Bull. Environ. Contam. Toxicol. (2005) 75:421–428

DOI: 10.1007/s00128-005-0770-4

Bulletin of Environmental Contamination and Toxicology

Inhibitory Effect of a Herbicide Formulation on Toxicity of Malathion to the Worm *Eisenia fetida*

D. W. Butler,[1] P. A. Verrell[2]

[1] Program in Environmental Science and Regional Planning, Washington State University, Pullman, WA 99164-4430, USA
[2] School of Biological Sciences and Center for Reproductive Biology, Washington State University, Pullman, WA 99164-4236, USA

Received: 5 March 2005/Accepted: 19 July 2005

As recognized by Darwin (1881), earthworms play a crucial role in the production and subsequent maintenance of fertile soils (see also Edwards 2004). It is recognized that this crucial ecological service provided by earthworms cannot be taken for granted. Pesticides used to control unwanted animal and plant species may affect non-target taxa in negative ways, and earthworms may be vulnerable (Grieg-Smith et al. 1992; Reinecke and Reinecke 2004). Significant amounts of pesticides are used in the gardens and back yards of the developed world. Could such residential use exert negative effects on earthworms?

ORTHO MALATHION PLUS and ORTHO WEED B GON are an insecticide and herbicide formulation, respectively, registered by the Environmental Protection Agency for residential use in the United States; they are often found on the same shelf in retail outlets. ORTHO MALATHION PLUS is 50% malathion formulated in petroleum distillate. ORTHO WEED B GON is a mixture that includes mecoprop (5.3%), 2, 4-D (3.05%) and dicamba (1.3%), all as dimethylamine salts. Pesticide residues from both of these formulations could be present in the same confined area. For example, the insecticide might be applied to control insects in a vegetable bed, and the herbicide used to control weeds in an adjacent lawn. Drift of spray from either the lawn onto the soil of the vegetable garden or vice versa would cause the co-occurrence of residues of the two formulations.

Much of the literature on the safety of these two formulations and their constituent components for annelid worms pertains to aquatic species (EPA Acquire Database). Aquatic oligochaetes exposed to malathion or 2, 4-D seem to be only moderately susceptible, with LC50s in the part per million range (generally > 1 mg/L). However, one study of the terrestrial worm *Eisenia fetida* classified malathion and 2, 4-D as very toxic (LC50 range 10-100 ug/cm^2: Roberts and Dorough 1984). We found no studies of the toxicity of dicamba and mecoprop to annelids. Furthermore, malathion has not been tested in combination with 2, 4-D, a plausible environmental exposure given the registered uses of the product formulations.

Like other organophosphate insecticides, malathion affects nerve signal

Correspondence to: P. A. Verrell

modulation via inhibition of acetylcholinesterase both at neuromuscular junctions and within the central nervous system. We hypothesized that MALATHION PLUS likely is toxic to earthworms. Also, we hypothesized that WEED B GON likely is toxic due to the presence of 2, 4-D. It is generally accepted that chemicals with different modes of action exhibit joint toxicity at less than simple concentration-addition (Broderius et al. 1995). For both pesticides applied together, we proposed the null hypothesis that any interaction between them would be additive; the alternatives would be some form of antagonism or synergy (i.e., effects less than or greater than additive).

Here we report the results of a series of experiments designed to test for the effects of acute exposure to MALATHION PLUS and WEED B GON, both singly and in combination, on the earthworm *Eisenia fetida*, a model species in studies of annelid toxicology (Kula and Larink 1997). We addressed two end-points of likely ecological significance: lethality (for both formulations) and behavioral avoidance (for MALATHION PLUS only).

MATERIALS AND METHODS

Eisenia fetida is a small clitellate earthworm that we maintain as a large breeding colony in our laboratory. The colony resides in a closed plastic bin of 50 L in volume at a temperature of 18 C. The substrate consists of commercial potting soil that pesticide-free, and domestic vegetable material regularly is buried in the substrate to provide worms with ample access to food.

The experiments reported here were preceded by a pilot study in which worms were exposed to: (i) three concentrations of the two pesticides applied singly (nominal = the dilution recommended by the manufacturer for use in the field, and additional dilutions of 0.1and 0.01 of nominal); (ii) the same concentrations of the two pesticides applied in combination, and (iii) distilled water (control). Three replicates of each of these 10 treatments were included ($N = 20$ worms per replicate).

Our first experiment was conducted to determine on a finer scale the dose-response relationship between MALATHION PLUS and mortality. Twenty worms were placed into a plastic box that contained 1 L of commercial potting soil. Twenty-two such boxes were created, and each was randomly assigned to one of two treatments: application of 100 ml of distilled water (control) or 100 ml of a MALATHION PLUS solution. Concentrations used in the latter treatment ranged between nominal and a dilution of 1/10th in decrements of 0.1 ($N = 2$ replicates per concentration, plus control). The volume of added solutions resulted in a final soil moisture content of approximately 45% by mass, with soil pH ranging between 6.3 and 7.0. Boxes were left for 96 hr at a temperature of 20 C, at which time they were emptied and the soil carefully examined for live worms.

We conducted a second experiment to test whether *E. fetida* avoids soil that is

contaminated with MALATHION PLUS. A plastic box was taken and separated into two halves by a cardboard divider. On one side was placed 1 L of soil moistened with 100 ml of distilled water, and on the other side was placed 1 L of soil moistened with 100 ml of nominal solution of MALATHION PLUS. The cardboard divider was then removed and a piece of white thread was placed on the surface of the soil to mark the division between the clean and contaminated sides. Thirty worms were then placed at the mid-point between the insecticide-treated and untreated soil. Boxes were left undisturbed for 24 hr ($N = 8$ replicates), at which time the numbers of worms present in each soil type were counted. This experiment was repeated twice using the same design for 0.1 and 0.01 dilutions of MALATHION PLUS.

Our third experiment explored the apparent partial 'protective' effect of nominal concentrations of WEED B GON on the acute toxicity of nominal MALATHION PLUS. Twenty worms were placed into a plastic box that contained 1 L of commercial potting soil. Sixteen such boxes were created, and each was randomly assigned to one of four treatments: (i) application of 100 ml of distilled water (control, $N = 2$ replicates), (ii) application of 100 ml of nominal MALATHION PLUS ($N = 2$ replicates), (iii) application of 100 ml of nominal WEED B GON ($N = 2$ replicates), and (iv) application of nominal concentrations of both MALATHION PLUS and WEED B GON in a total volume of 100 ml ($N = 10$ replicates). These boxes were left for 96 hr, at which time they were emptied and the soil carefully examined for live worms.

RESULTS AND DISCUSSION

Mortality after 96 hr was the only end-point examined in our pilot study (Table 1). All 60 worms died in the nominal MALATHION PLUS treatment, and all 60 survived in the 0.01 dilution. Mortality in $1/10^{th}$ was intermediate, with 33% of 60 worms dying ($H = 9.35$, 3 df, $P = 0{,}025$, two-tailed Kruskal-Wallis one-way ANOVA: Siegel and Castellan 1988). In contrast, WEED B GON was essentially nontoxic at all concentrations tested ($H = 0.69$, 3 df, $P > 0.80$, two-tailed Kruskal-Wallis one-way ANOVA). Finally, combined application of the two pesticides yielded mixed results. As might be expected, mortality was low at 0.1 and 0.01 dilutions (one of 120 worms died). Thirty eight (64%) of 60 worms died at nominal concentrations ($H = 6.58$, 3 df, $0.10 < P > 0.05$, two-tailed Kruskal-Wallis one-way ANOVA); recall that, when applied singly, nominal MALATHION PLUS exerted complete mortality. In terms of acute toxicity, these pilot data suggested that: (i) MALATHION PLUS is lethal to *E. fetida* at high concentrations, (ii) WEED B GON is quite innocuous, and (iii) the lethal effect of the former may be ameliorated by the presence of the latter.

In our dose-response experiment, all worms died when exposed to the 0.7 dilution and higher concentrations. No control worms died in the distilled water control treatment nor in soil treated with dilutions less than 0.7 of nominal. We conclude that the lowest observable adverse effect concentration of MALATHION PLUS for acute toxicity lies above a concentration of approximately 70% of nominal.

Table 1. Numbers of *Eisenia fetida* alive after 96 hr of exposure to soil treated with distilled water (control), and three concentrations of two pesticide formulations applied alone and in combination.

Treatment	Dilution	Nos. worms surviving
Water only (control)		20, 20, 20
MALATHION PLUS alone	Nominal	0, 0, 0
	0.1	12, 13, 15
	0.01	20, 20, 20
WEED B GON alone	Nominal	20, 20, 20
	0.1	17, 20, 20
	0.01	20, 20, 20
Both	Nominal	0, 5, 12
	0.1	19, 20, 20
	0.01	20,20,20

We did not conduct a dose-response experiment for WEED B GON because of the lack of acute toxicity observed in our pilot study.

Exposure to MALATHION PLUS, even at concentrations that are lethal within 96 hr, may stimulate an avoidance response in worms if uncontaminated soil is accessible. As shown in Table 2, our second experiment provides strong evidence that *E. fetida* will move into clean soil within 24 hr if adjacent soil is contaminated with a lethal concentration of nominal MALATHION PLUS ($T = 36$, $N = 8$, $P = 0.004$, two-tailed Wilcoxon test against a null expectation of 15 worms in clean soil). Interestingly, a similarly strong avoidance response was shown by worms exposed to the 0.1 dilution of MALATHION PLUS ($T = 36$, $N = 8$, $P = 0.004$). Recall that this concentration of pesticide did not result in acute mortality, yet it was sufficient to induce avoidance. Finally, worms treated soil contaminated with 0.01 dilution of MALATHION PLUS as no different from clean soil in terms of their spatial distribution ($T = 18$, $N = 7$, $P = 0.29$).

Avoidance is a common response of mobile animals exposed to contaminated areas (Smith and Logan 1997), and may result in greater-than-expected survival. Avoidance has been observed in studies of earthworms exposed to several soil contaminants (Reinecke et al. 2002; Slimack 1997; Verrell and Van Buskirk 2004; Yeardley et al. 1996). This response might be expected given the rich sensory innervation of oligochaete skins (Jamieson 1981). That avoidance also occurs when levels of contaminants are not directly lethal suggests that additional end-points not addressed in this study may be affected.

Our third experiment further explored the possibility suggested in our pilot study that the toxicity of MALATHION PLUS could be ameliorated by simultaneous exposure to WEED B GON. As shown in Table 3, exposure to nominal

Table 2. Numbers of *Eisenia fetida* retrieved from clean soil after 24 hr when the latter was adjacent to soil contaminated with one of three concentrations of MALATHION PLUS.

Treatment	Nos. worms in clean soil: Median	Range
Nominal MALATHION PLUS	25.5	21-28
0.1 dilution	22.5	18-28
0.01 dilution	17.5	8-21

concentrations of MALATHION PLUS and WEED B GON alone for 96 hr resulted in 100% mortality and 100% survival, respectively. In addition, no worms died in the control soil. However, exposure to soil treated with a nominal concentration of both pesticides (but in a volume of 100 ml to keep soil moisture constant among treatments) resulted in only 25% mortality ($H = 11.16$, 3 df, $P < 0.025$, two-tailed Kruskal-Wallis one-way ANOVA).

THE SAS GLM (general linear models) procedure was used to make pairwise comparisons among treatments in our third experiment (SAS Institute 1999). As expected, worm survivorship was greater in water only than with exposure to nominal conentrations of MALATHION PLUS ($P < 0.05$). Survivorship was greater with exposure to nominal concentrations of WEED B GON than to nominal MALATHION PLUS ($P < 0.05$). Survivorship with simultaneous exposure to nominal concentrations of both pesticide formulations also was statistically different than that observed with MALATHION PLUS alone $P < 0.05$). Thus, our data strongly suggest that WEED B GON exposure ameliorates the acute toxicity of malathion.

The literature on the toxicity of mixtures is dominated by studies of aquatic organisms and presents a number of quantitative tools for determining joint effects. One such method involves analysis of toxic units, in which the combined action of a mixture is evaluated by comparing its EC50 value with the corresponding sum of EC50 values of single components (e.g., Der Geest et al. 2000). Such an analysis could not be undertaken for the formulations that we used because WEED B GON exerted no acute toxicity on worms. The interaction that we have documented is best described as inhibition according to the EPA-based classification provided in Hertzberg and MacDonnell (2002). With inhibition, a substance that is toxic when alone is rendered less toxic when combined with a second substance that is innocuous when alone.

How might this inhibition operate at a mechanistic level? Manifestation of negative effects due to exposure to malathion follows a series of complex biochemical reactions. The parent compound (which itself has little neurotoxic activity) is metabolized to the anticholinesterase compound malaoxon by the microsomal enzyme cytochrome P-450. However, the hydrolase enzyme

Table 3. Numbers of *Eisenia fetida* alive after 96 hr of exposure to soil treated with distilled water (control) and nominal concentrations of two pesticides applied singly and together simultaneously.

Treatment	Nos. worms alive
Water (control)	20, 20
Nominal MALATHION PLUS alone	0, 0
Nominal WEED B GON alone	20, 20
Nominal both	Median = 15.0, range 11-18 (N = 10)

carboxyesterase can biotransform the parent compound into innocuous malathion carboxylic acids. Also, the degree of toxicity is modulated by the rate at which acetylcholinesterase spontaneously dephosphorylates after initial inhibition (Matsumura 1975; Zakrzewski 1997). We hypothesize that the inhibitory effect of nominal WEED B GON on nominal MALATHION PLUS is due to changes in the activities of components of the mixed-function oxidase system that: (i) prevent conversion of malathion to malaoxon, (ii) convert malathion into its carboxylic acids, and/or (iii) alter acetylcholinesterase phosphorylation by malaoxon. The plausibility of the first mechanism is indicated by data that show 2, 4-D and dicamba (both of which are components of WEED B GON) to alter activities of components of the detoxifying mixed-function oxidase system in insects (Kao et al. 1995) and mammals (Moody et al. 1991). The complexity of these enzyme systems (and associated isozymes) surely provides opportunities for complex interactions among xenobiotic compounds.

In summary, our data suggest that residential use of MALATHION PLUS may compromise the survival of worms in affected areas. Application of WEED B GON seems to have no acutely negative consequences, and may indeed be beneficial to worms exposed simultaneously to organophosphate insecticides.

Acknowledgments. This study was completed in partial fulfillment of requirements for an M.S. degree by the first author. We thank Professor Allan Felsot for help with data analysis and literature, and Professors Felsot, Rod Sayler, Paige Lawrence and Rolf Ingermann for comments.

REFERENCES

Broderius SJ, Kahl MD, Hoglund MD (1995) Use of joint toxic response to define the primary mode of action for diverse industrial organic chemicals. Environ Toxicol Chem 9: 1591-1605

Darwin C (1881) The formation of vegetable mould through the action of worms, with observations on their habits. John Murray, London

Der Geest HMV, Greve GD, Boivin M-E, Kraak MHS, van Gestel CAM (2000) Mixture toxicity of copper and diazinon to larvae of the mayfly (*Ephoron virgo*) judging additivity at different effect levels. Environ Toxicol Chem 19: 2900-2905

Edwards CA (2004) The importance of earthworms as key representatives of the soil fauna. In: Edwards CA (ed) Earthworm ecology, CRC Press, Boca Raton, Florida, pp 3-11

Greig-Smith PW, Becker H, Edwards, PJ, Heimbach F, eds (1992) Ecotoxicology of earthworms. Intercept, London

Hertzberg RC, MacDonnell MM (2002) Synergy and other ineffective mixture risk definitions. Sci Total Environ 288: 31-42

Jamieson BGM (1981) The ultrastructure of the Oligochaeta. Academic Press, New York

Kao LM, Wilkinson CF, Brattsten LB (1995) In-vivo effects of 2, 4-D and atrazine on cytochrome P-450 and insecticide toxicity in southern armyworm (*Spodoptera eridania*) larvae. Pestic Sci 45: 331-334

Kula H, Larink O (1997) Development and standardization of test methods for the prediction of sublethal effects of chemicals on earthworms. Soil Biol Biochem 29: 635-63

Matsumura F (1975) Toxicology of insecticides. Plenum Press, New York

Moody DE, Narloch BA, Shull LR, Hammock BD (1991) The effect of structurally divergent herbicides on mouse liver xenobiotic-metabolizing enzymes (P-450-dependent mono-oxygenases, epoxide hydrolases and glutathione S-transferases) and carnitine acetyltransferase. Toxicol Lett 59: 175-185

Reinecke AJ, Maboeta MS, Vermeulen LA, Reinecke SA (2002) Assessment of lead nitrate and Mancozeb toxicity in earthworms using the avoidance response. Bull Environ Contam Toxicol 68: 779-786

Reinecke AJ, Reinecke SA (2004) Earthworms as test organisms in ecotoxicological assessment of toxicant impacts on ecosystems. In: Edwards CA (ed) Earthworm ecology, CRC Press, Boca Raton, Florida, pp 299-320

Roberts BL, Dorough HL (1984) Relative toxicity of chemicals to the earthworm *Eisenia foetida*. Environ Toxicol Chem 3: 67-78

SAS Institute Inc (1999) SAS/STAT user's guide, version 8. SAS Institute Inc, Cary, North Carolina

Siegel S, Castellan NJ, Jr (1988) Nonparametric statistics for the behavioral sciences. McGraw Hill, San Francisco

Slimack KM (1997) Avoidance response as a sublethal effect of pesticides on *Lumbricus terrestris* (Oligochaeta). Soil Biol Biochem 29: 713-715.

Smith EH, Logan DT (1997) Linking environmental toxicology, ethology, and conservation. In: Clemmons, JR, Buchholz R (eds) Behavioral approaches to conservation in the wild, Cambridge University Press, New York, pp 277-302

Verrell P, Van Buskirk E (2004) As the worm turns: *Eisenia fetida* avoids soil contaminated by a glyphosate-based herbicide. Bull Environ Contam Toxicol 72: 219-224

Yeardley RB, Lazorchak JM, Gast LC (1996) The potential of an earthworm avoidance test for evaluation of hazardous waste sites. Environ Toxicol Chem 15: 1532-1537

Zakrzewski SF (1997) Principles of environmental toxicology (ACS monograph 190). American Chemical Society, Washington, DC