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Comparative toxicity of chlorpyrifos, diazinon, malathion and their oxon derivatives to larval *Rana boylii*

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Laboratory tests on the toxicity of OP insecticides and their oxons suggest that they may be acutely lethal to amphibians at ecologically relevant concentrations.

Abstract

Organophosphorus pesticides (OPs) are ubiquitous in the environment and are highly toxic to amphibians. They deactivate cholinesterase, resulting in neurological dysfunction. Most chemicals in this group require oxidative desulfuration to achieve their greatest cholinesterase-inhibiting potencies. Oxon derivatives are formed within liver cells but also by bacterial decay of parental pesticides. This study examines the toxicity of chlorpyrifos, malathion and diazinon and their oxons on the foothill yellow-legged frog (*Rana boylii*). *R. boylii* is exposed to agricultural pesticides in the California Central Valley. Median lethal concentrations of the parental forms during a 96 h exposure were 3.00 mg/L (24 h) for chlorpyrifos, 2.14 mg/L for malathion and 7.49 mg/L for diazinon. Corresponding oxons were 10 to 100 times more toxic than their parental forms. We conclude that environmental concentrations of these pesticides can be harmful to *R. boylii* populations.

Keywords: Pesticides; Acute toxicity; Amphibians; Cholinesterase; California

1. Introduction

Organophosphorus (OPs) pesticides have long been of serious environmental concern. They form the largest group of chemicals used in the control of pests including invertebrates, vertebrates and, to a lesser extent, plants. There are some 200 OP pesticides available in this class that have been formulated into literally thousands of different products (Hill, 2003). These products are used in agriculture, forests, gardens, home and industrial sites, urban and rural areas. As one example, over 3.0 million kg of active ingredient OPs were used in California during 2004, the most recent reporting year (California Department of Pest Regulation, 2006). It is estimated that this accounts for about 25% of OP use nationwide (Kegley et al., 2000: http://www.panna.org/resources/documents/hooked.pdf). Organophosphorus pesticides function to inhibit cholinesterase. They bind with acetylcholinesterase, an enzyme that breaks down the neurotransmitter acetylcholine so that subsequent impulses can be transmitted across the synapse. Therefore, inhibiting acetylcholinesterase results in repeated, uncontrolled firing of neurons leading to death usually by asphyxiation as respiratory control is lost.

A few OPs such as acephate or monocrotophos are direct inhibitors of acetylcholinesterase. That is, they attach directly to the molecule without any transformation. Most OPs, however, must undergo oxidative desulfuration to achieve maximum anticholinesterase potency. Oxidative desulfuration is mediated by mixed-function oxidases (MFO) residing in the liver and results in either an oxon or a sulfon degradate, depending on the active moiety of the molecule (Tahara et al.,

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2005). These MFOs are also involved with metabolizing cellular wastes but, in the case of OPs, increase the toxicity of the pesticide involved. In addition to being metabolized internally, bacteria and other factors can convert pesticides into sulfons or oxons (Hill, 2003), thereby making them available in the environment (Domagalski, 1996; Whitehead et al., 2005; Schomburg et al., 1991).

The Central Valley of California is an important agricultural area producing a variety of crops. This intensive agriculture requires frequent and abundant application of pesticides. These pesticides have been implicated in the declines of several species of amphibians in the valley (Fisher and Shaffer, 1995; Davidson et al., 2001) and in downwind montane areas (Sparling et al., 2001; Fellers et al., 2003; Davidson, 2004). One of these species is the foothill yellow-legged frog (*Rana boylii*) which inhabits the transition zone between the Valley and the Sierra Nevada Mountains. The most commonly used OPs in the Central Valley include chlorpyrifos, malathion and diazinon (California Department of Pest Regulation, 2006) which are found in snow, air and surface waters (Schomburg et al., 1991; McConnell et al., 1998; LeNoir et al., 1999). However, while pesticide concentrations in the montane regions generally occur in the ng/L range, those in the Central Valley are in the μg/L or mg/L levels (Gruber and Munn, 1998; Brady et al., 2006). Other studies have examined the toxicity of chlorpyrifos (Richards and Kendall, 2002), malathion (Fordham et al., 2001) and diazinon (Harris et al., 1998) on amphibians but we have not found any studies that have compared the toxicity of the parent and oxon forms of these pesticides on amphibians. Because both parent and oxon derivatives can be found in the environment, it is important to evaluate their toxicity to declining amphibian species.

The purpose of this paper is to: (1) determine the acute (96 h or less) toxicity of chlorpyrifos, malathion and diazinon and their oxon derivatives on larval *R. boylii*; (2) determine the dose–response relationships between total cholinesterase depression and each of these chemicals; and (3) examine the risk of acute toxicity in *R. boylii* by comparing data on environmental concentrations of these pesticides to toxic concentrations determined in this study.

2. Materials and methods

Rana boylii eggs from three masses were collected 11 May 2005 from a Coast Range stream (39°25.71′ N, 122°58.60′ W) 70 km inland from Fort Bragg, California. Eggs were collected from this site since it is upwind of agricultural activities in the Central Valley, and the stream is not near any agricultural or other activities along the coast where pesticides are used in significant quantities. Eggs were stored in heavy plastic bags, packed in an ice chest with wet ice, shipped the same day, and arrived at the Illinois laboratory on 12 May 2005. After hatching, larvae were raised in medium soft reconstituted water (American Society for Testing and Methods (ASTM), 1989) in 78 L aquaria for several weeks prior to experimentation. During this phase water was filtered continuously through glass wool and activated charcoal and supplemented as levels dropped. Larvae were fed boiled, organic romaine lettuce and high-protein fish flakes ad libitum prior to the experiment.

Chlorpyrifos, diazinon, malathion and their respective oxon derivatives were reagent grade (99% pure) purchased from Acros Organics® (Morris Plains, NJ). They were dissolved in acetone to form stock solutions used for dosing. Initial concentrations of each pesticide (Table 1) were determined through range finding with a few tadpoles at various concentrations until mortality was achieved. Doses were made by placing the amount of stock solution necessary to reach the desired final concentration in 7 L of water into amber vials and bringing doses up to 2 ml with additional acetone. Controls consisted of untreated tanks and acetone controls to which 2 ml of acetone were added. Aquaria (8 L) filled with 7 L of reconstituted water were randomly assigned to treatments and the appropriate amount of pesticide solution was electronically pipetted to each. All resulting concentrations were nominal. Immediately after treating the aquaria we added 9 same-aged *R. boylii* tadpoles ranging in development from Gosner 32 to Gosner 44 (Gosner, 1960). A mixture of stages tends to reflect what occurs in the wild due to differential rates of development observed in ponds. After the first 24 h of exposure tadpoles were fed a small amount of organic romaine lettuce obtained from a local grocery store to reduce stress. Tadpoles were checked two or three times per day for death or morbidity. Death was determined by gentle prodding and by faded coloration. All dead tadpoles were removed and frozen in liquid nitrogen. If results from the first effort were ambiguous (i.e. lacking a clear dose–response relationship) additional treatment concentrations were added until the supply of tadpoles was exhausted. At the end of 96 h all survivors were euthanized via flash freezing in liquid nitrogen and maintained at −80 °C until analyzed for cholinesterase.

Total cholinesterase activity was determined via the colorimetric method of Ellmann et al. (1961) modified for a multi-well plate reader. Prior to analysis, gut coils were dissected from tadpoles and each individual was staged for development. Based on a previous study (Sparling et al., 2001) and on unpublished data, we knew that total cholinesterase activity increases with developmental stage of tadpoles. To adjust for this, cholinesterase data were normalized to that of metamorphs (Gosner stage 46). Cholinesterase values were multiplied by 2.4, 1.9 and 1.6 for tadpoles falling into stages 32–36, 37–39, and 40–45, respectively. Statistical analyses were conducted with

Table 1
Percent of tadpoles dead by chemical, concentration and time

Chemical	*N*	Concentration (mg/L)	Time (h)			
			24	48	72	96
Chlorpyrifos	7	0.5	0	0	0	0
	9	1.19	22	100	100	100
	9	1.98	67	100	100	100
	9	3.3	44	100	100	100
	9	5.5	55	100	100	100
Chlorpyrifos oxon	9	0.005	100	100	100	100
	9	0.01	0	89	100	100
	9	0.04	89	100	100	100
Diazinon	9	1.25	0	0	0	0
	9	2.5	0	0	0	0
	9	5	0	0	0	0
	7	10	89	100	100	100
Diazinon oxon	9	0.0125	0	0	0	0
	9	0.025	0	0	11	22
	9	0.05	0	0	0	0
	9	0.10	0	11	11	11
	7	0.50	0	0	0	0
	7	1.0	0	0	0	75
	13	10	100	100	100	100
	5	20	100	100	100	100
Malathion	9	1.25	11	22	22	44
	9	2.5	0	33	55	55
	9	5	0	33	44	44
	9	10	0	100	100	100
Malathion oxon	9	0.0037	0	0	0	0
	9	0.0075	0	0	0	22
	9	0.015	0	0	33	33
	9	0.03	0	11	33	55

SAS (Cary, NC). Probit analysis using the normal cumulative distribution (default) option was used on mortality data. Analyses of variance (ANOVA) were used to determine which treatments significantly depressed cholinesterase activity compared to controls and analyses of covariance (ANCOVA) using pesticide concentration as the covariate were used to evaluate the relative effectiveness of different pesticides on cholinesterase depression. Mortality rates and cholinesterase activity met the assumption of parametric statistics and did not need to be transformed.

3. Results

Mortality varied via chemical, time, and dose (Table 1). For chlorpyrifos all tadpoles subjected to 0.5 mg/L survived while those at all other concentrations died by 48 h of exposure. Thus probit analysis was conducted on conditions at 24 h of exposure. The median lethal concentration (LC_{50}) was 3.005 mg/L (Table 2). All of the test animals except one exposed to chloroxon died within the first 24 h regardless of exposure concentration and an LC_{50} could not be determined. No mortality with diazinon was observed until the test concentration reached 10 mg/L, and the resulting LC_{50} was 7.488 mg/L but confidence limits could not be calculated. Lethal concentrations of diazoxon were difficult to discern at first but, with added treatments, we were able to calculate an LC_{50} of 0.760 mg/L, indicating a toxicity approximately 10 times greater than the parent form. The 96 h LC_{50} for malathion was 2.137 mg/L but, because the proportion of individuals dying in lower concentrations was relatively constant, no confidence intervals could be calculated. Maloxon, with an LC_{50} of 0.023 mg/L, proved to be approximately 100 times more toxic than its parent form.

Each pesticide significantly depressed normalized cholinesterase activity compared to controls. For malathion, maloxon, chlorpyrifos and chloroxon ANOVAs resulted in p values of <0.006 and controls had higher activities than all other treatments. For diazinon, normalized cholinesterase values differed between controls and those exposed to 5 mg/L or higher ($p = 0.0125$). Controls had higher values than all other treatments except 0.025 mg/L ($p < 0.0001$).

Regressions of normalized cholinesterase activity on exposure concentrations were significant for each pesticide. (Table 3). The concentration-response relationships were significantly different among pesticides as determined by ANCOVAs. When all pesticides were included, there was a significant chemical ($p = 0.006$), concentration ($p < 0.0001$) and chemical by concentration interaction ($p < 0.0001$). Oxon forms showed steeper declines by concentration than their respective parental forms. When oxons were compared (Fig. 1a), maloxon and chloroxon had steeper downward slopes than diazoxon ($p < 0.008$). For parent compounds (Fig. 1b) chlorpyrifos decreased cholinesterase activity more rapidly than did malathion ($p = 0.0201$).

4. Discussion

Our data on parent compound lethality are comparable to the few previously published studies that exist. As reviewed by Cowman and Mazanti (2000), reported 96 h LC_{50}s for chlorpyrifos ranged from 1 μg/L in *Bufo americanus* to 3 mg/L in *Rana pipiens*. For malathion substantial mortality (no LC_{50} reported) was observed in *Bufo arenarum* at 47.3 mg/L after 5 days. Relyea (2004) found that some mortality occurred among four species of amphibians exposed to 2 mg/L diazinon but that LC_{50}s for all species exceeded this concentration. Our sequence of toxicity: chlorpyrifos > malathion > diazinon is consistent with studies on fish. In mature trout the 96 h LC_{50}s for the three pesticides are 9 μg/L, 0.1–0.3 mg/L, and 2.6–3.2 mg/L, respectively. For fathead minnows (*Pimephales notatus*) the respective LC_{50}s are 0.331 mg/L, 8.6 mg/L and 15 mg/L (Ecotoxnet. http://extoxnet.orst.edu/ghindex.html).

In this study the oxon derivatives of chlorpyrifos, malathion and diazinon were significantly more toxic than their respective parental forms. Chloroxon killed all of *R. boylii* tadpoles and was at least 100 times more than the lowest concentration of chlorpyrifos which resulted in no mortality. Maloxon was nearly 100 times more toxic than malathion and diazoxon was approximately 10 times more toxic than its parent. This is consistent with other studies that have compared parent and oxon forms. Tsuda et al. (1997) found that the 48 h LC_{50} for diazinon in the killifish (*Oryzias latipes*) was 4.4 mg/L whereas it was 0.22 mg/L for the oxon form. They also found that the LC_{50} was 1.8 mg/L for malathion and 0.28 mg/L for maloxon.

These OPs exert their toxicity through inhibition of cholinesterase. While there may be ancillary effects of pesticides (Schuh et al., 2002; Sparling, 2003), cholinesterase depression remains the primary mechanism. The rate of inhibition relative to exposure concentration is due to the rate of assimilation of the pesticide, rate of conversion to the oxon form, affinity of the OP molecule for cholinesterase and rate of cholinesterase regeneration. It is not unusual for pesticides to vary in their potency to reduce cholinesterase within a given species (Richardson et al., 2001; Kousba et al., 2004). Since the rate of cholinesterase depression was much more rapid for the oxon derivatives than their parental counterparts, conversion to oxon forms was an important factor in the relative toxicity

Table 2
Results of probit analysis-generated concentration-response curves on mortality in *Rana boylii*

Chemical	Period (h)	Intercept	Slope	p of slope	LC_{50} (mg/L)	95% CI (mg/L)
Chlorpyrifos	24	−0.5882	17.018	0.0339	3.005	0.993–157
Diazinon	96	−25.269	3.374	NS	7.488	NA
Diazinon oxon	96	0.1368	14.077	0.0001	0.760	0.336–3.212
Malathion	96	−0.494	31.477	NS	2.137	NA
Malathion oxon	96	3.452	133.659	0.011	0.023	0.014–0.180

CI, confidence interval; NS, not significant; NA, not available.

Table 3
Regression results of normalized cholinesterase activity against exposure concentrations for six pesticides[a]

Pesticide	N	Intercept	p value	Slope	p value	R^2
Chlorpyrifos	46	0.8499	<0.0001	−0.0330	0.0061	0.1383
Chloroxon	9	1.2525	<0.0001	−26.8088	0.0140	0.2547
Diazinon	20	1.2169	<0.0001	−0.0796	0.0094	0.1729
Diazoxon	45	0.8504	<0.0001	−0.0511	0.0113	0.0908
Malathion	28	1.0534	<0.0001	−0.1028	0.0015	0.2244
Maloxon	27	1.0193	<0.0001	−24.5409	0.0080	0.1557

[a] Regressions take the form: ChE = intercept + slope · Concentration, where ChE is the stage-normalized cholinesterase value measured in umol/min/g of tissue and Concentration is mg/L of pesticide.

of these pesticides. It appears that chlorpyrifos which had the highest toxicity of all the parental compounds must be rapidly converted by *R. boylii* and have a high affinity for the cholinesterase molecule. Because maloxon and chloroxon had similar rates of mortality and cholinesterase depression but the parental forms of these oxons differed significantly, the rate of conversion for malathion was probably slower than that for chlorpyrifos. Diazinon appeared to be converted most slowly and the significant difference between diazoxon and the other oxons suggest that diazoxon may have a lower affinity for cholinesterase. Together these factors led to lower toxicity for diazinon compared to the other pesticides and made it more difficult to define its median lethal concentration.

In the Central Valley of California diazinon is used extensively used as a dormant spray in orchards. Peak diazinon concentrations in run-off from orchard fields have been reported between 0.1 and 1 mg/L (Brady et al., 2006), sufficiently high to be potentially toxic. Similarly, diazinon and diazoxon were found in storm water run-off within the Sacramento River Basin (Domagalski, 1996). We could find no references to run-off concentrations of chlorpyrifos or malathion in the Central Valley but it is not unreasonable to consider run-off directly from agricultural fields as having some toxicity. Amphibians inhabiting ponds in the Central Valley of California could be simultaneously exposed to two or all three of these pesticides and their oxons. Whereas chlorpyrifos and malathion are typically applied to actively growing crops and diazinon is extensively used on dormant vegetation, all three may be applied in close proximity to each other both in time and space. The potential for interactive effects of these chemicals needs to be explored. The environmental concentrations of oxon derivatives are often lower than their parental forms.

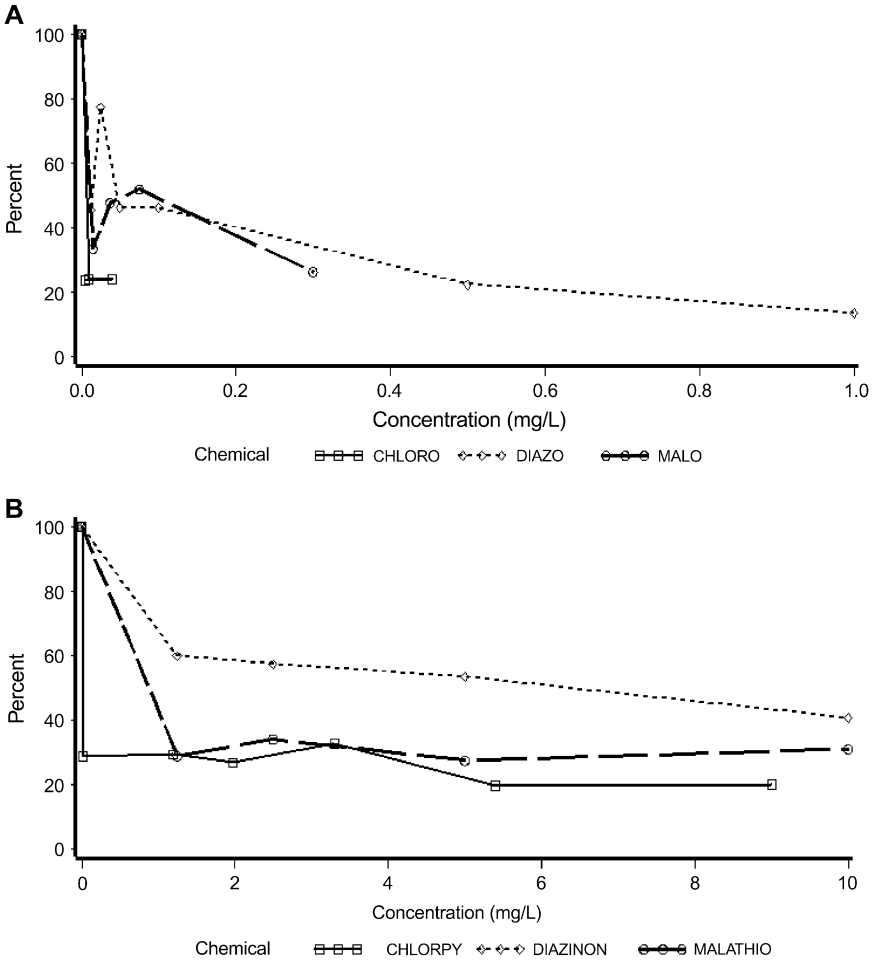

Fig. 1. Percent of control cholinesterase activity as functions of insecticide concentrations. (A) Oxon derivatives of chlorpyrifos, diazinon and malathion. (B) Parental forms of the each pesticide.

Under some conditions, however, they may equal to or even exceed parental forms (Schomburg et al., 1991). The dynamics of pesticide application and conversion in the Central Valley of California and environs suggest that agricultural run-off may indeed be toxic to amphibians.

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