utilized for Experiments

Dose	Concentrations of chemicals	
	TPA (mg/l)+2,4-D (mg/l)	R-11 (mg/l)+2,4-D (mg/l)
C1	0.00048 TPA + 0.00164 2,4-D	0.00089 R-11 + 0.00164 2,4-D
C2	0.0048 TPA + 0.0164 2,4-D	0.0089 R-11 + 0.0164 2,4-D
C3	0.048 TPA + 0.164 2,4-D	0.089 R-11 + 0.164 2,4-D
C4	0.48 TPA + 1.64 2,4-D	0.89 R-11 + 1.64 2,4-D

Note. Concentrations of TPA, R-11, and 2,4-D utilized for the dose-response experiments in Figure 3 and measured estradiol equivalents (black bars) (ng/l) in Figure 4.

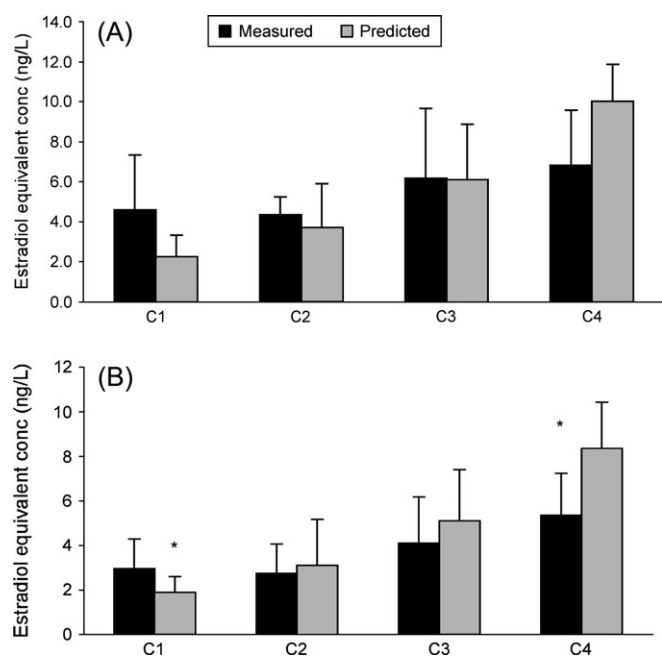


FIG. 4. Estradiol equivalent concentrations (EEQs) (ng/l) in fish exposed to mixtures of R-11 and 2,4-D (A) as well as TPA and 2,4-D (B). Data are expressed as mean \pm standard deviation. Black bars (Measured) were EEQ values determined from the Vtg concentrations of Figure 3, while dashed bars (Predicted) were EEQ values predicted using the additive responses of Vtg after exposure to each surfactant and 2,4-D determined from Figure 2. Concentration of each chemical is provided in Table 3.

tested for estrogenicity, 2,4-D was the only compound that showed estrogenic 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 with the ER (Nishihara *et al.*, 2000). Previous studies in our laboratory have shown *in vivo* assays are more sensitive and robust than the Yeast Estrogen Screen *in vitro* assay in detecting the estrogenic activities of chemicals (Huggett *et al.*, 2003;

Thompson *et al.*, 2000; Tilton *et al.*, 2002; Xie *et al.*, 2005). This is likely due to 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 that possess more disruptive characteristics. In aqueous solutions, 2,4-D undergoes rapid photolysis and hydrolysis to 2,4-dichlorophenol (Crosby and Tutass, 1966). However, it is slowly metabolized within fish to amino acid conjugates, with up to 90% of the parent being eliminated in the urine unchanged (James *et al.*, 1976; Plakas *et al.*, 1992). Although 2,4-dichlorophenol was not active (>1 mM) in the *in vitro* MCF-7 cell line assay for ER activity (Korner *et al.*, 1998), it did slightly bind quail ER α , but not ER β (Maekawa *et al.*, 2004). In trout, 2,4-dichlorophenol was shown to be an

TABLE 4
Concentrations of TPA and Triclopyr for the Estimation of Estradiol Equivalent Concentrations

Dose	Conc. of chemicals		Dose	Conc. of chemicals
	TPA (μ g/l)	Triclopyr (μ g/l)		TPA (μ g/l)+triclopyr (μ g/l)
S1	0.013	1	M1	0.013TPA + 1 triclopyr
S2	0.13	10	M2	0.013 TPA + 10 triclopyr
S3	1.3	100	M3	0.13 TPA + 100 triclopyr
S4	13	1000	M4	13 TPA + 1000 triclopyr

Note. Concentrations of TPA and triclopyr for the estimation of estradiol equivalent concentrations (ng/l) in Figure 5.

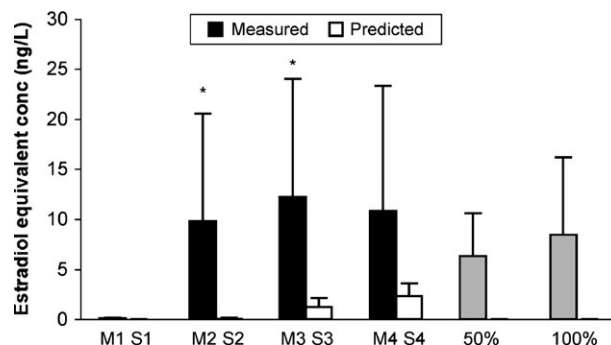


FIG. 5. Estradiol equivalent concentrations (EEQs) (ng/l) in fish exposed to mixtures of triclopyr and TPA and water from Anderson Pond following a TPA/triclopyr application. Data are expressed as mean \pm standard deviation. Black bars are the measured values of EEQs determined from the Vtg response, while empty bars are the predicted EEQ value for the mixture of triclopyr and TPA based upon additive Vtg responses following exposures to TPA and triclopyr as separate stressors. Dashed bars represent EEQs (ng/l) of fish following exposure to 50% and 100% of Anderson Pond Water after mixture treatment. * Indicates significant difference between the predicted value and the measured value at $p < 0.05$. Concentrations of each chemical are provided in Table 4.

ER antagonist (Jobling *et al.*, 1995). 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. Alternatively, it is well documented that 2,4-D also contains several dioxin-like compounds which may affect the estrogenic response. However, most aryl hydrocarbon receptor (AhR) agonists tend to repress rather than enhance estrogenic activity (Safe and Wormke, 2003). However, this may be a dose-dependent phenomenon, as previous studies comparing the estrogenic response in rainbow trout hepatocytes with varied doses of the AhR agonist, β -naphthoflavone, indicated both enhancement and antagonism of Vtg expression following E2 treatment (Anderson *et al.* 1996).

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 (Petty *et al.*, 2001), which is the major metabolite of the slightly estrogenic organophosphate chlorpyrifos (Andersen *et al.*, 2002). 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 with low potencies. The LOEC values for Vtg induction (in terms of NP) for TPA and R-11 were similar to the values ($\sim\mu\text{g/l}$) observed from other studies (Thorpe *et al.*, 2001) and similar to concentrations of alkylphenol ethoxylate and their degraded products in various surface waters (Rodgers-Gray *et al.*, 2000). Within one day of exposure to 10–150 $\mu\text{g/l}$ NP, vitellogenin mRNA was detected in liver of rainbow trout, with maximum production detected after 72 h of exposure (Lech *et al.*, 1996). 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. 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 less 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 surfactant 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, NPEs were approximately 20% higher in the TPA (data not shown). 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 inverse sigmoidal 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 (Rajapakse *et al.*, 2001, 2002). U-shaped dose-response curves have been documented in many biological, toxicological, and pharmacological studies (Calabrese and Baldwin, 2001). In one study, it was shown that certain phytoestrogens were aromatase (CYP19) inhibitors at low concentrations ($<1\ \mu\text{M}$) diminishing estrogen synthesis but ER ligands at high concentrations ($>1\ \mu\text{M}$) (Almstrup *et al.*, 2002). 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 (Katzenellenbogen *et al.*, 1996). 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 (Nimrod and Benson, 1998). 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-h LC50 for 50- to 200-g rainbow trout of 4-NP was 150 to 250 $\mu\text{g/l}$ (Lech *et al.*, 1996). 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 h. 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-h LC50 for the dimethylamine salt of 2,4-D was reported to be 100 mg/l (Tomlin, 1994). 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.

In summary, 2,4-D and 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 an inverse 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.

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