

PESTICIDES IN MOUNTAIN YELLOW-LEGGED FROGS (*RANA MUSCOSA*) FROM THE SIERRA NEVADA MOUNTAINS OF CALIFORNIA, USA

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**Abstract**—In 1997, pesticide concentrations were measured in mountain yellow-legged frogs (*Rana muscosa*) from two areas in the Sierra Nevada Mountains of California, USA. One area (Sixty Lakes Basin, Kings Canyon National Park) had large, apparently healthy populations of frogs. A second area (Tablelands, Sequoia National Park) once had large populations, but the species had been extirpated from this area by the early 1980s. The Tablelands is exposed directly to prevailing winds from agricultural regions to the west. When an experimental reintroduction of *R. muscosa* in 1994 to 1995 was deemed unsuccessful in 1997, the last 20 (reintroduced) frogs that could be found were collected from the Tablelands, and pesticide concentrations in both frog tissue and the water were measured at both the Tablelands and at reference sites at Sixty Lakes. In frog tissues, dichlorodiphenyldichloroethylene (DDE) concentration was one to two orders of magnitude higher than the other organochlorines ( $46 \pm 20$  ng/g wet wt at Tablelands and  $17 \pm 8$  Sixty Lakes). Both  $\gamma$ -chlordane and *trans*-nonachlor were found in significantly greater concentrations in Tablelands frog tissues compared with Sixty Lakes. Organophosphate insecticides, chlorpyrifos, and diazinon were observed primarily in surface water with higher concentrations at the Tablelands sites. No contaminants were significantly higher in our Sixty Lakes samples.

**Keywords**—Pesticide Amphibian Sierra Nevada Mountains Organophosphate *Rana muscosa*

## INTRODUCTION

Researchers have observed severe declines in several amphibian populations in the Sierra Nevada Mountains of California, USA [1–4]. Disease, habitat loss, ultraviolet radiation, predation, and pollution are factors that have been hypothesized to be involved with the disappearance of amphibians over the last two decades [1,5–14]. Frogs, toads, and salamanders are an important part of the terrestrial ecosystem of the Sierra Nevada Mountains, an alpine habitat located in California and Nevada. *Rana muscosa* (mountain yellow-legged frog) is of special interest because southern California populations have been listed as endangered by the U.S. Fish and Wildlife Service [15], and the listing of the Sierran populations has been determined to be warranted, but precluded (due to lack of budget and personnel) by the U.S. Fish and Wildlife Service [16]. *Rana muscosa* is especially vulnerable to pollutants in the water because two to four years are required for tadpoles to metamorphose, and three to four years for frogs to reach sexual maturity [17]. Furthermore, this species inexplicably has disappeared from national park areas where scientists previously had documented flourishing populations.

Amphibian surveys conducted by U.S. Geological Survey field biologists in the Sixty Lakes Basin (Kings Canyon National Park) from 1992 to 1995 indicated that there were at least 62 populations of *R. muscosa*. Many of these populations were large and apparently healthy. Population counts showed that a minimum of 1,700 adult frogs, 1,200 subadults, and 16,000 tadpoles occupied the basin (Fellers, unpublished data). As recently as 1980, there also were large populations of *R. muscosa* in the Tablelands, Sequoia National Park, only 30

km to the southwest. Surveys of all 44 ponds in the Tablelands area during the summer of 1980 indicated that there were approximately 1,000 frogs and 10,000 tadpoles (D. Bradford, U.S. Environmental Protection Agency, Las Vegas, NV, USA, personal communication). By the mid-1980s, *R. muscosa* could no longer be found at the Tablelands, and repeated searches during 1992 indicated that this frog had been extirpated from the area (Fellers et al., unpublished data). The cause of this extirpation was not apparent. The decline occurred in a large national park, an area that was protected from habitat loss. The absence of introduced fish in all but a few lakes in the lowest portions of the watershed precludes fish predation as a causative factor.

In 1994 and 1995, Fellers et al. (unpublished data) conducted an experimental reintroduction to evaluate potential causes of the extirpation in the 1980s. *Rana muscosa* eggs, tadpoles, subadults, and adults were translocated from several sites in the Sixty Lakes Basin to a series of ponds at a similar elevation in the Tablelands. By 1997, there had been only minimal reproduction by introduced frogs, and recruitment from the introduced eggs and tadpoles had not been sufficient to maintain the adult population. Based on these observations, we believed that the reintroduced frogs were not going to persist, so the remaining individuals were collected and analyzed for contaminants. A comparable sample of *R. muscosa* was collected from the original donor sites in the Sixty Lakes Basin.

Several lines of evidence support the hypothesis that amphibian declines in the Sierra Nevada may be caused, at least in part, by contaminants drifting into the mountains from the adjacent agricultural areas of the Central Valley [3,14,18]. Because the Tablelands are exposed directly to the prevailing westerly winds, it seems plausible that contaminants might be

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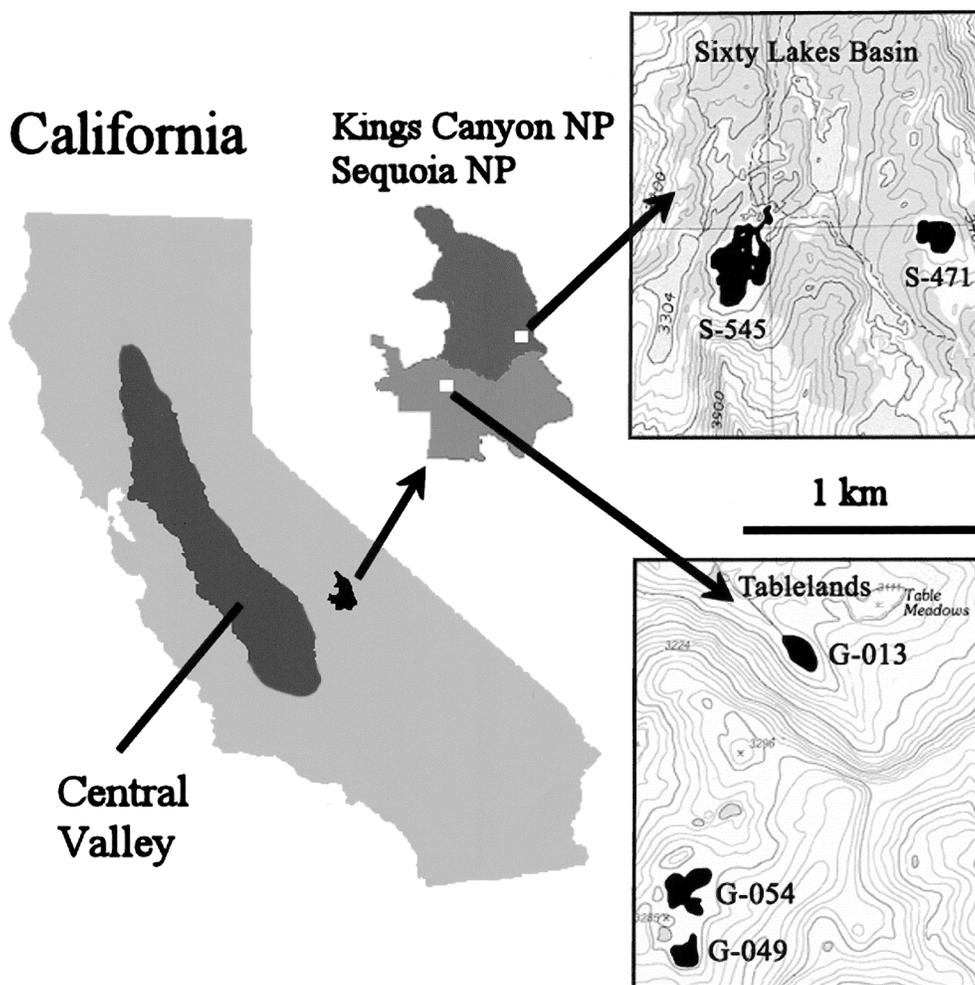


Fig. 1. Location of *Rana muscosa* tissue and surface water collection sites at the Sixty Lakes Basin, Kings Canyon National Park (NP), and the Tablelands, Sequoia National Parks, California, USA.

playing a significant role in both the original and subsequent loss of *R. muscosa* in the Tablelands. This paper provides data on the concentrations of pesticide residues in frogs from two sites in the Sierra Nevada Mountains and adds support to the idea that pesticide drift from the Central Valley of California is a significant contributing factor in Sierran amphibian declines.

## MATERIALS AND METHODS

### Design

The Tablelands area lies east of the highly agricultural Central Valley of California. The Tablelands sites are located in a west-facing drainage near the crest of the Sierra Nevada Mountains (36°48.737'N, 118°38.805'W; site G-049; Fig. 1), and are not protected from the prevailing winds from the west. By contrast, sites in the Sixty Lakes Basin are in a north-facing watershed (36°48.850' N, 118°25.584' W; site S-545, Fig. 1) and, hence, not likely to be as directly influenced by atmospheric transport of contaminants from the Central Valley. Although there has been no comparison of pesticide deposition in these two areas, it is reasonable to hypothesize that concentrations of pesticides would be higher at the Tablelands. Given the persistence of *R. muscosa* in the Sixty Lakes Basin and the complete loss of this frog from the Tablelands, we compared pesticide levels in both frogs and pond water to

evaluate whether there might be differences that would explain the observed loss.

A number of the analytes we detected are banned as pesticides in the United States and in most other countries, but they were used historically and are known to be persistent global contaminants that bioaccumulate. These are:  $\alpha$ - and  $\gamma$ -hexachlorocyclohexane,  $\alpha$ - and  $\gamma$ -chlordane, *trans*-nonachlor, two parent DDT isomers, and the metabolite dichlorodiphenyldichloroethylene (DDE). Other chemicals we examined currently are registered for use by United States farmers [19] and are applied in the Central Valley agricultural region of California [20]. These include diazinon and chlorpyrifos (organophosphate insecticides [OPs]), the two endosulfan isomers (a chlorinated insecticide), and the sulfate degradation product of endosulfan. These chemicals have differing toxicities and modes of action; hence, the compound classes must be considered separately.

### Sample collection

From August 18th through the 20th, 1997, we were able to find only 20 *R. muscosa* that had survived the experimental reintroduction at the Tablelands (Fellers et al., unpublished data). All 20 frogs were collected, including 12 adult males, three adult females, and five subadults. Most of the frogs were collected at an unnamed pond we designated site G-049 (Fig.

1). Two frogs were collected from G-054 and one was collected from site G-013. Each frog was anaesthetized in a 0.02% solution of benzocaine [21], weighed, measured, and frozen in liquid nitrogen.

On August 22, 1997, 20 *R. muscosa* from the Sixty Lakes reference sites were collected (10 adult males, five adult females, and five subadults) and processed in the same way. All frogs came from one of two unnamed ponds that we designated as sites S-545 and S-471. Frog samples were shipped on dry ice to the University of Nevada (Reno, NV, USA) for processing. Analyses were conducted at the U.S. Department of Agriculture in Beltsville (MD, USA).

At each site, concurrent 4-L water samples also were collected. Water was filtered through a glass fiber filter (Whatman, Clifton, NJ, USA) and extracted using a 4-g C18 solid-phase extraction cartridge (Jones Chromatography, Lakewood, CO, USA). A detailed description of the sample collection, processing, and analysis methods used for these water samples has been published by LeNoir et al. [20]. However, LeNoir et al. presented data only on currently used pesticides and not the organochlorines, which also were analyzed in these samples. In addition, concentration values from the two samples collected at the Tablelands and Sixty Lakes sites were given only as averages. Here we present concentrations of all analytes found in each water sample. Values that are below quantitation limit indicate that the chemical was detected, but concentrations were below our quantitation criteria. Values below detection level indicate that the compound was not detected at all.

The analytes included in our study represent several major compound classes of both currently used agricultural pesticides and persistent organochlorine insecticides. Information on agricultural pesticide use in the San Joaquin Valley region has been discussed in detail in previous publications [20,22]. Though the analyte list is not exhaustive, the chemicals included represent those that have been observed in previous studies of atmospheric transport and deposition of pesticides to the Sierra Nevada Mountain Range [20,22–23]. The same set of target analytes was included in both the water and tissue sample analysis methods.

#### Chemical analysis

Tissue samples from the preserved frogs were extracted following the procedures of Ribick et al. [24], a method that has been shown to be effective in extracting pesticides from tissue samples. Individual whole frog samples were homogenized in a blender with anhydrous tissue: $\text{Na}_2\text{SO}_4$  (1:4 wt/wt) until a flowing powder was achieved. Each sample was ground into a finer powder using a mortar and pestle. The powder was packed into a  $2.5 \times 53$ -cm glass column and extracted with 100 to 250 ml chromatographic-grade methylene chloride. The solvent volume was increased for the larger mass samples. The eluant was concentrated and cleaned-up on a 30-g Florisil column (Aldrich [now Sigma-Aldrich], St. Louis, MO, USA) to remove lipids, and pesticide residues were eluted with 50 ml pesticide-grade anhydrous diethyl ether in petroleum ether (70:30 v/v). The eluant was concentrated to 0.5 ml and exchanged into 1.0 to 1.5 ml of isoctane for final analysis. Four procedural blank samples using only the drying agent also were extracted and analyzed in the same manner as the samples.

Extracts were analyzed using Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA, USA) equipped with a Hew-

lett-Packard 5989A mass spectrometer operating in negative chemical ionization mode with selected ion monitoring. Details of chromatographic conditions have been published elsewhere by Sparling et al. [14] and McConnell et al. [22]. Sample extracts were spiked with 2,2',3,4,4',5,6,6'-octachlorobiphenyl (PCB-204) as an internal standard, and calibration standards with a minimum of three calibration points spanning the concentration levels of the samples were used for each analyte to ensure a high standard of accuracy in quantitation. The analytes were  $\alpha$ - and  $\gamma$ -hexachlorocyclohexane (HCH), diazinon, chlorpyrifos,  $\alpha$ - and  $\gamma$ -chlordane, *trans*-nonachlor,  $\alpha$ - and  $\beta$ -endosulfan, *o,p'*-DDT, *p,p'*-DDT, and *p,p'*-DDE.

Quantitation limits were set at three times the instrumental detection limit for each chemical, and ranged from 5 ng for  $\alpha$ -HCH to 20 ng for diazinon. No interfering peaks were observed in the procedural blank sample extracts, and only small traces of  $\alpha$ -endosulfan were observed in one blank extract at levels well below quantification limits.

#### Data analysis

All statistical tests were performed with Statistix (Ver 8, Analytical Software, Tallahassee, FL, USA; www.statistix.com/home.html). We used  $\alpha = 0.05$  to evaluate statistical significance. For statistical comparisons, contaminants that were not detected were treated as zeros. Contaminants that were detected, but were below quantitation limits, were entered as one-half the quantitation limit of that sample.

## RESULTS

### Organophosphate insecticides

Chlorpyrifos was observed in water samples at all four sites, with the highest concentration at one Tablelands site (G-049), and much lower concentrations at the other Tablelands site and the two Sixty Lakes sites (Table 1). Diazinon was found at similar concentrations at the two Tablelands sites, but at much lower concentrations at one of the Sixty Lakes sites (Table 1). Diazinon was not detected in any of frog tissue samples, and chlorpyrifos was present above the quantitation limits (0.06 ng/L) in only one of the Sixty Lakes samples (S-471–9, 0.67 ng/g wet wt).

### Endosulfan

Residues of both endosulfan isomers and the sulfate degradation products were present in water from both Tablelands and Sixty Lakes. Only small differences occurred in the levels of the  $\alpha$ - and  $\beta$ -isomers from the Tablelands and Sixty Lakes (Table 1), but the sulfate concentrations were almost an order of magnitude higher at the Tablelands compared with Sixty Lakes (Table 1). In tissue samples, only the  $\alpha$ -endosulfan isomer was observed at levels above our quantitation limits and concentrations at the Tablelands sites were not significantly different from the Sixty Lakes sites ( $H = 1.06$ ,  $df = 1$ ,  $p = 0.30$ ).

### Chlordanes

In surface water, low concentrations ( $<1$  ng/L) of  $\gamma$ -chlordane and *trans*-nonachlor were observed only at Sixty Lakes site S-545. However, in frog tissue there was strong evidence of higher levels of chlordane exposure at the Tablelands. *trans*-Nonachlor and  $\gamma$ -chlordane were found at significantly higher levels at the Tablelands (*trans*-nonachlor,  $H = 16.49$ ,  $df = 1$ ,  $p = 0.000$ ;  $\gamma$ -chlordane,  $H = 16.93$ ,  $df = 1$ ,  $p = 0.000$ ; Table 2). Furthermore, subadult frogs at the Tablelands

Table 1. Concentration of pesticides (ng/L) in surface water samples collected at the Tablelands (Sequoia National Park), and the Sixty Lake Basin (Kings Canyon National Park) California, USA, from August 18th through the 22nd, 1997

Compound	Log $K_{ow}$	Limit of detection <sup>a</sup>	Tablelands		Sixty Lakes	
			G-049	G-054	S-545	S-471
Chlorpyrifos	4.3 <sup>b</sup>	0.03	12	0.72	0.22	0.17
Diazinon	3.4 <sup>b</sup>	0.06	3.1	3.4	1.8	
$\alpha$ -Endosulfan	3.6 <sup>c</sup>	0.03	0.78	1.0	0.30	0.37
$\beta$ -Endosulfan	3.6 <sup>c</sup>	0.05	0.40	0.42	1.8	0.17
Endosulfan sulfate	3.6	0.03	2.9	2.2	0.33	0.40
$\alpha$ -Chlordane	5.2 <sup>b</sup>	0.03				
$\gamma$ -Chlordane	5.2 <sup>b</sup>	0.03			0.27	
<i>trans</i> -Nonachlor	6.1	0.04			0.31	
$\alpha$ -HCH <sup>d</sup>	3.81 <sup>c</sup>	0.03				0.29
$\gamma$ -HCH		3.8 <sup>c</sup>		0.03		
<i>o, p'</i> -DDT <sup>e</sup>		6.8 <sup>b</sup>		0.10		0.46
<i>p, p'</i> -DDT		6.9 <sup>f</sup>		0.10		
<i>p, p'</i> -DDE <sup>g</sup>		6.9 <sup>f</sup>		0.10		0.31

<sup>a</sup> Analytical detection limit was three times the standard deviation of blank levels [20].

<sup>b</sup> Selected values from Finizio et al. [41].

<sup>c</sup> Selected values from Suntio et al. [42].

<sup>d</sup> HCH = Hexachlorocyclohexane.

<sup>e</sup> DDT = Dichlorodiphenyltrichloroethane.

<sup>f</sup> de Bruijn et al. [43].

<sup>g</sup> DDE = Dichlorodiphenyldichloroethylene.

had significantly higher levels than adult frogs for both *trans*-nonachlor ( $H = 3.87$ ,  $df = 1$ ,  $p = 0.049$ ) and  $\gamma$ -chlordane ( $H = 5.44$ ,  $df = 1$ ,  $p = 0.020$ ). Sixty Lakes frogs had a similar but nonsignificant trend for subadult frogs to have higher concentrations (*trans*-nonachlor,  $H = 3.68$ ,  $df = 1$ ,  $p = 0.052$ ;  $\gamma$ -chlordane,  $H = 3.34$ ,  $df = 1$ ,  $p = 0.067$ ). The  $\alpha$ -chlordane isomer was not detected in any samples.

#### Hexachlorocyclohexanes

The  $\alpha$ -HCH was found in only one water sample from the Sixty Lakes (site S-471) and the  $\gamma$ -isomer was below quantitation limits in all water samples (Table 1). However, both isomers were detected in tissue samples from the Tablelands and Sixty Lakes (Table 2). Statistically, there was no difference in concentration of either isomer between the Tablelands and Sixty Lakes ( $\alpha$ -HCH,  $H = 1.11$ ,  $df = 1$ ,  $p = 0.30$ ;  $\gamma$ -HCH,  $H = 0.13$ ,  $df = 1$ ,  $p = 0.71$ ). Comparing the two Sixty Lakes sites,  $\alpha$ -HCH concentrations were significantly higher at site S-471 ( $H = 6.87$ ,  $df = 1$ ,  $p = 0.009$ ) and  $\alpha$ -HCH also was significantly higher at site S-471 compared with the two Tablelands sites ( $H = 7.94$ ,  $df = 1$ ,  $p = 0.005$ ).

#### DDTs

Two DDT components were observed in surface water at one Tablelands site (S-545, 0.31 ng/L for *p, p'*-DDE and 0.46 ng/L for *o, p'*-DDT); however, levels were below detection limits at the other three sites. In tissue samples, only the metabolite *p, p'*-DDE was found at quantifiable concentrations (Table 2). The DDE was the largest organochlorine component in the tissue samples for the analytes included in our study (Fig. 2). Concentrations of DDE were one to two orders of magnitude greater than the other organochlorines, DDE concentration at the Tablelands sites was significantly higher than the Sixty Lakes sites, and there was a highly significant difference between concentrations in the Tablelands and Sixty Lakes ( $H = 21.04$ ,  $df = 1$ ,  $p = 0.000$ ).

### DISCUSSION

Our results support the hypothesis that contaminants have played a significant role in the decline of *R. muscosa* in the

Tablelands of Sequoia National Park. None of the compounds that we analyzed were found at significantly greater concentration in the Sixty Lakes Basin where *R. muscosa* continued to thrive. Chlorpyrifos, diazinon,  $\alpha$ -endosulfan,  $\beta$ -endosulfan, and endosulfan sulfate were found at higher levels in surface water samples from the Tablelands. In tissue samples, *p, p'*-DDE,  $\gamma$ -chlordane, and *trans*-nonachlor were at significantly greater concentrations in the tissue of frogs.

Organophosphate insecticides undergo hydrolysis in surface waters with highly variable reaction rates dependent on pH, temperature, and other water quality parameters [25]. Residues may be entering these ponds in pulses with precipitation events, followed by slow degradation. In order to better evaluate impacts of OPs on frogs, more information is needed on the hydrolysis rates of OPs under alpine lake conditions. In particular, sampling throughout the year and measurements of pesticide concentrations in air, rain, and snow samples in the two regions would be needed to evaluate actual loads in more detail.

Diazinon was not detected in any of the frog tissue samples, and chlorpyrifos was detected only in one sample from Sixty Lakes. However, OPs are more polar than the organochlorines and their physical properties do not favor bioaccumulation. Organophosphates and carbamate insecticides are cholinesterase-inhibitors. They bind with cholinesterase, thereby disrupting neural function [14,26]. In frogs, exposure results in reduced activity, uncoordinated swimming, increased vulnerability to predators, depressed growth rates, and greater mortality [27].

Of the pesticides detected by LeNoir et al. [20] near Crescent Meadow (2,040 m elevation, 11 km southwest of the Tablelands), and at the lower elevation sites, it appears that the OPs were the most likely to be having an adverse effect on *R. muscosa*. LeNoir et al. calculated that the combined exposure to chlorpyrifos, diazinon, and malathion was approximately equal to the 96-h (4-d) median lethal concentration (LC50) for the amphipod *Gammarus fasciatus* [20]. Further implicating the organophosphates at those sites, Sparling et al. [14] found that *Hyla regilla* (Pacific tree frog) cholinesterase

Table 2. Concentration of pesticides (ng/g wet wt) in *Rana muscosa* tissue collected at the Tablelands (Sequoia National Park) and the Sixty Lakes Basin (Kings Canyon National Park) California, USA, from August 18th through the 22nd, 1997

Sample	Sample size (g)	Age class <sup>a</sup>	ng/g wet wt					
			$\alpha$ -HCH <sup>b</sup>	$\gamma$ -HCH <sup>b</sup>	$\gamma$ -Chlordane	<i>trans</i> -Nonachlor	$\alpha$ -Endosulfan	<i>p,p'</i> -DDE <sup>c</sup>
Tablelands, Sequoia National Park								
G-049-1	3.25	J	BQL <sup>d</sup>	ND <sup>e</sup>	1.5	5.5	1.4	31
G-049-2	2.08	J	BQL	ND	2.8	8.7	BQL	51
G-049-4	9.42	F	2.0	BQL	0.65	2.0	0.74	46
G-049-5	12.88	M	BQL	ND	1.00	1.5	BQL	54
G-049-6	16.31	M	0.92	BQL	BQL	0.44	BQL	17
G-049-7	14.21	M	1.0	BQL	0.52	1.9	0.67	47
G-049-8	12.83	M	0.43	ND	0.35	1.2	BQL	37
G-049-9	14.68	M	4.9	0.70	0.77	2.5	1.2	100
G-049-10	12.00	M	2.7	BQL	0.38	0.95	0.55	47
G-049-11	11.61	M	1.8	ND	0.42	1.2	0.39	40
G-049-12	14.41	F	1.2	BQL	1.1	2.5	0.49	56
G-049-13	11.19	M	2.5	0.55	1.2	2.5	0.52	61
G-049-14	12.44	M	3.0	0.51	1.0	1.6	0.51	71
G-049-15	15.92	M	2.4	0.30	0.80	1.8	0.50	57
G-049-16	10.17	M	1.5	BQL	BQL	1.1	0.49	58
G-049-17	3.10	J	ND	ND	2.5	7.5	BQL	30
G-049-18	2.55	J	ND	ND	BQL	8.1	BQL	24
G-054-1	12.22	F	1.2	ND	BQL	1.4	BQL	35
G-054-2	8.34	M	0.67	ND	BQL	0.88	BQL	36
G-013-1	2.48	J	ND	ND	ND	ND	ND	13
Sample size			20	20	20	20	20	20
Median			1.16	0.07	0.71	1.72	0.50	46.72
Mean			1.41	103.1	0.84	2.66	0.56	45.69
Standard deviation			1.24	221.0	0.75	2.60	0.36	19.92
Sixty Lakes Basin, Kings Canyon National Park								
S-545-1	16.5	F	0.36	BQL	BQL	BQL	BQL	6.3
S-545-2	15.95	F	0.97	BQL	BQL	0.48	0.42	14
S-545-3	14.97	M	1.1	BQL	BQL	0.49	0.40	20
S-545-4	13.44	M	1.5	BQL	BQL	0.71	BQL	25
S-545-5	13.37	M	1.1	BQL	BQL	0.40	BQL	23
S-545-6	4.15	J	ND	ND	ND	ND	ND	3.4
S-545-7	2.71	J	ND	ND	ND	1.7	ND	8.7
S-545-8	3.56	J	0.43	ND	ND	BQL	BQL	6.1
S-545-9	16.80	M	1.1	BQL	BQL	0.59	BQL	26
S-545-10	12.40	M	1.7	BQL	BQL	0.40	0.37	22
S-471-1	2.58	J	BQL	ND	BQL	BQL	BQL	4.9
S-471-2	2.87	J	BQL	BQL	BQL	2.0	BQL	7.9
S-471-3	17.63	F	11	0.71	BQL	0.78	0.61	24
S-471-4	15.95	F	8.4	1.1	BQL	ND	0.39	18
S-471-5	13.98	F	1.1	ND	BQL	ND	0.48	24
S-471-6	9.57	M	6.9	ND	ND	ND	BQL	18
S-471-7	8.37	M	11	1.4	ND	ND	0.69	27
S-471-8	9.59	M	12	1.1	ND	ND	0.47	17
S-471-9	10.11	M	6.5	ND	ND	ND	BQL	21
S-471-10	8.78	M	12	1.2	BQL	1.3	BQL	21
Sample size			20	20	20	20	20	20
Median			1.12	0.15	0.14	0.46	0.41	19.34
Mean			3.95	0.37	0.17	0.53	0.45	16.96
Standard deviation			4.56	0.48	0.24	0.59	0.24	7.88
<i>p</i> -Value <sup>f</sup>			0.29	0.71	0.000	0.000	0.31	0.000

<sup>a</sup> Frogs were classified as: J = Juvenile, M = Male, or F = Female.

<sup>b</sup> HCH = Hexachlorocyclohexane.

<sup>c</sup> DDE = Dichlorodiphenyldichloroethylene.

<sup>d</sup> BQL indicates the chemical was identified in the sample with ion ratios in the proper proportions, but signal levels were below the quantification limit (BQL).

<sup>e</sup> Chemical was not detected.

<sup>f</sup> Kruskal-Wallis *p*-value using Chi-squared approximation.

levels were depressed in samples collected at Crescent Meadow, as would be expected after exposure to organophosphate pesticides. Sparling et al. [14] also analyzed *H. regilla* tissues for the organophosphate insecticides chlorpyrifos and diazinon, and found average concentrations of 7 and 2 ng/g wet wt of tissue, respectively.

Whether organophosphates have played a role in the loss of *R. muscosa* from the Tablelands is unclear. The concentrations we found were nearly 20 times less than at the sites of Sparling et al. [14], but the work of Relyea and Mills [28] shows that our understanding of longer-term exposures and the additive role of predators (and possibly other stressors) is

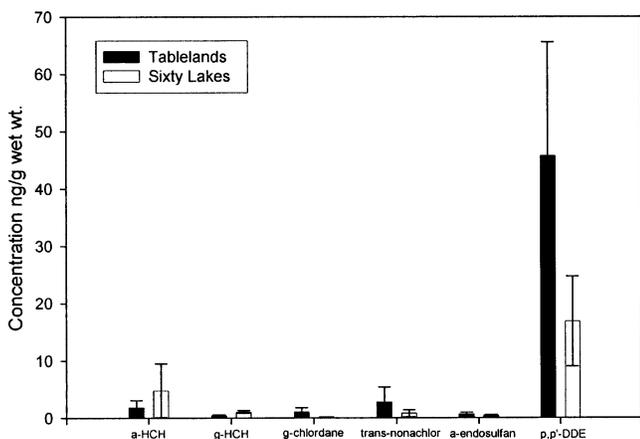


Fig. 2. Mean concentration of pesticides in *Rana muscosa* tissue at the Sixty Lakes Basin, Kings Canyon National Park, and the Tablelands, Sequoia National Parks, California, USA. Results are based on  $n = 20$  for each bar; see Table 2 for  $p$ -values. Error bars represent standard deviation (SD), DDE = dichlorodiphenyldichloroethylene; HCH = hexachlorocyclohexane.

largely unknown. At this time, we cannot state whether organophosphates are impacting *R. muscosa*. Long-term exposure experiments and additional field data are needed. Also, because the use of pesticides is not constant throughout the year, it is highly unlikely that the concentrations we measured represent peak values that these frogs are exposed to each year.

Concentrations of endosulfan sulfate in water and  $\alpha$ -endosulfan in frog tissue were greater in the Tablelands compared with Sixty Lakes. Toxicity levels for endosulfan have been reported for *Rana tigrina* (Indian bullfrog) tadpoles (1.8  $\mu\text{g/L} = 96$  h LC50; [29]), *Rana clamitans* (green frog) tadpoles (15  $\mu\text{g/L} = 13$ -d LC50; [30]), and *Rana sylvatica* (wood frog) tadpoles (32  $\mu\text{g/L} = 96$ -h exposure induced paralysis; [31]).

The Canadian Water Quality guidelines [32] set a limit of 0.02  $\mu\text{g/L}$  as a "guideline for the protection of freshwater aquatic life." The concentrations of endosulfan at our sites were an order of magnitude below the Canadian guidelines, but the work of Relyea and Mills [28] suggests that long-term exposure and/or the presence of natural predators have the potential of greatly reducing the concentration that can have lethal effects. Long-term and sublethal effects have not been studied.

Low concentrations of  $\gamma$ -chlordane and *trans*-nonachlor in water were observed only at Sixty Lakes. However, in frog tissue *trans*-nonachlor and  $\gamma$ -chlordane were found at significantly greater concentration in the Tablelands. The absence of the  $\alpha$ -chlordane isomer may be because it makes up a smaller fraction of the technical chlordane mixture than  $\gamma$ -chlordane or *trans*-nonachlor [33]. A comparison of *trans*-nonachlor concentrations revealed that levels at the Tablelands were significantly higher than those at Sixty Lakes. Further examination of *trans*-nonachlor concentrations in individual frogs at the Tablelands showed that subadult frogs had significantly higher levels when compared with adult frogs. A similar, but non-significant, trend also was seen for  $\gamma$ -chlordane. This suggests that exposure occurs in the early life stages followed by dilution as frogs grow. This is an area where additional research is needed to assess exposure pathways.

Hexachlorocyclohexanes were nearly absent from our water samples, but both isomers were detected in tissue samples from the Tablelands and the Sixty Lakes. Though there was no sig-

nificant difference between the two areas, the concentration was greater at Sixty Lakes. Frog tissue from Sixty Lakes site S-471 had significantly higher concentrations of  $\alpha$ -HCH than the other Sixty Lakes site, as well as significantly higher concentrations compared with the two Tablelands sites. This indicates that  $\alpha$ -HCH was not the cause of the frog decline at the Tablelands. In a review of organochlorine contaminant toxicity to amphibians, Sparling [34] reported that in toxicity studies with Fowler's toad larvae (*Bufo fowleri*) and one-week-old chorus frogs (*Pseudacris triseriata*), lindane ( $\gamma$ -HCH) was less toxic than toxaphene, heptachlor, dieldrin, aldrin, or DDT.

Walker et al. [35] reviewed the factors influencing the distribution of lindane and other hexachlorocyclohexanes and noted that HCH isomers have a relatively high vapor pressure (volatility) and a relatively high solubility in water compared to other organochlorine pesticides. Hexachlorocyclohexane residues, therefore, tend to volatilize (evaporate) into the atmosphere, dissolve into precipitation, and end up in surface waters, especially in high latitudes where it is cold. As a result, HCH has become the most abundant organochlorine insecticide in the Arctic, an area where it has never been used. Blais et al. [36] suggested that HCH and other semi-volatile organochlorine compounds also should accumulate preferentially at high altitudes, because of the cold climates there. In the Canadian Rockies, the HCH increased nearly 100-fold in samples from elevations of 770 to 3,100 m, with the sharpest increases coming at elevations over 2,000 m. The increases were due to both increased amount of snow at the higher elevations and a higher concentration of HCH in that snow.

Two DDT components (*p,p'*-DDE and *o,p'*-DDT) were observed in surface water at one Tablelands site and *p,p'*-DDE was found at significantly greater concentrations in Tablelands frog tissue. The absence of the parent DDT congeners suggests that *R. muscosa* metabolizes the parent material to DDE form, or that the contaminant signal has been weathered in the environment such that the majority of the parent has been converted to DDE.

The DDE levels that we found compare to average levels of 9 ng/g for *H. regilla* tadpoles from Sycamore Creek (610 m elevation, 22 km southwest of the Tablelands) and 258 ng/g from an agricultural area at Davis, California on the floor of the Central Valley [23]. Currently there are no data available of the toxicity of DDE to *R. muscosa*. However, our results suggest that DDE is persistent in *R. muscosa* and that DDE exposures have been greater at the Tablelands.

Our understanding of the impact of pesticides on amphibians is rudimentary at best. Most toxicological tests do not include native amphibians, and most tests are run for only a short period of time (e.g., 1–4 d) [37–39]. Only recently have more realistic exposure regimes been utilized with native species of amphibians. Relyea and Mills [28] found that gray tree frog (*Hyla versicolor*) tadpoles exposed to <3% of published LC50 levels of the carbamate pesticide carbaryl for that species, suffered up to 97% mortality within one week. (An LC50 is the concentration that will kill 50% of the test animals as a single exposure, typically 1 or 4 h.) This effect increased two- to four-fold when tadpoles were exposed to cues from caged predators (spotted salamander larvae, *Ambystoma maculatum*) that naturally occur within the range of *H. versicolor*.

The effect of pesticides appears to be more pronounced with longer exposures, and the effect on *R. muscosa* is likely to be much greater than with other frogs. *Rana muscosa* tadpoles typically take three years to metamorphose. This exposes

them to pesticides in the water for a much longer period of time than other anurans. Also, adult *R. muscosa* are closely associated with water throughout their lives. Unlike many anurans that move away from breeding sites during much of the year, adult *R. muscosa* remain in the immediate vicinity of ponds and lakes all year. This would increase their exposure to water-borne contaminants and increase the effects of potentially lethal compounds.

### CONCLUSION

Our results show that organochlorines residues, especially DDE, are accumulated in *R. muscosa* tissues. Organochlorines residues probably enter these lakes via precipitation or through dry deposition. The differences in amphibian tissue concentrations of HCH isomers from nearby ponds suggest that small differences in lake characteristics and positioning in areas that receive prevailing winds from agricultural areas are critical factors in exposure to aquatic wildlife. Organophosphate insecticides were observed in surface waters at higher concentration at the Tablelands than at Sixty Lakes, indicating atmospheric inputs from up-wind agricultural areas, but they were not observed in frog tissues. The potential toxicity of the OP insecticides to native amphibians requires additional study under controlled laboratory conditions to determine the toxicological significance of pesticides. Based on traditional toxicological studies, it would seem unlikely that the extirpation of *R. muscosa* was caused by pesticides, but there are several lines of evidence that suggest that conclusion is wrong. First, most of the pesticides included in this study were found at higher levels in the Tablelands, where *R. muscosa* was extirpated in the early 1980s, and where nearly all frogs involved in an experimental reintroduction (1994–95) died out by 1997. Second, Relyea and Mills [28] demonstrated that levels of carbaryl once thought to be nontoxic actually caused high levels of mortality when exposure times were increased to 7 d.

The unusual life history characteristics of *R. muscosa* (e.g., tadpoles that take two to four summers to metamorphose, and adults that spend nearly all their time in the water) make them especially susceptible to pesticide poisoning. The unusually long tadpole period extends the time they are exposed to water-borne contaminants, and by being in the water two or more entire years, they are exposed to the entire range of aerial deposition caused by seasonal usage of pesticides. It becomes apparent that even low levels of contaminants, if toxicologically significant, play a significant role in the widespread amphibian declines that are being observed throughout the Sierra Nevada. Sublethal effects are difficult to detect, yet they may be the most likely to occur [40].

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