Bull. Environ. Contam. Toxicol. (2002) 68:801–808

DOI: 10.1007/s00128-002-0026-5

Bulletin of Environmental Contamination and Toxicology

Acute Toxicity of Mixtures of Chlorpyrifos, Profenofos, and Endosulfan to *Ceriodaphnia dubia*

M. Woods,[1] A. Kumar,[1] R. Correll[2]

[1] School of Pharmaceutical, Molecular, and Biomedical Sciences, University of South Australia, Adelaide, SA, 5000, Australia
[2] Mathematical and Information Sciences, CSIRO, Urrbrae, SA, 5064, Australia

Received: 21 September 2001/Accepted: 31 December 2001

Combinations of chemicals occur in the environment as many compounds persist for long periods (e.g., organochlorine pesticides), while others are discharged repeatedly (e.g., organophosphorus pesticides). Agricultural runoff water and effluent discharges therefore often contain complex mixtures of contaminants. Additionally, pesticides are frequently applied as mixtures. In Australia, multiple chemical residues have been detected in surface waters throughout various catchment areas (Leonard et al. 1999). Therefore, aquatic organisms are typically exposed to mixtures of chemicals rather than to single substances. Despite this, ecotoxicological studies in Australia have been limited to assessing the impact of individual toxicants only.

As the potential for multiple chemical exposure increases, the question raised is whether the toxicity of mixtures of chemicals is simply additive or whether there is an enhancement of toxicity (Johnston et al. 1994). The general consensus has been that chemicals interact by concentration addition, however past studies have demonstrated that concentration addition of the components of a mixture does not always reflect the overall effect of a mixture (Forget et al. 1999). Combinations exhibiting synergistic behaviour (greater than additive) have been reported. Forget et al. (1999) reported that binary and ternary combinations of pesticides (carbofuran, dichlorvos, malathion) and metals (arsenic, cadmium, copper) exhibited synergistic lethal effects to the marine microcrustacean *Trigriopus brevicornis.* Synergism was also observed by Pape-Lindstrom and Lydy (1997) when larvae of the aquatic midge (*Chironomus tentans*) were exposed to pairwise combinations of atrazine with chlorpyrifos, malathion and trichlorfon. The combinations of toxicants that have enhanced toxicity are of greatest concern in ecotoxicology because the toxicity predicted from the individual components would under estimate the overall toxicity (Forget et al. 1999).

The aim of the current study was to assess the acute mixture toxicity of three commonly used pesticides, chlorpyrifos, profenofos and endosulfan on a cladoceran species, *Ceriodaphnia dubia.* These pesticides were chosen as all three have been detected in the same catchment area (Leonard et al. 1999). Also, Batley and Peterson (1992) identified these pesticides to be of high environmental concern in the cotton industry.

Correspondence to: M. Woods

MATERIALS AND METHODS

Technical grade chlorpyrifos (*O,O*-diethyl-*O*-(3,5,6-trichloro-pyridyl phosphorothioate) of 99.8% purity was donated by Dow AgroSciences LLC (Indianapolis, Indiana). Technical grade profenofos (*O*-[4-bromo-2-chlorophenyl] *O*-ethyl *S*-propyl phosphorothioate) of 95.2% purity was obtained from Sigma Aldrich (New South Wales, Australia). Technical grade endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a - hexahydro-6,9-methano-2,4,3-benzodioxathiepin 3 oxide) of 96% purity was donated by Centre for Ecotoxicology, Environment Protection Authority (New South Wales, Australia). Main stock solutions were prepared by dissolving the nominal amount of pesticide into HPLC-grade acetone. Stock solutions were stored at 4°C in foil wrapped Schott bottles.

Mass cultures of *Ceriodaphnia dubia* were maintained at 23±1°C with a 16 hour light and 8 hour dark photoperiod using cool white fluorescent lamps. *Ceriodaphnia dubia* were reared in formulated moderately hard water (hardness 80-100 mg/L as $CaCO_3$) according to the guidelines recommended by the U.S. Environmental Protection Agency (USEPA) (USEPA 1989). Moderately hard water was enriched with 2 µg/L selenium (as Na_2SeO_4). The culture was fed a tri-algal mix consisting of *Ankistrodesmus* sp., *Chlamydomonas* sp. and *Pseudokirchneriella subcapitata* and a mixture of yeast, cereal leaves and trout chow (YCT). The culture water was replaced and the culture was fed three times per week.

Tests were conducted in 20 mL glass scintillation vials containing 18 mL of test solution to avoid volatilisation of the pesticides. The highest acetone concentration in the exposure vials was less than 0.1% which is well below the concentration to produce acute toxicity to *C. dubia.* A control and a negative and positive control were established the same way as the test concentrations. Copper sulfate (as $CuSO_4.5H_20$) was used as a positive control to monitor the health of the test organisms. Concentrations of copper sulfate ranged from 5 to 20 µg/L. Pesticide concentrations used in the definitive tests were based on range finding tests. The nominal pesticide concentrations ranged between 0.004-0.128 µg/L for chlorpyrifos, 0.005-0.08 µg/L for profenofos and 10-320 µg/L for endosulfan.

The concentrations used in the mixture toxicity tests were based on LC50 values derived from tests conducted with individual pesticides. The pesticides were added in equitoxic concentrations (identical fractions of their individual LC50 values for each pesticide). Four concentrations below the LC50 value ($^1/_{16}$ LC50, $^1/_8$ LC50, ¼ LC50 and ½ LC50), one at the LC50 value and one concentration above the LC50 value (2×LC50) were established using a 50% dilution increment. All three binary and the ternary mixtures of pesticides were used for the toxicity tests. The nominal amounts of pesticides were pipetted into the test water and were mixed prior to addition of the test organism.

The test procedures followed the standard test methods for acute testing outlined by USEPA (1989). Four replicates containing 5 neonates (<24-h old) were used

for each test concentration. The test solutions were not renewed and the test organisms were not fed for the duration of the test. Mortality was monitored at 24 and 48-h. Tests were conducted under the same controlled conditions used for culturing. Water quality parameters dissolved oxygen (ppm), pH, conductivity (μS/cm) and temperature (°C) were measured at test commencement and at the end of the 48-h exposure period. These parameters were measured using a TPS 90-FL Field Lab electrode water quality meter (Analytical Equipment Company, Australia).

All statistical analyses were performed using TOXSTAT version 4 (West Inc and Gulley 1994) unless stated otherwise. Prior to analysis all data were subjected to a Sharpiro-Wilks test for normality and to Barlett's test for homogeneity. All significant differences were determined at α=0.05 (Zar 1984). For *C. dubia* the total number of organisms dead (from four replicates) at each concentration was calculated. These data were used to calculate LC50 values and 95% confidence intervals using the trimmed Spearman-Karber method (Hamilton et al. 1977). A trimming value of 10% was used where possible.

Toxic interactions were evaluated by converting the LC50 values associated with the mixtures to toxic units (TU's). This is based on the LC50 values of the individual pesticides in the mixture compared to the LC50 values established from tests on each pesticide alone. Toxic unit is the sum of the toxic contributions of each component in the mixture. The TU for a binary mixture is given by the following equation:

$$TU = \frac{LC50A(mix)}{LC50A(alone)} + \frac{LC50B(mix)}{LC50B(alone)} \qquad [1]$$

where A and B are toxicants, LC50 (mix) is the toxicity of each component in the binary mix and LC50 (alone) is the LC50 of A and B applied as single components. By this model if TU equals one, the toxicity of the mixture is additive. If TU is greater than one, the toxicity is less than additive (antagonistic) and if the TU is less than one the toxicity of the mixture is more than additive (synergistic) (Bailey et al. 1997). For individual pesticides and for pesticide mixtures the triplicate trials were pooled to calculate an overall variance for the average LC50 estimate.

The estimate of TU is derived from a meta-analysis of experiments that determined the LC50 of each of the components and of all four mixtures. An estimation of the between experiment error variance was determined by performing an analysis of variance on the LC50s obtained using each toxicant or mixtures of toxicants. The errors observed from this analysis were approximately normally distributed, and the estimate of the between experiment variance was 0.103. This large experimental error meant that the first order approximations, which are used in the estimation of the variance of a ratio, were potentially unreliable.

A Monte Carlo simulation (Gentle 1985) was used to estimate the SE of the TUs. In the Monte Carlo simulation, it was assumed that all the LC50s had a log normal distribution with a variance of s^2/r, and a mean equal to the natural log of the observed LC50s, where $s^2 = 0.103$ and r is the number of replicate experiments (in this case $r = 3$). The estimation of the LC50s of each of the pure toxicants were assumed to have independent errors, but the components of the mixtures were given the same random error as these LC50s were measured in the same experiment. The simulation was repeated 1000 times and the mean and standard deviation of each series of the 1000 TUs were estimated.

The statistical methods used in this paper make many assumptions, including the slopes of the bioassays do not vary between toxicants. A more general approach to that problem is required and will be the subject of a separate paper.

RESULTS AND DISCUSSION

Control survival exceeded the minimum level of 90% in all tests. Water quality remained within the guidelines established by the USEPA (1989). The average (± standard deviation) water quality parameters measured were: dissolved oxygen: 7.6 ± 0.5 ppm; pH: 8.0 ± 0.1; conductivity: 326 ± 23 µS/cm and temperature: 24.5 ± 1.6 °C. The average LC50 value for the copper references tests throughout the testing period was 7.05 ± 0.34 µg/L (± standard deviation). The acute toxicity of the individual pesticides, expressed as an average nominal LC50 value and the standard error (in parenthesis) for chlorpyrifos, profenofos and endosulfan are summarised in Table 1. Chlorpyrifos and profenofos, organophosphorus pesticides (OPs), exhibited comparable toxicity to *C. dubia* with average LC50 values that ranged from 0.032 to 0.072 µg/L and 0.027 to 0.061 µg/L respectively. Previous studies have indicated that OPs are highly toxic to aquatic invertebrates (Bailey et al. 1997; Forget et al. 1999). In contrast, endosulfan, an organochlorine pesticide (OC), was significantly less toxic to *C. dubia* with a LC50 value ranging from 35.6 to 79.8 µg/L which was 1100 and 1300 times less toxic than chlorpyrifos and profenofos respectively.

Based on the LC50 values estimated from the individual pesticide and pesticide mixture exposure tests (Table 1), the values were converted to TUs as shown in Figure 1. For the binary mixture of chlorpyrifos and profenofos the average estimated TU was 0.25 (± 0.06). This value was more than three times less than the hypothetical value of 1 TU (which assumes additive effects), denoting that the two pesticides exhibited synergistic behaviour when present together. Pesticides that have a common site of action will be at least additive in their combined effects (concentration addition). Consequently, each component in the mixture acts like a dilution of the other and can be replaced by the other without changing the overall toxicity (Altenburger et al. 2000). Given this, additivity between chlorpyrifos and profenofos is reasonable, as both these compounds are metabolically activated OPs and act similarly with respect to the binding with the target site, acetylcholinesterase (Bailey et al. 1997). Forget et al. (1999) reported

synergistic lethal effects of two OPs, dichlorvos and malathion (TU=0.007) to the marine microcrustacean *Tigriopus brevicornis*. Whereas, a study by Bailey et al. (1997) testing the interactive toxicity of two OPs, chlorpyrifos and diazinon, to *C. dubia* suggest that this combination was additive with respect to acute toxicity (TU=1.13).

Table 1. Average acute median lethal concentration (LC50) estimates (μg/L) for chlorpyrifos, profenofos and endosulfan when present individually and in binary and ternary mixtures.

Exposure	Pesticide	LC50 and 95% Confidence Interval (μg/L)
Individual exposure	Chlorpyrifos	0.048 (0.032, 0.072)
	Profenofos	0.041 (0.027, 0.061)
	Endosulfan	53.3 (35.60, 79.80)
Combination exposures	Chlorpyrifos + Profenofos	0.0059 (0.0039, 0.0088) 0.0050 (0.003, 0.007)
	Chlorpyrifos + Endosulfan	0.0067 (0.0045, 0.01) 7.48 (5.00, 11.20)
	Profenofos + Endosulfan	0.033 (0.022, 0.049) 43.24 (28.89, 64.72)
	Chlorpyrifos + Profenofos + Endosulfan	0.0037 (0.0025, 0.0055) 0.0034 (0.0023, 0.0051) 4.30 (2.90, 6.40)

The confidence intervals were derived from the between trial estimate of the variance, and used a *t* value of 12 degrees of freedom (n=3).

There are two currently recognised mechanisms that may increase the toxicity of a pesticide when present in a mixture. Firstly, the presence of one compound may cause an increase in the rate of enzymatic activation of the other compound. Secondly, inhibition of enzymes that are responsible for detoxification will result in compounds remaining in their toxic form and more available to interact with target sites (Johnston et al. 1994). Chlorpyrifos and profenofos require metabolic conversion from the parent compound (P-S form) to their corresponding oxygen analogs (P-O form) to become potent inhibitors in cholinesterase and hence cause toxicity. This is achieved through oxidative and hydrolytic pathways by the cytochrome P450 system (Chambers and Carr 1995). Given this, the presence of both these chemicals may cause one chemical to increase the rate of activation to its active metabolite or prevent the detoxification of the active metabolite (Pape-Lindstrom and Lydy 1997). Both these processes would result in an enhanced level of active compound to interact with the target site and cause increased toxicity. Although the individual components in a mixture have same primary toxic mechanisms, they may still have secondary effects that contribute to the overall toxicity of a compound. It is unlikely that the toxic action is limited to a

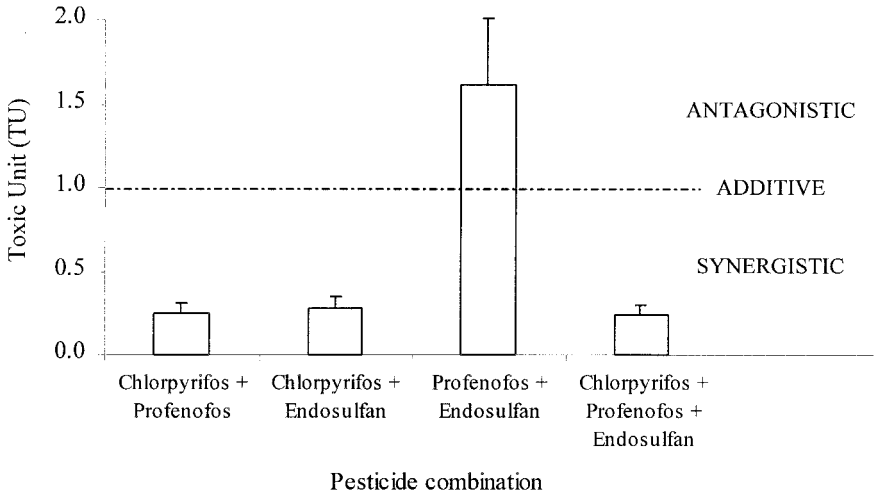

Figure 1. Calculated Toxic Unit (TU) values for *Ceriodaphnia dubia* exposed to binary and ternary combinations of chlorpyrifos, profenofos and endosulfan. Each bar represents the mean of three replicates (± SEM).

specific mode of action or specific receptor, therefore this may lead to overall enhanced toxicity (Forget et al. 1999).

The binary combination of chlorpyrifos and endosulfan and the ternary combination of chlorpyrifos, profenofos and endosulfan also produced synergistic effects to *C. dubia* (Figure 1). The TU for these two combinations were 0.28 (± 0.06) and 0.24 (± 0.05) respectively. A study by Johnston et al. (1994) assessed the interactive effects between ergosterol–biosynthesis-inhibiting (EBI) fungicides (prochloraz, propiconazole and penconazole and OP pesticides dimethoate, chlorpyrifos and diazinon in the hybrid red-legged partridge. This study found that pre-treatment of the red-legged partridge with an EBI fungicide followed by exposure to an OP resulted in overall enhanced toxicity. The reasoning suggested by the authors was due to the EBI fungicide inducing forms of cytochrome P450 responsible for the activation of the OP and hence resulted in enhanced toxicity. This may also be the case in the current study where increased toxicity was observed in three of the four pesticide combinations. However, further investigation into cytochrome P450 activation needs to be addressed to confirm this theory.

The binary combination of profenofos and endosulfan exhibited less than additive behaviour (antagonism) with a TU=1.83, nearly twice that predicted by additivity. Therefore, the overall toxicity of this combination equals the toxicity of either of its counterparts. Profenofos and endosulfan exert their toxic effects through different mechanisms. Therefore it would be expected that as they act on different

biological systems or different aspects of the same system, the presence of one compound might interfere with the activity of the other. Previous studies with pesticide mixtures have also reported less than additive effects. Pape-Lindstrom and Lydy (1997) reported less than additive effects in *Chironomus tentans* exposed to pesticides with different modes of action, methyl-parathion (OP) and atrazine (herbicide). According to Marking (1985) there are four main types of antagonism: functional, chemical, dispositional and receptor antagonism. Functional antagonism occurs when two chemicals counter balance one another by eliciting opposite effects on the same physiological function. Chemical antagonism is a chemical reaction between two chemicals to produce a less toxic product. Dispositional antagonism occurs when the absorption, biotransformation, distribution, or excretion of a chemical is changed so that the concentration or duration of the chemical at the target site is decreased. Receptor antagonism occurs when two chemicals that bind to the same receptor site produce less of an effect when together than the sum of their individual effects, or when one chemical antagonises the effect of the second. The antagonistic effects of the binary combination of profenofos and endosulfan may be due to functional, dispositional or chemical antagonism. Further research is needed on the metabolism of these two pesticides when present in a mixture to determine which is the case here.

Combinations that result in greater than additive toxicity are of greatest concern in ecotoxicology as the toxicity cannot be predicted based on the effect of the individual components in the mixture. This was highlighted in the current study as the two combinations of pesticides with one OP and one OC (chlropyrifos + endosulfan and profenofos + endosulfan) resulted in completely different toxicities when present in combination. Therefore, caution needs to be taken when predicting combination toxicity based on chemical class, as it is not always truly indicative of the actual toxicity that may occur.

More emphasis on the biochemical interactions responsible for the synergistic responses observed in mixtures of pesticides needs to be addressed. Measuring actual enzyme activity of those enzymes involved in the activation and detoxification processes (cytochrome P450s) and the target enzymes (acetylcholinesterase in the case of OPs) would be a valuable tool to determine why enhanced toxicity actually occurs when organisms are exposed to more than one pesticide. In the present study, the effects of binary and ternary combinations of chlorpyrifos, profenofos and endosulfan was addressed using an invertebrate model *C. dubia*. Future work determining the effects of pesticide combinations to non-target organisms with higher levels of organisation such aquatic vertebrates (fish and frogs) is needed. The target site for most pesticides is the nervous system which is more advanced in higher organisms therefore the effect of pesticides and combinations in an invertebrate may not be truly representative of the effects that may occur in higher organisms. Therefore, it is important to assess these effects in more advanced systems.

Acknowledgments. This study was supported by the Australian Technology Network Small Research Grants. The authors would like to thank Dr Quanxi Shao for his valuable comments.

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