Comparative evaluation of the effects of pesticides in acute toxicity luminescence bioassays

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Abstract

Acute toxicity of pesticides in water was assessed singly and in mixtures using the responses of the luminescent bacterium *Vibrio fischeri* (BioToxTM), the aquatic invertebrate *Daphnia magna* (DaphtoxkitTM), and the MitoScanTM assay. The latter utilized fragmented mitochondria to enzymatically convert β -nicotinamide adenine dinucleotide (NADH) to its oxidized form, NAD⁺. The rate of the conversion being sensitive to type and concentration of toxicants. The pesticides tested were Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate), Cyromazine (*N*-cyclopropyl-1,3,5-triazine-2,4,6-triamine), Fenamiphos (ethyl 4-methylthio-*m*-tolyl isopropylphosphoramidate), and Formetanate (3-dimethylaminomethyleneiminophenyl methylcarbamate). The toxicity bioassays were characterized in terms of relative sensitivity, reproducibility, range of the linear response, and the ability to reveal synergistic/antagonistic interactions among toxicants. The *D. magna* assay was the most sensitive and best able to detect toxic interactions of mixtures. Also, unlike the other assays used, the response of the daphnid system was linear over a 10-fold change in pesticide concentration. Relative to the BioToxTM, the MitoScanTM was 2- to 11-fold more sensitive for the compounds and mixtures tested. The EC₅₀ reproducibility of all tests was within $\pm 20\%$ coefficient of variation; however, the lowest observable effect concentration (LOEC) were only reproducible to $\pm 35\%$ on average. Cyromazine was the least toxic of the pesticides tested. To test the predictive value of the concept of concentration addition, toxicities of binary and quaternary mixtures of four different pesticides were analyzed. Synergistic/antagonistic responses were most frequently observed in testing with *D. magna*. Synergistic/antagonistic effects were seen only in 25 and 50% of the cases with the BioToxTM and the MitoScanTM assays, respectively. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: *Vibrio fischeri*; *Daphnia magna*; MitoScanTM; Carbofuran; Cyromazine; Fenamiphos; Formetanate; Mixtures; EC₅₀; LOEC; Toxicity

1. Introduction

Hundreds of new chemicals are introduced into commerce every year and may eventually end up in the environment. Many of these new and existing

compounds are toxic to the biosphere. Compounds that are deliberately released into the environment in large amounts are of special concern, because of the nature of their application. Pesticides used in agriculture are examples of such compounds. Pesticides are necessarily toxic, at least to a part of the biosphere, as a requirement of their function; however, a broader ecotoxicity of a compound and its persistence are

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matters of extreme public concern. Pesticides are commonly encountered singly and as mixtures in drinking water, rivers, lakes and other aquatic bodies. In such instances of contamination, the quantification of the concentration of the contaminant(s) is plainly insufficient unless the concentration can be satisfactorily related to the toxicity. In addition to having an impact on the discharge regulations, the toxicity considerations are important because a compound's toxicity can affect its rate of degradation in natural and manmade waste treatment processes [1].

The toxicity of pesticide contaminated effluent depends on the amounts and types of the individual pesticides present; however, even for pure compounds, concentration–toxicity relationships are complex. Mixtures of compounds may pose bigger problems because the toxicity of a mixture is not easily linked to individual toxicities of components in the mixture; synergistic and antagonistic response are known to occur [2–4]. Thus, for predicting the impact of a wastewater stream on the ecology of a receiving body such as a biological wastewater treatment facility, or a lake, the toxicity of the contaminated water needs to be determined with single and multiple contaminants and with a selection of assay methods. Multiple bioassays are necessary because no single method can reveal the possible interactions of a toxicant with the great variety of biochemistries of living systems. So far, toxicity of compounds has been assessed as single compounds because of a lack of standardized methods for assessing synergistic toxicity [5]; nevertheless, the existence of synergistic effects is well-known [5–9] and there is a clear need to quantify the aggregate toxic impact of pesticide mixtures. For example, a recent report of the US Geological Survey on the quality of the aquatic environment emphasized the need to consider synergistic effects of chemical mixtures on aquatic toxicity [7].

The subject of whole effluent (i.e. one containing a mix of chemicals) toxicity testing has been reviewed recently [7]. Whole effluent toxicity (WET) testing is useful for assessing the potential impact of the effluent on the biotic environment; however, the WET methods are subject to inherent variability, species differences, and response modification caused by physicochemical and biological factors in the receiving environment [7]. Variability is higher for moderately toxic effluent samples than those with low

or high toxicity [7]. With regards to species, genetic differences between different populations are known to affect the toxic response. Also, differences in diet, temperature and water quality can alter the response of daphnids and other organisms used in testing [7].

Except over a narrow range of concentrations, the toxicity response plots are generally nonlinear. Nontraditional concentration response curves in which a small amount of a toxicant actually produces a beneficial response (a phenomenon known as hormesis) have been demonstrated [7]. Although, the so called beneficial response to a toxic substance is really an upset of the system relative to the control and any upset whether beneficial or not should be viewed as an unwanted abnormal effect on the ecosystem. Furthermore, the results of the laboratory toxicity tests do not necessarily correlate with the impact that an effluent has on the biosphere; nevertheless, such tests do provide an indication of the relative hazard posed by a discharge. Inferring risk from hazard is certainly not always correct [7] but is a safer approach to establish the effluent discharge guidelines.

This study presents a detailed analytical assessment of several bioassays for toxicity. Identified are important factors such as the range of linear response of the assay, the relative sensitivity, reproducibility, and the ability to discern synergistic and/or antagonistic effects of pesticide mixtures. Three rapid and relatively inexpensive toxicity bioassays were used: (i) the luminescent bacterium *Vibrio fischeri*; (ii) the aquatic invertebrate *Daphnia magna* is a preferred test system for aquatic toxicology [10]; and (iii) the MitoScanTM mitochondria-derived assay [11,12]. The toxicity end-point (EC₅₀) for each bioassays was determined as the concentration of a test sample that causes a 50% reduction in the biological response studied. Acute toxicity was evaluated of water samples contaminated with various amounts of the following pesticides: Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate), Cyromazine (*N*-cyclopropyl-1,3,5-triazine-2,4,6-triamine), Fenamiphos (ethyl 4-methylthio-*m*-tolyl isopropylphosphoramidate), and Formetanate (3-dimethylaminomethyleneiminophenyl methylcarbamate). These compounds are commonly used in agricultural activities as systemic insecticide, acaricide and nematicides. Formetanate, Fenamiphos and Carbofuran are cholinesterase inhibitors and Cyromazine interferes in the growth of insects.

2. Materials and methods

The following pesticides were tested singly and in mixtures: Carbofuran, Cyromazine, Fenamiphos, and Formetanate (Fig. 1). Pure pesticides (>98.0% pure) were purchased from Riedel-de-Haën (Seelze, Germany). Stock solutions of the pesticides and mixtures were prepared in distilled water for *V. fischeri* and MitoScanTM assays and in culturing medium for *D. magna* assays. Mixtures of pesticides were prepared by combining various stock solutions of pure components in 1:1 ratio. When using pesticide mixtures, the concentration of the toxicant was defined as the sum of concentrations of all the pesticides in solution. In those cases, where the compounds had low solubility in water, 0.5% v/v of dimethyl sulfoxide (DMSO) was used to facilitate dissolution. Pesticide-free aqueous solutions of DMSO (0.5% v/v) exhibited no observable toxic response in the bioassays used. For *V. fischeri* assay, the osmolality of solutions was adjusted to 2% NaCl for its optimal performance.

2.1. Toxicity analyses

2.1.1. Bacterial bioluminescence assay

A commercial assay marketed as BioToxTM (Bio-Orbit Oy, Turku, Finland) was used. The assay, based on the luminescent bacterium *V. fischeri* NRRL B-1117, is in principle similar to the better known Microtox[®] test [13–15]. The effect of test compounds on the luminescent bacterium *V. fischeri* was

evaluated at 5, 15 and 30 min of exposition determining the EC₅₀. The bacteria were purchased as freeze-dried reagents. They were stored at –20 °C and rehydrated prior to testing. Light emitted from bacterium is a result of the interaction of the enzyme luciferase, reduced flavin, and a long-chain aldehyde in the presence of oxygen. The metabolic energy generated in this pathway is converted to chemical energy, through the electron transport system, into visible light. This metabolic pathway is intrinsically linked to cellular respiration, so disruption of normal cellular metabolism causes a decrease in light production. The toxicity end-point (EC₅₀) was determined as the concentration of a test sample that causes a 50% reduction in light output. The acute bioluminescence assay was carried out according to ISO 11348 [16]. Light production from luminescent bacteria was measured with a photomultiplier in a luminometer equipped with a constant temperature water bath (15 °C).

In addition to the EC₅₀ value, the lowest observable effect concentration (LOEC) was also determined. The LOEC is analogous to the “limit of detection” of the conventional methods of analysis. In keeping with the established practice, the LOEC was obtained as follows:

$$\text{LOEC} = \frac{3S}{m} \quad (1)$$

where S and m were the standard error in the intercept and the slope of the linear response–dose plot, respectively.

Carbofuran

Cyromazine

Fenamiphos

Formetanate

Fig. 1. Structures of pesticides analyzed by toxicity bioassays: Carbofuran, Cyromazine, Fenamiphos and Formetanate.

2.2. *Daphnid motility assay*

The toxicity of the compounds to the crustacean species (*D. magna*) was assessed using commercially available DaphtoxkitTM (Creasel, Belgium). The toxicity studies were performed in accordance with testing conditions prescribed by OECD Guideline 202 and ISO 6341 [17]. Acute toxicity was assessed by noting the effects of the test compounds on the motility of *D. magna*. This bioassay using dormant eggs (ephippia) treated as described in the standard operational procedure to induce hatching and the experiments was carried out on less than 24-h-old daphnids. The ephippia were incubated in standard freshwater at $21 \pm 1^\circ\text{C}$ under continuous illumination of 6000 lx. Between hatching and the test, the daphnids were fed on the dried cyanobacterium *Spirulina*. For the test, the animals were incubated for various periods with the specified concentrations of pesticides. All the test were conducted in the dark at a constant temperature of $20 \pm 1^\circ\text{C}$. The neonates are considered immobilized, after 24 and 48 h of incubation, if they lay on the bottom of the multi-well test plate and do not resume swimming within 15 s of observation. The toxicity end-point (EC_{50}) was determined as the concentration estimated to immobilize 50% of the daphnids after 24 and 48 h of exposure.

For the daphnid test, the LOEC was determined as the concentration of a test sample that produced a 10% change in the biological response studied (motility).

2.3. MitoScanTM mitochondrial assay

MitoScanTM toxicity assay (BioRenewal Technologies, Madison, WI, USA) utilizes fragmented mitochondria or submitochondrial particles (SMP) that are functionally equivalent to the whole organelle. SMPs prepared from bovine heart mitochondria were stored frozen (-80°C) until use.

The test monitored the appearance of NAD^+ via oxidation of β -nicotinamide adenine dinucleotide (NADH). The rate of appearance of NAD^+ was reduced relative to controls when an interfering toxicant was present. The test was carried out in 96-well microplates using a volume of 300 μl per well. The final concentrations in the test medium were: 50 mM HEPES buffer, pH 7.5, 6 mM MgSO_4 , and 0.017 mg/ml SMP protein. The reaction was initiated

by adding 0.137 mM of NADH. The appearance of NAD^+ with time was monitored spectrophotometrically at a wavelength of 340 nm (Labsystems Multiskan RC microplate reader). For each test, vial containing a given dilution of the toxicant, the appearance of NAD^+ was monitored. A least-square fit of the linear portion of the data provided the slope, i.e. the rate of generation of NAD^+ . The rates obtained in the various vials were plotted against the concentration of the pesticide to obtain a dose–response curve.

3. Results and discussion

The toxicity end-point was determined as EC_{50} , i.e. the effective concentration required for a 50% change in the biological response studied (bioluminescence, motility, enzyme activity). The pesticides used covered different classes of compounds with different modes of pesticide action, thus the various toxicity assays could be assessed for their abilities to respond to a range of possible interactions of the toxicant with the assay system. The assays used were relatively rapid and simple, yet covered a broad range of life biochemistries, including two whole organism assays (BioToxTM and DaphtoxkitTM) and a multi-enzyme assay based on fragmented mitochondria. The organisms used included a prokaryote (*V. fischeri*) and a multi-cellular eukaryote, *D. magna*. The latter was biochemically the most complex test system used and was also found to be the most sensitive.

The EC_{50} values obtained for various compounds, mixtures, and assays are noted in Table 1 which also lists the coefficient of variation for the measurements. In both the BioToxTM and the daphnid tests, the EC_{50} values declined with increasing duration of exposure (5, 15, 30 min for *V. fischeri*; 24 and 48 h for *D. magna*) to pesticides. This is understandable, as a larger amount of a toxicant is needed to produce the same response over a shorter incubation period. The *D. magna* test was by far the most sensitive; the EC_{50} values obtained with this test were in the parts per billion range, except for the relatively nontoxic Cyromazine. *D. magna* was sensitive to 0.1 ppb total pesticide concentration in quaternary mixtures. The EC_{50} values determined with the BioToxTM and the MitoScanTM assays were in the milligram per liter range; the MitoScanTM test was 2- to 11-fold more

Table 1

EC₅₀ values (mg/l CV%) for single pesticide and mixtures of pesticides analyzed by toxicity bioassays for different exposure times

Pesticides and mixtures	BioTox TM			MitoScan TM	DaphtoxKit TM	
	5 (min)	15 (min)	30 (min)	40 (min)	24 (h)	48 (h)
Formetanate	18.8 (33)	7.4 (37)	4.8 (40)	3.3 (6)	0.1 (22)	0.077 (34)
Carbofuran	33.3 (5)	31.2 (7)	28.9 (17)	7.6 (30)	0.096 (16)	0.018 (20)
Cyromazine	None	None	None	26.1 (26)	None	10.7 (20)
Fenamiphos	38.4 (14)	35.1 (22)	33.2 (22)	2.9 (15)	0.025 (15)	0.005 (5)
Formetanate–Carbofuran	31.1 (14)	16.7 (18)	11.4 (20)	3.6 (6)	0.1 (18)	0.03 (20)
Formetanate–Fenamiphos	27.7 (20)	11.9 (17)	8.7 (20)	9.5 (30)	0.09 (18)	0.001 (30)
Carbofuran–Cyromazine	61.3 (23)	50.8 (13)	46.5 (26)	5.6 (20)	0.05 (18)	0.005 (20)
Formetanate–Carbofuran– Cyromazine–Fenamiphos	22.3 (12)	7.4 (25)	4.4 (20)	22.8 (14)	0.01 (18)	1 × 10 ^{−5} (10)

sensitive than the BioToxTM for the compounds and mixtures tested.

For pure compounds in the BioToxTM test, toxicity followed the order, Cyromazine, Fenamiphos, Carbofuran, and Formetanate, irrespective of the duration of the exposure. This pattern was different for the other assays used; however, in all the cases Cyromazine proved to be the least toxic of the pesticides tested. The EC₅₀ values for this compound were 10.7 and 26.1 mg/l with the daphnid and the MitoScanTM assays, respectively. No toxic effect was detected for Cyromazine with the BioToxTM test. In fact, the light emission was measurably enhanced during contact with Cyromazine, possibly because it was being consumed as a nutrient by the cells [9]. Because the highest Cyromazine concentration in the incubation vial was 50 mg/l, it may be concluded that the BioToxTM EC₅₀ value for Cyromazine exceeds 50 mg/l.

The LOEC data are shown in Table 2. As expected, the EC₅₀ values were always greater than the corresponding LOECs: 10- to 150-fold greater for the BioToxTM; 2- to 30-fold greater for the MitoScanTM; and 5- to 100-fold greater for the *D. magna* test. In addition to the EC₅₀ value, a consideration of the LOECs can be useful in establishing the effluent discharge limits. If the LOEC is much less than the EC₅₀, the prudent practice is to base the limit on the LOEC value. The EC₅₀ is merely an index of relative toxicity whereas the LOEC provides compound-specific data that is more directly relevant.

Compared to conventional chemical analyses, toxicity bioassays are much less reproducible. Variability within a factor of two is generally considered acceptable for toxicity results on a given effluent and using a given toxicity test [7]. In the present work, the EC₅₀ reproducibility was within ±20% (coefficient of

Table 2

LOEC values (mg/l CV%) and their coefficients of variation for single and mixtures pesticides analyzed by various toxicity bioassays for different exposure times

Pesticides and mixtures	BioTox TM			MitoScan TM	Daphtoxkit TM	
	5 (min)	15 (min)	30 (min)	40 (min)	24 (h)	48 (h)
Formetanate	1.4 (14)	1.2 (9)	1.2 (12)	0.86 (25)		0.01 (30)
Carbofuran	2.4 (30)	1.38 (21)	1.5 (16)	0.24 (30)	0.018 (30)	0.0018 (28)
Cyromazine	None	None	None	3 (25)	None	1.07 (30)
Fenamiphos	1.5 (35)	1.38 (35)	1.6 (30)	0.3 (26)	9 × 10 ^{−4} (30)	9 × 10 ^{−4} (30)
Formetanate–Carbofuran	1.4 (24)	1.7 (35)	1.7 (35)	1.7 (30)	0.01 (40)	0.01(40)
Formetanate–Fenamiphos	0.18 (30)	0.18 (30)	0.2 (21)	1.26 (30)	0.001 (40)	0.001(40)
Carbofuran–Cyromazine	0.47 (30)	0.21 (30)	0.26 (30)	3.2 (30)	0.001 (40)	0.001 (40)
Formetanate–Carbofuran– Cyromazine–Fenamiphos	0.31 (40)	0.23 (30)	0.25 (30)	10 (30)	1 × 10 ^{−4} (31)	1 × 10 ^{−5} (31)

variation) for all the tests (Table 1). The reproducibility data given are based on three replicates each for the BioToxTM and the MitoScanTM tests; the *D. magna* data are for four replicates. Generally the EC₅₀ value was more reproducible than the LOEC. The average reproducibility of the LOEC data was 27, 28, and 34% (coefficient of variation) for the BioToxTM, the MitoScanTM, and the *D. magna* assays. For the EC₅₀ data, the average reproducibility was 20, 18, and 19% for the BioToxTM, the MitoScanTM, and the *D. magna* measurements. The generally better than normal reproducibility is attributable to the fact that the ‘whole effluent’ used was relatively simple and clean (i.e. solution of one to four pure compounds in ultrapure water).

Except for the BioToxTM assay, the dose–response relationships were generally logarithmic both for the pure compounds and the mixtures tested. The linear response range was generally quite narrow for the BioToxTM and the MitoScanTM assays but at least 10-fold for the *D. magna* test. Some representative data on the linear ranges are given in Table 3 for various test compounds. The narrow range of linear response for the BioToxTM and the MitoScanTM suggest the involvement of multiple enzymes in determining the toxicity response of those tests. A larger range of linear response at extremely low concentrations of toxicants (generally < 1 mg/l) suggests that the response of *D. magna* is likely associated with one enzyme necessary for some critical metabolic function. Of course, the one enzyme being affected could be different for the different toxicants. The data in Table 3 were measured at the following range of concentrations: 1.50–50.00 mg/l for the BioToxTM

(except for cyromazine when the concentration range was 3–212 mg/l); 0.5–100.0 mg/l for the MitoScanTM (1–10 mg/l when Cyromazine was used); and 0.1 ppb to 1 mg/l for *D. magna* (1–10 mg/l when Cyromazine was used).

3.1. Toxicity of pesticide mixtures

No method exists for relating the toxicities of individual components of a mixture to the mixture’s toxicity, except when synergistic and antagonistic effects are absent. In the latter event, when compounds act independently, the toxicities are sometimes additive. When this occurs, a mixture’s toxicity may be estimated as a weighted sum of toxicities of the individual components present [8,16]. Another approach to estimating the mixture toxicity when the components are noninteracting is the use of toxicity units. A toxicity unit is the concentration of a component *i* divided by its EC₅₀ value; thus,

$$TU_i = \frac{C_i}{EC_{50_i}} \quad (2)$$

The mixture toxicity is then given by

$$TU_M = \sum_{i=1}^n TU_i \quad (3)$$

If the measured toxicity is greater or less than the predicted value, synergistic and antagonistic interactions among components are revealed; however, it is impossible to say a priori whether the TU_M calculated with Eq. (3) will be equal to, greater than, or less than the measured toxicity of the mixture. The toxicity unit values of the mixtures and components were calculated and compared with the measured mixture toxicity to reveal the interactions shown in Table 4. Note that TU_i and EC₅₀ are not comparable quantities as one is dimensionless and the other is a concentration. Similarly, the toxicity units of a mixture cannot be compared to the EC₅₀ value for the mixture. As shown in Table 4, synergistic/antagonistic responses were most frequently observed in testing with *D. magna*; in fact all mixtures tested produced such responses with this test. With the same mixtures, synergistic/antagonistic effects were seen only in 25 and 50% of the cases with the BioToxTM and the MitoScanTM assays, respectively.

Table 3

Typical linear response range of the pesticides analyzed by toxicity bioassays

Pesticides	Linear response range (mg/l)		
	BioTox TM	MitoScan TM	Daphtokit TM
Formetanate	1.60–25.75	0.50–4.10	0.10–1.00
Fenamiphos	6.50–52.00	0.50–2.20	0.096–0.96
Carbofuran	4.00–35.25	0.70–3.10	0.018–0.18
Cyromazine	Non toxic	1.06–4.60	1.07–10.07
Quaternary mixture	6.40–25.60	2.05–8.90	0.001–0.010

Table 4
Interactions of toxicity response

Mixture of pesticides	Bioassays		
	BioTox TM	MitoScan TM	Daphtoxkit TM
Formetanate–Carbofuran	Additive	Additive	Antagonistic
Formetanate–Fenamiphos	Additive	Antagonistic	Synergistic ^a
Carbofuran–Cyromazine	Additive	Additive	Synergistic ^a
Formetanate–Carbofuran– Cyromazine–Fenamiphos	Synergistic	Antagonistic	Synergistic

^a The observed behavior depended on the duration of exposure (24 or 48 h); the response shown is for 48 h exposure. For 24 h exposure, the Formetanate–Fenamiphos response was antagonistic and the Carbofuran–Cyromazine response was additive.

As noted above, no methods are currently available for predicting the toxicity response generated by a mixture of compounds from toxicity data for individual components of the mixture; however, predictive methods can be developed, at least in principle. Attaining a predictive capability requires a detailed knowledge of the kinetics of all essential metabolic reactions of a given biosystem and a quantitative understanding of how the individual toxicants interact with the various metabolic enzymes and intermediates. An understanding of the mutual chemical interaction of the compounds irrespective of the biosystems is also necessary. The metabolic network could then be modeled mathematically and such models could be used to simulate the effects of any combination of type, number, and quantity of toxicants on the response of the biosystem. Considering the already available capacity for modeling complex networks (e.g. ecosystems, the global environment, and indeed many aspects of metabolism), metabolic modeling for predicting the toxicity response is undoubtedly feasible at least for the simpler systems and the known toxicants.

4. Conclusions

Toxicity is a biological response and this needs to be taken into account in selection and usage of toxicity assays. As documented here, different pesticides in mixtures interact to produce synergistic and antagonistic effects on toxic response. Of the three toxicity assays evaluated, the *D. magna* is clearly the best in discerning a toxic effect. This assay is by far the most sensitive and it is good at detecting toxicity synergisms. The EC₅₀ reproducibility of the *D. magna*

assay is comparable to that of the other tests examined. Also, the daphnid test has a significantly broader range of linear response than the other assays. In comparison with the BioToxTM and the MitoScanTM, the *D. magna* assay is cumbersome and slow; however, it requires minimal initial investment, operating expense, and technology. Furthermore, the toxicity data obtained with a relatively complex aquatic species such as *D. magna* perhaps provide a more meaningful indication of aquatic toxicity of a compound than the other tests used. Toxicity testing has a clear role in safeguarding environmental quality but a considered selection of testing methods is essential for obtaining results that are relevant. Effluent discharge criteria need to consider not only the EC₅₀ values of pure compounds, but also the data for whole effluent (i.e. synergistic effects) and the LOEC of potential pollutants.

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