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Vineyard Pesticides and Their Effects on Invertebrate Biomarkers and Bioindicator Species in New Zealand

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There is general concern that pesticides and other agrochemicals may be damaging agroecosystems and wildland resources throughout New Zealand and globally. Vineyards can have high pesticide inputs, and organophosphorus insecticides have been used to manage insect pests in New Zealand for over 30 years (Suckling 1994). However, the use of these insecticides has prompted concerns with food safety, and due to their broad-spectrum nature, they impact on both pests and beneficial species. These concerns have resulted in the development of integrated pest management strategies that utilize alternative insecticides, e.g., insect growth regulators, such as tebufenozide (Mimic) (Valentine et al. 1996; Gurr et al. 1999). Insect growth regulators are typically used at very low application rates and may be very specific. Therefore they are perceived to be less environmentally harmful than are organophosphates. Tebufenozide is gaining acceptance as a replacement for the organophosphate, chlorpyrifos, as it acts specifically on Lepidoptera as an ecdysone agonist (Gerard and Ruf 1994), and is therefore thought to have minimal nontarget effects.

To compare the nontarget effects of chlorpyrifos and tebufenozide, three beneficial invertebrate species were evaluated; the common pasture earthworm *Aporrectodea caliginosa* (Savigny), the wolf spider *Lycosa hilaris* (Koch), and the brown lacewing *Micromus tasmaniae* (Walker). *A. caliginosa* is found in arable and pasture land where it is important for maintaining soil productivity (Springett 1992). The wolf spider and brown lacewing are important polyphagous predators (Leathwick and Winterbourn 1984). Furthermore, the wolf spider and particularly the brown lacewing are sensitive to organophosphates (Hodge et al. 2000; van Erp et al. 2002).

Nontarget effects of pesticides are typically evaluated using mortality as the end point, but mortality can be an insensitive predictor of long-term adverse effects on the organism. Measurement of biomarkers that provide data on the potential adverse impacts of contaminants can act as 'early warning' signs of impending ecosystem harm. In this study, two biomarkers, the neutral red retention assay (NRRA) and cholinesterase (ChE) activity, were evaluated alongside mortality to compare the ecological effects of chlorpyrifos and tebufenozide. The NRRA

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provides a measure of membrane stability by measuring the retention time of neutral red (NRRT) within lysosomes. Healthy, unstressed cells will retain the dye for long periods after uptake, which results in a high NRRT. In contrast, in stressed cells, dye will leak from the lysosomes into the cytoplasm more rapidly, leading to a lower NRRT value. This assay has been used as a biomarker of heavy metal exposure (Weeks and Svendsen 1996) and it has also proven useful for detecting exposure to organophosphates (Booth and O'Halloran 2001).

Cholinesterases are inhibited by organophosphates, and these enzymes have been used as biomarkers in invertebrates to diagnose organophosphate poisoning (Edwards and Fisher 1991). Cholinesterase activity has previously been evaluated in wolf spiders (van Erp et al. 2002) and lacewings (Hodge et al. 2000) exposed to diazinon and chlorpyrifos. While van Erp et al. (2002) found ChE activity to be a sensitive indicator in wolf spiders, ChE activity in lacewings was less useful, due to high mortality that occurred with organophosphate exposure (Hodge et al. 2000). Therefore, in this research, ChE activity and mortality were measured in wolf spiders, and mortality alone was monitored in lacewings.

MATERIALS AND METHODS

The insecticides used in this study were chlorpyrifos (Lorsban; 750 g active ingredient (a.i.)/kg, DowElanco (NZ), New Plymouth, NZ) and tebufenozide (Mimic; 200 g a.i./kg, Rohm and Haas, Philadelphia, PA, USA). Both pesticides were applied at rates recommended for vineyards: chlorpyrifos at 0.75 kg a.i./ha, and tebufenozide at 0.26 kg a.i./ha. Both insecticides were wettable powder formulations, and were applied at 250 L water/ha. Residues in soil samples were analysed by gas chromatography with mass spectroscopy by LincLab, Lincoln, New Zealand. The following fungicides were also applied to all treatments: Dithane/Mancozeb (800 g mancozeb/kg) applied at 2 kg/ha; Switch (375 g cyprodinil/kg and 250 g fludixonil/kg) applied at 0.8 kg/ha; and Topas (100 g pencanzole/L) applied at 0.2 l/kg.

Field experiments were conducted in a vineyard on the Kaituna Estate, Montana Wines, Marlborough, New Zealand in early summer 2000. Chlorpyrifos, tebufenozide and a water control were applied to the grapevines at flowering with a Turbo-col 2000 sprayer (Croplands Equipment, Adelaide, South Australia) with T-Jet nozzles rated at 12-15 L/min. For each treatment there were two blocks, each of six rows, and the middle two rows of each block were used for monitoring. Both monitoring rows were divided in half at the midway point (approximately 220 m), and from this center point a 50-m buffer was established between each section. This resulted in two pairs of half rows in each block, and within each of these pairs, one half row was randomly allocated for earthworm deployment and the other for spider deployment. Within each half row there were three sampling sites, each 50 m apart, and there was a minimum of 25 m between the end sampling site and the row end. This resulted in four independent replicates, with three sampling sites in each replicate for each treatment for each bioassay.

Earthworms were obtained from a laboratory culture derived from adult earthworms collected in Canterbury, New Zealand. Ten adult earthworms were deployed in mesh bags (45 × 15 cm) containing soil from the vineyard, approximately 12 hr before spraying. Soil type was a Wairau sandy loam. One bag per sampling site (total of 12 bags per treatment) was buried between irrigation drippers to a depth of 10 cm. The irrigation was operated for 1.5 hr at 2.5 L/hr on every second day during the experiment. After 7 d, the bags were removed, mortality determined, and the NRRA was determined in two randomly selected live earthworms from each bag, i.e., 24 per treatment.

Male wolf spiders were collected from a pesticide-free area using dry pitfall traps. They were acclimated for a minimum of 7 d in the laboratory in glass aquaria (37 × 38 × 75.5 cm) containing clean moist soil, uncontaminated bark and leaf matter, and were fed laboratory-reared pea aphids (*Acyrthosiphon pisum* (Harris)). Wolf spiders were deployed in mesocosms comprising a cut plastic bucket (24 cm id × 12 cm height) covered with a plastic mesh (1.6 × 1.2 mm). One mesocosm per sampling site (total of 12 per treatment) was placed directly under the grapevines before spraying and one spider was placed on to the soil surface inside each mesocosm 3, 24, and 48 hr after spraying. Mortality was monitored 24, 48, or 96 hr, respectively, after spraying, i.e., spiders were exposed for 21, 24, and 48 hr. Surviving wolf spiders were collected and frozen at -80°C for ChE activity analysis.

Lacewings were collected from a pesticide-free area. They were maintained in plastic boxes in the laboratory at 20°C and 16 hr light, 8 hr dark until required, and fed a mixture of honey and brewer's yeast and pea aphids. Adult lacewings were deployed in pairs in a Terylene bag (18 × 24 cm), and attached to the vines by closing the drawstring of the bag around the petiole of a leaf in the vine canopy. One bag for each each sampling site (total of 12 per treatment) was placed in the canopy 3 hr after spraying. Mortality was monitored 24 hr after spraying. Lacewing and spider mortality data were analysed by fitting logistic regression using function glm in S-PLUS, with treatment, time and their interaction included as effects in the model.

Duplicate soil samples (n = 4) to a depth of 1 cm were collected from each treatment for residue analysis, 3, 24, 48, and 96 hr after spraying to coincide with the collection of the invertebrate samples. Data were analysed using ANOVA in S-PLUS, with time as a factor.

A neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) working solution of 80 mg/mL was prepared in physiological ringer solution (Lockwood, 1963). Coelomic fluid was collected from the earthworm by inserting a 25-gauge needle containing 50 µl of ringer into the coelomic cavity posterior to the clitellum. The coelomic fluid was placed on to a clean glass slide and mixed with an equal volume of neutral red solution. Slides were scanned for 2 min at 5-min intervals and the number of stained and unstained cells counted until 50% of the cells were red; this time was recorded as the neutral red retention

time (NRRT). Data were analysed using ANOVA in S-PLUS. The dependent variable, NRRT, was averaged over the six worms in each replicate (n = 4). Adjusted treatment means were compared using two-sided Bonferroni tests.

Frozen spider samples were defrosted, and spiders were homogenized individually in 200 µl of ice-cold 0.02 M phosphate buffer, pH 7.5, and left to sit for 1 hr. Cholinesterase activity was measured based on the method of Ellman et al. (1961), adapted for spiders and microtitre plates. In summary, the reaction mixture containing 5 µL homogenate, 15 µL buffer, and 140 µL 0.5 mM 5,5',-dithio-bis-2-nitrobenzoic acid (DTNB) was incubated at 37°C for 5 min. The reaction was started by addition of 20 µL of 15 mM acetylthiocholine iodide (final concentration). Each sample was measured in triplicate, and was repeated if the coefficient of variation was > 20%. A control containing no homogenate was used as an analytical blank. Samples were analysed for protein content using the Bradford method (Bradford 1976), with bovine serum albumin (BSA, Sigma Chemical Co., St Louis, MO, USA) as a standard. Each sample was measured in triplicate, and the coefficient of variation was < 5%. Cholinesterase activity is expressed as nmoles acetylthiocholine hydrolysed/min/mg protein. Data were analysed using ANOVA in S-PLUS, with time and treatment as factors.

RESULTS AND DISCUSSION

There was no earthworm mortality in any treatment, indicating that neither chlorpyrifos nor tebufenozide were acutely toxic to earthworms at the application rates used in vineyards and under these experimental conditions. Neutral red retention time, however, was significantly reduced in earthworms exposed to both insecticides, from 28 (± 0.75) min in the control to 18 ± 1.5 and 14 ± 1 min for tebufenozide and chlorpyrifos, respectively ($F_{2,3}$ = 40.2, P = 0.007), but there was no evidence that the two insecticides differed in this respect (P > 0.05). The NRRA is a general stress biomarker and reduced NRRT indicates that exposure has induced a physiological response, but in this instance this response is unlikely to be toxicologically significant. For example, earthworms exposed to chlorpyrifos at 4 mg a.i./kg soil (a laboratory-simulated field rate equivalent to a field application rate of 0.8 kg a.i./ha) exhibited no adverse effects on earthworm mortality, growth, or fecundity (Booth and O'Halloran 2001). The route of exposure of earthworms in the mesh bags to the pesticides is unclear, but is likely to be due to leaching of insecticides through the soil, facilitated by the irrigation system. Soil was also disturbed during burial of the bags, which may have assisted movement of the pesticides through the soil profile. Small amounts of leaching by chlorpyrifos have previously been demonstrated (Konda and Pasztor 2001), while tebufenozide is reported to bind strongly to soil organic matter. However, total carbon was low in the vineyard soil (2.24%), in comparison to trials in forest litter (Addison 1996) where binding will be much greater.

In previous laboratory exposure experiments where *A. caliginosa* were exposed to chlorpyrifos at 28 mg a.i./kg soil (equivalent to 5.6 kg a.i./ha), growth and fecundity were significantly impaired (Booth and O'Halloran 2001). In contrast,

when Addison (1996) exposed the forest earthworm *Dendrobaena octahedra* (Savigny) in the laboratory to tebufenozide in leaf litter at the equivalent of 7 kg a.i./ha, no effects on mortality, growth, or fecundity were detected. These results and those in this study suggest that it is unlikely that either chlorpyrifos or tebufenozide sprayed at recommended application rates for vineyards would have adverse effects on earthworm survival and fecundity, and therefore earthworm numbers. These results also suggest that tebufenozide is less likely to lead to adverse effects on earthworms than is chlorpyrifos. However, the bioavailability and also the degradation profiles of these insecticides may vary considerably in different soils, and should be taken into account when considering potential nontarget impacts.

Wolf spider ChE activity was inhibited by exposure to both insecticides compared with the controls ($F_{2,2} = 39.8$, $P = 0.024$), but there was no difference between the pesticides (Table 1). There was also no evidence for an effect on mortality ($\chi^2_2 = 1.12$, $P = 0.57$) (Table 2). However, variability was high, indicating that this result should be treated with caution. Enzyme activity was measured 24, 48, and 96 hr after spraying, but there was no apparent effect of time on enzyme activity. Chlorpyrifos inhibited ChE activity by 39% compared with control activity 24 hr after spraying, but 48 and 96 hr after spraying, activity had returned to 90% (10% inhibition) and 105% (no inhibition) with respect to control activity. Cholinesterase activity in spiders exposed to tebufenozide was inhibited by 15, 46, and 5% compared with controls, 24, 48, and 96 hr, respectively, after spraying. Van Erp et al. (2002) exposed wolf spiders in the laboratory to chlorpyrifos at 0.8 kg a.i./ha, and observed a 39% inhibition in ChE activity after 24 hr exposure. This result is consistent with the 39% inhibition of activity observed in wolf spiders exposed in this study to 0.75 kg a.i./ha. However, van Erp et al. (2002) found no mortality at this concentration.

Table 1. The effect of field exposure to chlorpyrifos and tebufenozide on ChE activity in wolf spiders (Mean ± SE).

Time after application (h)	Exposure duration (h)	ChE activity (nmoles/min/mg protein) Control	Chlorpyrifos	Tebufenozide
24	21	66.0 ± 12.8	40.6 ± 13.3	55.9 ± 14.5
48	24	58.5 ± 13.3	52.8 ± 14.0	31.7 ± 7.7
96	48	46.6 ± 9.7	49.4 ± 13.8	44.4 ± 12.8

Table 2. The effect of field exposure to chlorpyrifos and tebufenozide on wolf spider mortality (Mean ± SE).

Time after application (h)	Exposure duration (h)	Mortality (%) Control	Chlorpyrifos	Tebufenozide
24	21	25 ± 8	58 ± 16	42 ± 16
48	24	25 ± 16	25 ± 17	46 ± 21
96	48	36 ± 14	33 ± 24	21 ± 13

The diagnostic relationship between ChE activity and mortality varies greatly between species and for each compound, but 40-60% inhibition of enzyme activity has been suggested as a critical level for detection of neurological effects (Grue et al. 1992). Van Erp et al. (2002) found no mortality when ChE activity was inhibited by 53%, but 80% inhibition resulted in almost total mortality. Van Erp et al. (2002) also monitored the duration of the response in spiders exposed to diazinon in the field, and found that ChE activity returned to normal rapidly (after 8 d), even though diazinon soil concentrations remained unchanged. These results and the results of the present study suggest that neither chlorpyrifos nor tebufenozide are likely to have any lasting effects on spiders when insecticides are applied at the recommended application rates. However, as stated before, this may depend on soil type and degradation rates, and also the frequency of application of the insecticides.

Adult lacewing survival was not significantly reduced by exposure to insecticides ($F_{2,9} = 1.87$, $P = 0.210$), despite 92 ± 17% and 100 ± 0% mortality in lacewings exposed to tebufenozide and chlorpyrifos, respectively. However, mortality was also very high in the controls (71 ± 34%), making it difficult to determine pesticide-induced effects. The air temperature exceeded 28°C during the lacewing exposure period and is likely to be responsible for this high mortality. Hodge et al. (2000) previously found that lacewings were sensitive to environmental conditions. The fungicides applied to all treatments may also have been responsible for this mortality. Chlorpyrifos has previously been shown to cause mortality of up to 80% 24 hr after exposure to 0.3 kg a.i./ha (less than half the application rate used in this research) in the field (Hodge et al. 2000). However, chlorpyrifos-induced mortality disappeared by 48 hr, indicating toxicity, and hence adverse effects are likely to be short-lived for this insecticide. Rumpf et al. (1997) exposed lacewing larvae in the laboratory to tebufenozide at 0.8% a.i. (eight times higher than the concentration used in this research), and also found no effects on mortality, or on fecundity and other life-table parameters (Rumpf et al. 1998). However, fecundity was reduced in the F1 generation, indicating possible long-term population effects (Rumpf et al. 1998).

Chlorpyrifos soil residues ranged from 0.1 to 1.51 mg/kg, and declined from 1.15 ± 0.35 mg/kg, 3 hr after spraying, to 0.42 ± 0.17 mg/kg, 96 hr after spraying ($F_{3,11} = 3.78$, $P = 0.044$). In contrast, tebufenozide concentrations showed no decline in soil concentrations over the 96-hr sampling period, indicating that this pesticide may be more persistent in soil than is chlorpyrifos ($F_{3,11} = 0.06$, $P = 0.980$). Soil residues ranged from 0.58 to 2.37 mg/kg, with a mean of 1.4 ± 0.78 mg/kg, 3 hr after spraying, and 1.33 ± 0.84 mg/kg at 96 hr after spraying.

In conclusion, exposure to chlorpyrifos and tebufenozide in New Zealand vineyards is likely to cause minimal short-term adverse effects on earthworms or wolf spiders when the manufacturers' recommendations are followed. However, the effect of chlorpyrifos and tebufenozide on lacewings was less clear than that for earthworms and wolf spiders, and should be further investigated. Overall, this study did not find any obvious differences in the nontarget effects of chlorpyrifos

and tebufenozide when these insecticides are applied at recommended application rates.

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