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Toxicity of Organophosphorus Pesticides to *Dugesia dorotocephala*

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In addition to their use as a sentinel species (NRC 1991), planaria have been proposed as a biological control agent in integrated mosquito eradication operations. Both uses of planaria require data on the concentrations of organophosphorus pesticides (OPs) which produce toxicity. However, the susceptibility of planaria to organophosphorus pesticides is not well known. Levy et al. (1978) found that *Dugesia dorotocephala* tolerated the "relatively high" concentrations of organophosphorus pesticides (0.001 mg/L temephos; 0.004 mg/L fenthion, chlorpyrifos; 0.1 mg/L malathion) used in mosquito control programs better than did most other aquatic invertebrates, especially insect larvae. Our study determined the concentrations of OP's which produced mortality, behavioral toxicity, and developmental abnormalities during head regeneration in *D. dorotocephala*. It also examined the correspondence between the various bioassay endpoints.

MATERIALS AND METHODS

Exposure was accomplished using a shell-coating procedure that worked well with polychlorinated biphenyls (Schaeffer et al. 1991) and other water insoluble compounds. The standard test medium (STM) was 7.5 g $CaCl_2$, 3 g $MgSO_4$, 1.5 g $NaHCO_3$, 0.27 g KCl/25 L distilled water. The positive control was *p*-nitrophenol (Yoshioka et al. 1986) in STM (Table 1). Aliquots of technical grade pesticides (gift from Dr. Robert Metcalf, University of Illinois or purchased from Ultra Scientific, Kingstown, RI; see Table 1) were dissolved in acetone and pipeted into 400 ml beakers and the acetone was evaporated under an air stream while rotating the beaker to coat the bottom and bottom 2 cm of the walls. The amounts of pesticide used were below the solublity, and are given as the concentrations (mg/L) expected assuming complete solubility in the medium.

In the first experiment, groups of 10 asexual *D. dorotocephala* weighing 20-25 mg/animal (Carolina Biological Supply, Gladstone, Oregon) were exposed to pesticide dissolved in 100 ml of STM. "Intact" animals and "bodies" of animals decapitated behind the auricles were used in this experiment. These animals are referred to as the light-colored ("light") race. In the second experiment, animals from a dark-colored ("dark") race (Connecticut Valley Biological Supply, Southampton, MA) were similarly exposed. This experiment used "intact" animals, "bodies" from animals decapitated behind the auricles, and "head" and "tail" pieces from animals sectioned anterior to the pharynx. In both experiments, the animals were maintained at 19 °C and fed beef liver twice weekly for several weeks prior to their use. They were not fed at least 48 h before use. Most exposures were carried out in at least 2 independent trials at both 19 °C and 27 °C for 7 d. Mortality was recorded

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daily. Dead animals were removed and survivors were placed into clean beakers at the same concentration. The LC_{50} at 7 d was estimated using the trimmed logit method (Sananthanan et al. 1987).

Four classes of behavioral responses (locomotive, morphological, neurological, morbidity) were assessed in intact animals at 1-5, 10-30 and 40-60 min (Grebe and Schaeffer 1991).

Head regeneration was scored using a scheme based on Henderson and Eakin (1959) under a dissecting microscope at 16X between days 3-7 following decapitation behind the auricles. The abnormalities scored were death and head abnormalities as defined by Child (1911):

Normal: Head triangular with two symmetrically placed eyes and auricles at lateral margins.
Teratophthalmic: Head normal in shape and auricles in normal position but with eyes showing all degrees of approximation to the median plane from two distinct eyes with pigment connected to complete cyclopia.
Teratomorphic: Head more or less rounded in outline with single, or apparently single, median eye; auricles more or less anterior and showing all degrees of approximation to the median plane to a single median auricle.
Anophthalmic: Head rudimentary without eyes and with or without a single median auricle.
Acephalic: Head completely absent.

The frequency of the various forms that reconstitute from lots consisting of pieces of a certain length and from a certain body-level of animals of the same length is termed the "head frequency." If a certain lot of pieces gives a preponderance of normal and near-normal animals, the lot has a high head frequency. If a lot gives a majority of headless or strongly inhibited head-forms, it has a low head frequency. Child (1911) designated each type of head by an arbitrary number representing the degree of inhibition, and summed these as the "head frequency index" (HFI):

HFI = (100 #Normal + 80 #Teratophthalmic + 60 #Teratomorphic + 40 #Anophthalmic + 20 #Acephalic)/ #Animals

We define an analogous tail frequency index (TFI) using: normal tail = 100, abnormal tail (including deformed or stunted) growth = 80, blastema formed but not developed = 60, no blastema = 40, bipolar head = 20 and death = 0.

Representative animals were relaxed, fixed, embedded and sectioned for microscopic examination. Examples of gross lesions and abnormal outgrowths in regenerating survivors were preserved by embedding the entire animal in plastic.

RESULTS AND DISCUSSION

The lethalities (LC_{50}) for intact animals are given in Table 1. The LC_{50} for *p*-nitrophenol was 10.2 mg/L for *D. dorotocephala*; Yoshioka et al. (1986) reported 20 mg/L for *Dugesia japonica*. Table 1 shows that the LC_{50} concentrations of OPs decreased by about 50% in the "light" strain, and 10-50% in the "dark" strain as the temperature was increased from 19 °C to 27 °C. The lethality of OPs to both planaria strains tended to be "all-or-none" within a narrow concentration range; i.e., there was an exposure threshold. For example, all "dark" animals survived ≤ 2.8 mg/L of methyl parathion at 19 °C, whereas all died at > 2.8 mg/L. Furthermore, the three OPs caused sudden deaths of most animals by days 5-7 at both temperatures without any significant signs on preceding days.

Table 1. Amount of compound tested and 7-d LC_{50}.

Chemical	Exposure (mg/L) 19°C	Exposure (mg/L) 27°C	LC_{50} (mg/L) 19°C	LC_{50} (mg/L) 27°C
"Light" Strain				
Chlorpyrifos	0.25 - 6	0.5 - 4.5	4.3	2.0*
Malathion	5 - 19	2 - 12	13.1	6.9*
Methyl parathion	2 - 6	1 - 2.5	4.2	2.0*
p-Nitrophenol	4 - 22	2 - 9	10.2	>9
"Dark" Strain				
Chlorpyrifos	0.2 - 3	0.2 - 2.8	2.5	2.2*
Malathion	5 - 15	1 - 8	8.6	6.0*
Methyl parathion	2 - 3.5	1 - 2	2.9	1.3*
p-Nitrophenol	8 - 21	8 - 22	16.9	>22.0*

*LC_{50}s at 19°C and 27°C differ significantly at $P < 0.05$.

Chronic toxicity to intact animals differed for each OP, as represented by the results for the "light" strain. Chlorpyrifos ($\geq$ 0.5 mg/L) was the only pesticide which produced depigmentation of the head of intact animals during the 7 d of exposure. Malathion, after the first day, produced invagination of the neck. This was followed by head loss and then either by formation of abnormal tissue at the wound surface or by death. Methyl parathion (5 mg/L), after 24 h exposure, produced a characteristic depigmented line in $\geq$60% of the animals. This line started at the eyes and progressed posteriorly past the auricles. The number of animals increased with time and steeply with concentration: at 4.5 and 3.5 mg/L, 2/10 and 1/10, respectively.

p-Nitrophenol (5 mg/L) produced head swelling and ulceration by day 11 in intact animals. Histopathology showed the ulceration was epidermal, and that it exposed the underlying gastroderm and stroma. There was no proliferation of any single cell population associated with the lesion. Swelling of the region was due to increased water in the tissue and disorganization and dissociation of the cells of the GI tract. The GI tract occasionally sent a stream of fluid out of the ulcerated end.

The characteristic behavioral signs of each pesticide in intact animals differed. Each produced nervous signs. They all produced head contractions whose types differed for each compound. For the "light" strain, chlorpyrifos produced no apparent behavioral responses during the first 5 min. For the remainder of the hour, they exhibited coiling and ornamentation (i.e., crenelated edges). This was accompanied by severe depression (unconsciousness), a response not found with the other compounds. The head keeps moving even when they adopt a coiled position, and it is contracted toward the median line of the animal. The lowest concentration tested which manifested clinical signs during the first hour was 0.75 mg/L.

During the first 30 min, malathion induced restless movements at all concentrations. Concentrations $\geq$11 mg/L produced severe nervous signs such as fast changes in direction and constant head waving to both sides. During the last half hour, all concentrations produced abnormal movement. Concentrations $\geq$11 mg/L also produced morphological changes such as "banana curl," elongation, and head/nose twist (Grebe and Schaeffer 1991). Concentrations of methyl parathion $<$3.5 mg/L did not produce behavioral effects during the 7-d exposure. At or above 3.5 mg/L, characteristic effects in the first 5 min included frequent switches in the direction of movement and backward contractions of

the head. During the last 30 min, movement was abnormal and they maintained a characteristic head/nose contraction. The typical signs of exposure to *p*-nitrophenol were restlessness during the first 5 min (≥ 8 mg/L) and elongation at all concentrations. During the next 25 min, concentrations ≥ 8 mg/L produced abnormal movement.

Representative results for head regeneration are given in Table 2 for "light" animals and Table 3 for "dark" animals. In these experiments, head parts developed normal or stunted tails, did not develop a blastema, or died. The compounds tended to be more toxic at 27 °C than at 19 °C (Tables 1-3), so lower concentrations were used in the 27 °C regeneration studies. The effects of *p*-nitrophenol were complex. Regenerating bodies produced abnormalities such as teratophthalmic and teratomorphic heads (Yoshioka et al. 1986) in both the "light" and "dark" strains at 19 °C but not at 27 °C. Supernumerary eyes were produced in intact "light" animals at 27 °C but not at 19 °C. Supernumerary eyes were not produced in heads regenerating from "light" or "dark" bodies over 7 d.

All OPs delayed head regrowth in a concentration-related manner and concentrations near the LC_{50} for the intact animal produced total inhibition. Head regeneration at 27 °C was approximately 1.8 times faster than at 19 °C, confirming the report by Henderson and Eakin (1959). A stepwise analysis of covariance of the arcsin transformed HFIs for "bodies" in Tables 2 and 3 used the concentration of the compound (nM) as a covariate to adjust for differences in the concentration ranges. The adjusted mean HFI (arcsin) was higher at 19 °C (0.70) than at 27 °C (0.44), and for the "light" (0.77) than for the "dark" (0.37) strain. The adjusted mean HFI was lowest for chlorpyrifos (0.12), intermediate for methyl parathion (0.33) and highest for malathion (1.25). Mean HFIs did not differ for "dark" bodies and tails (Table 3), and were higher at 19 °C (0.83) than at 27 °C (0.56).

After using analysis of covariance to correct for concentration, mean corrected TFIs (arcsin) were lowest for chlorpyrifos (0.036), intermediate for methyl parathion (0.20), and highest for malathion (1.1). There was no overall temperature effect. However, for chlorpyrifos and malathion, the corrected TFIs were larger at 27 °C than at 19 °C, but the differences were not statistically significant. The TFI at 27 °C (-0.13) for methyl parathion was significantly below that at 19 °C (0.52).

For all of the toxicity endpoints in both strains, the order of toxicity was chlorpyrifos $\approx$ methyl parathion > malathion. The behavioral and head regeneration toxicities were concentration-dependent. The pesticides delayed or prevented head regeneration and prevented normal development of auricles but did not produce the types of abnormal eyes found with *p*-nitrophenol. The developmental toxicity of the compounds can be compared in several ways. Ranking the compounds in order of the lowest concentration (nM) giving at least one acephalic head, the order in the "light" strain is: *p*-nitrophenol < malathion < methyl parathion « chlorpyrifos. The same order obtains if the criterion is at least one anophthalmic head. In the "dark" strain, the order based on acephalic heads is: *p*-nitrophenol = methyl parathion < malathion < chlorpyrifos, whereas, the order based on the other criteria is the same as for the "light" strain.

The three OPs cause teratogenic effects in mammalian and/or avian species, and produced developmental defects in both planarian strains. Malathion caused teratogenic effects in hen (*Gallus* spp.) eggs (Dunachie and Fletcher 1970) and in developing fish eggs, *Oryzias latipes* (Solomon 1979), but not in rats (Khera et al. 1978). Chlorpyrifos and methyl parathion were not teratogenic to avian embryos (Kitos and Suntornwat 1992). Chlorpyrifos caused delayed ossification in mice (Deacon et al. 1980) and induced a reduction in egg production and hatchability in the snail *Biomphalaria alexandrina* (Ibrahim et al. 1992). Methyl parathion did not produce teratogenic effects in rats, but produced cleft palates in mice (Tanimura et al. 1967). However, some perinatal mortality

Table 2. Head abnormalities in regenerating "light" bodies[1].

Chemical mg/L (nM)	Number of Animals: Eye / Head Abnormalities: Ace-phalic	Anoph-thalmic	Terato-phthalmic	Dead	Other: Normal	HFI
Control (19°C/27°C)					20/10	100
Chlorpyrifos (MW 350.6)						
1.0 (2.8) (19°C)				1	19	95
1.7 (4.8)			2		8	96
2.0 (5.6)	3	5	7		6	68
3.0 (8.4)	3	2	1		4	62
0.5 (1.4) (27°C)					10	100
1.0 (2.8)					10	100
1.5 (4.2)			1		9	98
Malathion (MW 330.4)						
7.1 (22) (19°C)				1	9	90
8.1 (25)	1			2	17	86
9.1 (28)			1	4	15	79
10.1 (31)	1	1	1	5	12	67
11.1 (34)				7	3	30
12.1 (37)	6			10	4	26
13.1 (40)				9	1	10
14.1 (43)	2			6	2	24
15.1 (46)				10		0
3.0 (9.1) (27°C)					10	100
5.0 (15.1)	1			5	4	42
7.0 (21.1)				4	6	60
Methyl parathion (MW 263)						
3.5 (13) (19°C)		5	3		2	64
4.5 (17)	4	4		2		24
5.0 (19)	4			16		4
***p*-Nitrophenol** (MW 139.1)						
5.0 (36) (19°C)			2		18	98
8.0 (58)	1		3		16	93
10.0 (72)	1			9		2
8.0 (58) (27°C)					10	100

[1]For one (N = 10) or two replicates (N = 20) of 10 animals.

increase was seen in rats given 24 mg/kg on days 9 and 15 of gestation, and the growth rate after birth was reduced (Fish 1966). Our results for development of abnormalities in planaria agree more closely with mammalian data, which suggest that planaria might be a good organism for screening OPs prior to mammalian testing.

Table 3. "Dark" strain head (HFI) and tail (TFI) frequency indices.

Chemical mg/L (nM)	HFI for Bodies 19°C	HFI for Bodies 27°C	HFI for Tails 19°C	HFI for Tails 27°C	TFI for Heads 19°C	TFI for Heads 27°C
Chlorpyrifos						
Control	99	100	86	100	100	100
0.2 (0.57)	94	100	82	100	80	90
0.5 (1.43)	62	100	44	84	20	60
1.0 (2.85)	12	9	0	4	0	0
Malathion						
Control	99	100	86	100	100	100
4.0 (12.1)	--	100	--	100	16	68
6.0 (18.2)	70	86	52	67	6	39
8.0 (24.2)	45	44	16	20	12	8
10.0 (30.3)	6	--	--	--	--	--
Methyl parathion						
Control	97	100	97	99	100	100
1.0 (3.8)	--	98	--	100	--	100
1.1 (4.2)	--	78	--	100	--	0
1.7 (6.5)	100	--	94	--	100	--
2.0 (7.6)	97	0	74	0	81	0
2.3 (8.7)	56	--	54	--	52	--
2.6 (9.9)	48	--	48	--	0	--
4.0 (15.2)	0	--	4	--	0	--
***p*-Nitrophenol**						
3.0 (22)	76	--	72	--	92	--
5.0 (36)	100	100	96	100	92	80
8.0 (58)	98	100	76	100	60	100

Methyl parathion is metabolically converted in several species to *p*-nitrophenol by serum or tissue arylesterases and by cytochrome P-450 catalyzed oxidative dearylation (Wallace 1992). We have no analytical data on the conversion of methyl parathion in our tests. However, Table 2 shows important differences in the types of responses produced by methyl parathion and *p*-nitrophenol. Possibly, the concentration of any *p*-nitrophenol from the metabolism of methyl parathion was below that producing biological effects during head regeneration. It is also possible that by the time sufficient *p*-nitrophenol was produced by metabolism, the window for its effect on regeneration had passed. Published experiments with many other compounds have clearly shown the existence of such windows in head regeneration, and the temporal narrowness of a few minutes to several hours. Because the LC_{50} of methyl parathion at 19 °C (4.4 mg/L) is below the lowest concentration of *p*-nitrophenol showing an effect (5 mg/L), even complete metabolism might not produce *p*-nitrophenol concentrations high enough to produce abnormalities.

A 10 °C rise in temperature often doubles the rate of metabolic reactions, so an increase in temperature was expected to affect the lethality of the compounds tested. In fact, the 8 °C increase in temperature increased the acute toxicity by the expected factor of 1.7 to 1.8. However, there was no corresponding increase in the rate of head abnormalities during regeneration. On day 7 at the concentrations tested at 27 °C, 1 to 7 mg/L malathion produced deaths in 50% of the animals and normal heads in the remainder, and

0.5 to 1.5 mg/L chlorpyrifos and 4 to 8 mg/L *p*-nitrophenol produced 100% normal animals. To some extent, this may be due to the lower concentrations tested at this temperature. However, the same concentrations of *p*-nitrophenol which produced abnormalities at 19 °C did not produce abnormalities at 27 °C. This is the strongest evidence we have from the present studies of a temperature effect on head regeneration. It is possible that a higher metabolic rate could have had at least two effects which could have produced these results. First, the compounds could have been detoxified at a faster rate than was needed for them to exert a toxic effect. Second, Henderson and Eakin (1959) reported that head regeneration was complete in 48 h at 27 °C but required 96 h at 20 °C.

Organophosphorus pesticides are potential teratogens, but there is great variation in species susceptability. Chick embryos are generally very sensitive, effects are reproducible, and incidences of teratogenic effects are high. The biological effects of OP's on mammalian and amphibian embryos are not as well characterized as in avian embryos and the findings are more variable. Planaria have been proposed as a sentinel species for environmental monitoring (NRC 1991) and as a model system for teratogenesis studies (Best and Morita 1984; Collins 1987). Most of the approximately 150 mammalian teratogens tested in regenerating planaria since 1910 produced abnormalities and most of the mammalian nonteratogens did not produce abnormalities (Schaeffer, unpublished). However, organophosphorus pesticides have not been studied, and it was not known before the present study whether planarians more closely resembled chick embryos or mammalian and amphibian embryos in their responses to OPs.

Regenerating planaria develop head abnormalities, reproducibly and in a dose-dependent manner, when exposed to organophosphorus pesticides. Planaria are sensitive to the structure of the compound as evidenced by differences in the both the numbers of acephalic and anophthalmic abnormalities, and in the ratios of these abnormalities. Also, the *p*-nitrophenol results demonstrate other types of abnormalities that are likely to be found as more OP's are tested. These limited data suggest that planaria might be a good test system for evaluating the developmental toxicity of OPs.

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