

Toxicity of pesticides in wastewater: a comparative assessment of rapid bioassays

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Abstract

Acute toxicity of pesticides in water was assessed singly and in mixtures using various responses of the luminescent bacterium *Vibrio fischeri*, the aquatic invertebrate *Daphnia magna*, and the MitoScanTM assay. The latter utilized fragmented mitochondria to enzymatically convert oxidized β -nicotinamide adenine dinucleotide (NAD⁺) to its reduced form, NADH. The rate of the conversion being sensitive to the type and concentration of toxicants. The pesticides tested were carbofuran, cyromazine, fenamiphos, formetanate, and propamocarb. The aqueous solubility of all compounds exceeded 320 mg l⁻¹. All the toxicity bioassays were characterized in terms of relative sensitivity and complementarity. Synergistic and antagonistic toxicity effects were observed with pesticide cocktails relative to pure compound toxicities. The *D. magna* assay was the most sensitive and best able to detect toxic interactions of mixtures. Cyromazine was the least toxic of the pesticides tested. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Carbofuran; Cyromazine; *Daphnia magna*; Fenamiphos; Formetanate; MitoScanTM; Pesticides; Propamocarb; Toxicity; *Vibrio fischeri*

1. Introduction

Many tones of pesticides are used annually in agriculture and horticulture. Consequently, wastewater from greenhouses and runoff from agricultural land are nearly always contaminated with pesticides. Because of their toxicity, pesticides affect the ecology of the receiving bodies of water and contaminate drinking water supplies. Some pesticides bioaccumulate, affecting fish, birds, and other animals, and appearing in human food sources. Depending on the local cultivation practices, a waterbody may receive a

single pesticide or a varying cocktail of compounds. Toxicity of pesticides contaminated effluent depends on the amounts and types of the individual pesticides present; however, even for pure compounds, concentration–toxicity relationships are generally nonlinear. Cocktails of compounds pose bigger problems because toxicity of a mixture is not easily linked to individual toxicities of components in the mixture. 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. Few such data exist.

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An additional complicating factor is the absence of a precise definition of ‘toxicity’. Toxicity is a biological response. A variety of toxicity measurement systems exist, including those based on bacteria [1,2] and algae, animal cells, small mammals, fish fly, and zooplankton. Some of these systems, e.g., animals and fish larvae, are difficult to handle and they do not provide a rapid response. Also, use of some of these systems may raise ethical objections. Other systems, such as mammalian cells are expensive and not always consistent. In addition, the response of a single toxicity assay is an insufficient measure of the adverse biological impact of a compound in a generally diverse receiving ecosystem. Different toxicants act differently and not all life-forms are equally susceptible. Consequently, several assays need to be used simultaneously to assess toxicity adequately. Careful selection of assays is essential with regard to complementarity. This work provides such multifaceted toxicity data and a comparative evaluation of some toxicity bioassays. An attempt is made to correlate toxicities of mixtures to those of their constituents.

Acute toxicity was evaluated for water samples contaminated with various amounts of the following pesticides: carbofuran, cyromazine, fenamiphos, formetanate, and propamocarb. All these compounds are significant specially in greenhouse-based production of vegetables and fruits in southern Europe, particularly Spain. Pesticides were tested singly and as cocktails of two or more. Use of pesticide combinations ensured that any synergistic toxicity effects were identified. Three rapid and relatively inexpensive toxicity bioassays were used: (i) the luminescent bacterium *Vibrio fischeri* as being relevant to assessing a compound’s impact on the microbial ecology of biological wastewater treatment facilities; (ii) the aquatic invertebrate *Daphnia magna* (a microscopic shrimp), a preferred test system for aquatic toxicology [3]; and (iii) the MitoScan™ mitochondria-derived assay [4,5] for comparison. The toxicity was characterized as EC₅₀, i.e. the toxicant’s concentration causing a 50% change in the response parameter, calculated from the relevant dose–response curves. Use of multiple assays allowed a comprehensive and comparative assessment of the assay methods and provided data that could be potentially combined into a single weighted toxicity index which would

more realistically correlate the expected impact of the pollutants on a receiving ecosystem.

2. Materials and methods

Following pesticides were tested singly and in mixtures: carbofuran, cyromazine, fenamiphos, formetanate, and propamocarb (Fig. 1). The pesticides, purchased as pure compounds and not commercial formulations, were made into standard stock solutions by dissolving in ultrapure water that contained 5% (v/v) of methanol. The stock solutions were diluted with ultrapure water as needed. Pesticide mixtures were produced by combining various stock solutions of pure components. The water solubility of pesticides always exceeded 320 mg l⁻¹ (25°C). The amounts of pesticides in stock solutions and mixtures were confirmed by LC-MS. When using pesticide mixtures, the concentration of the toxicant was defined as the sum of concentrations of all the pesticides in the test vial or solution. Three toxicity assays were used as explained.

2.1. Toxicity analyses

2.1.1. *Vibrio fischeri* bioluminescence assay

A commercial assay marketed as BioTox™ (Bio-Orbit Oy, Turku, Finland) was used. The assay, based on the luminescent bacterium *V. fischeri* NRRL B-1117, is similar in principle to the better known Microtox® test that uses the marine bacterium *Photobacterium phosphoreum* [1,2,6–8]. Bioluminescence of *V. fischeri* was inhibited by toxicants. To monitor the inhibition, different dilutions of the test sample were mixed with the bacterial suspension made in 2% (w/v) aqueous sodium chloride. The decline in light emission was measured after contact periods of 5 and 15 min in separate experiments. The test was carried out at 15±1°C.

The inhibition effect of the dilutions was compared with a toxicant-free control to obtain percent inhibition (INH%); thus

$$\text{INH\%} = \left(1 - \frac{I_{S,x}}{\varphi I_{S,0}} \right) \times 100 \quad (1)$$

where $I_{S,0}$ and $I_{S,x}$ are the luminescence intensities of the sample initially and after contact time x min,

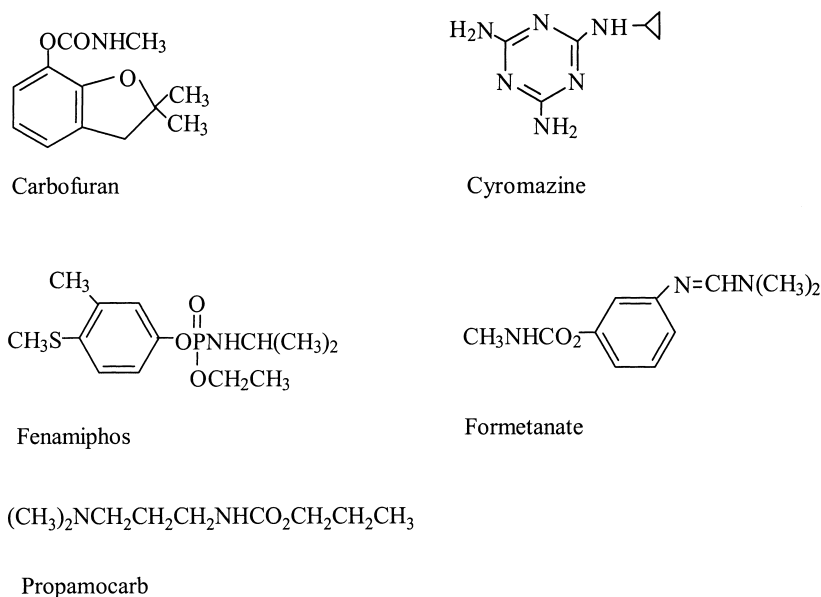


Fig. 1. Structures of pesticides.

respectively, and φ is a correction factor for the control. The latter is calculated as follows:

$$\varphi = \frac{I_{C,x}}{I_{C,0}} \quad (2)$$

where $I_{C,0}$ and $I_{C,x}$ are the luminescence intensities of the control initially and after contact time x min, respectively. Inhibition data were expressed in terms of the gamma function, i.e. the ratio of light lost to light remaining

$$\Gamma = \left(\frac{\varphi I_{S,0} - I_{S,x}}{I_{S,x}} \right) \quad (3)$$

Gamma is related to percent inhibition as follows:

$$\Gamma = \frac{\text{INH}\%}{100 - \text{INH}\%} \quad (4)$$

A double log plot of Γ versus concentration was used to calculate EC_{50} values, i.e. the concentration of toxicant where $\Gamma=1$.

Careful control of temperature was essential as noted, because light emission is sensitive to temperature. For example, for *P. phosphoreum* the light intensity changes by about 10% for every °C change in temperature [1]. The capabilities and limitations of

the BioTox™ assay are generally similar to those of the Microtox®. The latter is discussed by Ribo and Kaiser [1]. The positive and negative controls for the BioTox™ assay were a 26 ppm phenol solution and 2% (w/v) aqueous sodium chloride, respectively.

2.1.2. *Daphnia magna* motility assay

Daphnia magna is especially sensitive to dissolved metal ions and to organic toxicants. Acute toxicity was assessed by noting the effects of the various concentrations of pesticides on motility (swimming performance) of *D. magna*. The organisms were laboratory bred and less than 24 h old when tested. The animals were incubated for various periods with the specified concentrations of pesticides. Individuals that did not swim within 15 s of gentle agitation of the test container were considered nonmotile. The motility was observed under a magnifying lens. EC_{50} , the effective concentration of pesticides that reduced the motility to 50% of the animals after 24 or 48 h exposure, was calculated from data of motility versus concentrations of pesticides. The test temperature was $22 \pm 1^\circ\text{C}$. Animals in the normal culture medium without additives were used as a negative control. Potassium dichromate added at a concentration of $0.6\text{--}1.2 \text{ mg l}^{-1}$ was the

positive control and it killed all the animals. In some cases, the toxicity data were expressed as percent mortality defined as 100 (percent of motile animals).

2.1.3. MitoScanTM mitochondrial assay

MitoScanTM toxicity assay utilizes fragmented mitochondria or submitochondrial particles that are functionally equivalent to the whole organelle. Mitochondria, occurring in all eukaryotic cells, are essential to survival as they are the cell's principal energy generators. Mitochondrial energy generation from glucose and other compounds depends on concerted action of several dozen mitochondrial enzymes. Any toxicant that interferes with the action of one or more of these enzymes potentially disrupts the generation cascade and, hence, has a toxic effect. Because of the large array of enzyme needed for mitochondrial function, the likelihood that a toxicant will affect one or more is high and, consequently, mitochondrial biochemistry is responsive to a variety of toxicants. Mitochondrial enzymes serve a vital function. As a result, they have been conserved across species, largely unaltered by the evolutionary process. Thus, a compound that is toxic in a mitochondrial assay is likely to be broadly toxic to all kinds of eukaryotes, including higher animals. Compared to assays that measure a response of prokaryotic cells (e.g., MicrotoxTM), mitochondrial toxicity assays have better correlated with a substance's toxicity toward fish [4,5,9,10].

The test as used here monitors the appearance of NADH via reduction of NAD⁺. The rate of reaction and therefore the appearance of NADH are affected relative to controls when an interfering toxicant is present. The appearance of NADH is monitored spectrophotometrically (340 nm) as a function of time. Reduction of NAD⁺ requires a source of energy and electrons. These needs are met respectively by providing adenosine triphosphate (ATP) and succinate in the reaction mixture. In addition, antimycin A is added to prevent enzymatic oxidation of the generated β -nicotinamide adenine dinucleotide (NADH) back to the oxidized form of NADH (NAD⁺) through another existing pathway in mitochondrial fragments. Antimycin A also prevents electron siphoning to oxygen. In the MitoScanTM assay, the EC₅₀ value corresponds to a concentration of toxicant that causes a 50% inhibition of the enzyme activity.

For each test vial containing a given dilution of the toxicant, the appearance of NADH was monitored with time. A least-square fit of the linear portion of the data provided the slope, i.e. the rate of generation of NADH. The rates obtained in the various vials were plotted against the concentration of the pesticide to obtain a dose–response curve. The positive and negative controls were 15 ppm aqueous sodium azide and ultrapure water, respectively.

3. Results and discussion

A proper assessment of a compound's toxicity must necessarily use a battery of assays especially if the individual tests are based on simplified biochemistries or a narrow range of possible interactions of the toxicities with the test system. The latter would be the case with tests using purified enzymes and subcellular entities. Even whole organisms assays based on a single prokaryote, e.g. the *V. fischeri* BioToxTM and the *P. phosphoreum* based Microtox[®], are not sufficiently representative of possible toxicological impact of a compound at various levels of the ecosystem. Some simplified toxicity determinations may not satisfactorily detect synergistic and antagonistic phenomena. To cover a broad range of life biochemistries, while retaining simplicity and speed of analysis, this work used two whole organism assays and a multienzyme assay based on mitochondria, a subcellular organelle. The organisms used included the marine bacterium *V. fischeri*, a prokaryote, and *D. magna*, a multicellular eukaryote. *D. magna* was biochemically the most complex test system used and it was also the most sensitive. The pesticides used (Fig. 1) covered different classes of compounds with different modes of pesticidal action, thus the various toxicity assays could be assessed for ability to respond to a range of possible interactions of the toxicant with the assay system.

Fig. 2 shows representative curves of changes in BioToxTM light emission (expressed as INH%) with concentration of toxicant, a pure pesticide and various mixtures (binary, ternary, and quaternary). Light emission or bioluminescence declined and, correspondingly, percent inhibition increased with increasing concentration of toxicant (Fig. 2). The pattern of change was similar for the other compounds and mixtures tested. The EC₅₀ values were calculated from double

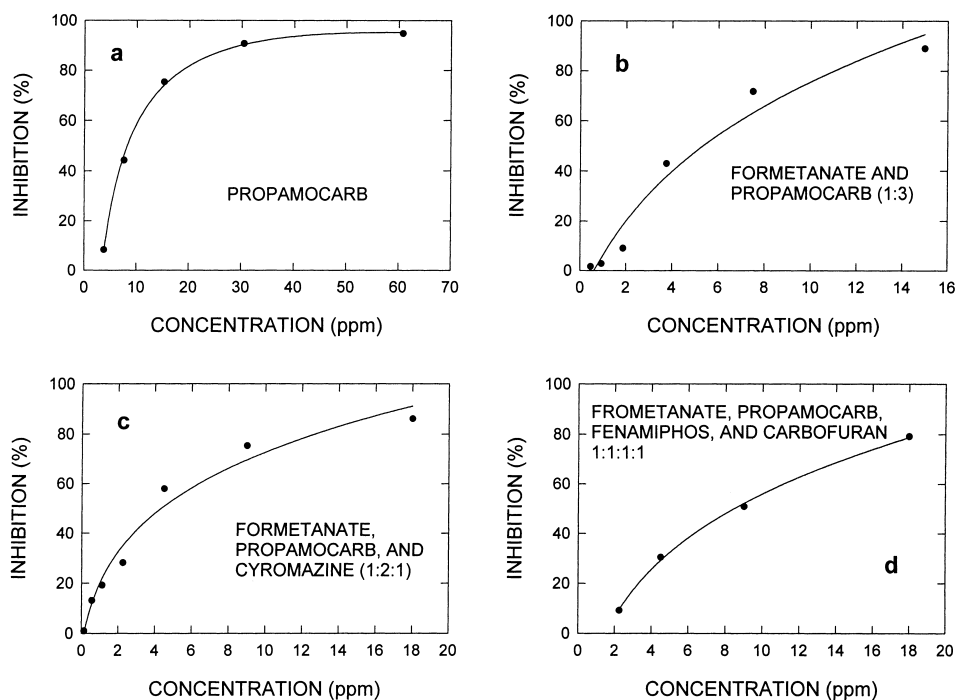


Fig. 2. Changes in *Vibrio fischeri* light emission (INH%) as a function of toxicant concentration for various constant incubation times: (a) pure propamocarb (5 min incubation); (b) formetanate and propamocarb 1:3 (5 min incubation); (c) formetanate, propamocarb, and cyromazine 1:2:1 (15 min incubation); and (d) formetanate, propamocarb, fenamiphos, and cyromazine 1:1:1:1 (5 min incubation).

log plots of the Γ function (Eq. (3)) versus concentration and these plots were linear as shown in Fig. 3 for some representative cases. The only exception was cyromazine. No toxic effect was observed for that compound with the BioToxTM test. Thus, as shown in Fig. 4, there was no loss of light emission with time. In fact, the light emission was measurably enhanced during contact with cyromazine, possibly because it was being consumed as a nutrient by the cells. Because the highest cyromazine concentration in the incubation vial was 50 ppm, it may be concluded that the EC₅₀ value for cyromazine exceeds 50 ppm.

Pure compound toxicities determined with the BioToxTM test are noted in Table 1, as EC₂₀, EC₅₀, and EC₈₀ values for 5 and 15 min incubation. Generally the EC_{xx} values for 5 min incubation are greater than for 15 min incubation (Table 1). This is sensible, as a larger amount of a toxicant is needed to produce the same response over a shorter incubation period.

The pattern of increasing toxicity with the BioToxTM was cyromazine, fenamiphos, carbofuran,

propamocarb, and formetanate. This pattern was consistent with that of the *Daphnia* assay (Table 2) where the order of increasing toxicity of compounds was cyromazine, formetanate, and carbofuran, using both 24 and 48 h variants of the test. However, the 48 h test period always showed a substantially higher toxicity of a compound than the 24 h test. This is expected, as a chronic toxicity effect probably comes into play over longer durations. The *D. magna* assay was much more sensitive than the BioToxTM (Tables 1 and 2). For example, the *D. magna* EC₅₀ (24 h incubation) for carbofuran was less than 0.1 ppm (Table 2) whereas the corresponding BioToxTM value (5 min incubation) was about a 100-fold greater (Table 1). Similarly, as previously noted, cyromazine up to 50 ppm failed to show a toxic response with the BioToxTM system, but the same compound was clearly toxic to *D. magna* at <25 ppm (24 h test) level (Table 2).

There is little published data on the BioToxTM test to allow a direct comparison with other work. For the *D. magna* assay, the measurements reported here were

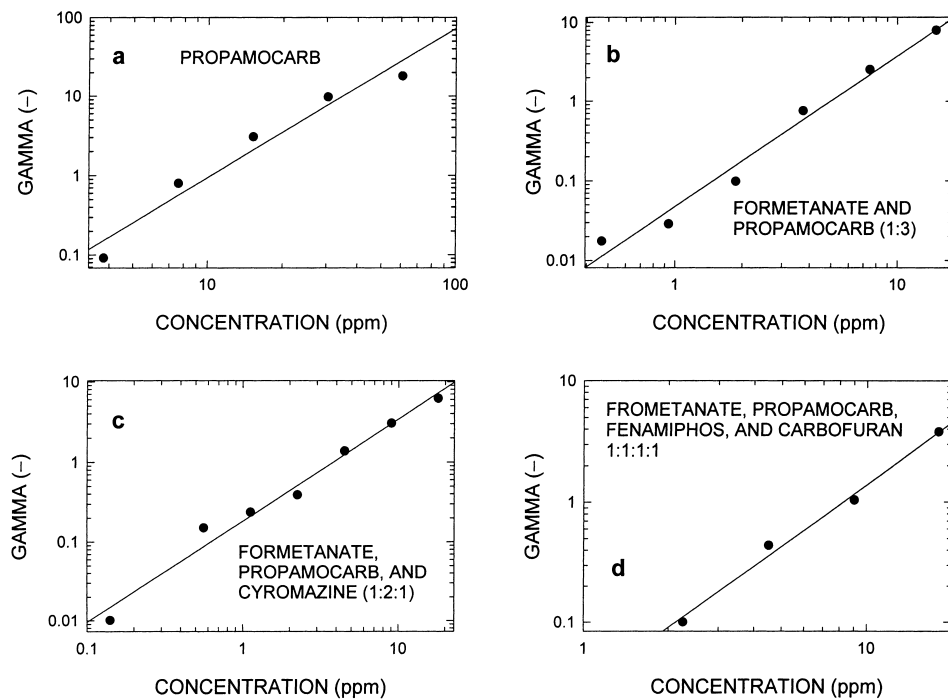


Fig. 3. Representative double log plots of Γ values vs. concentration for the BioTox™ assay: (a) pure propamocarb (5 min incubation); (b) formetanate and propamocarb 1:3 (5 min incubation); (c) formetanate, propamocarb, and cyromazine 1:2:1 (15 min incubation); and (d) formetanate, propamocarb, fenamiphos, and cyromazine 1:1:1:1 (5 min incubation).

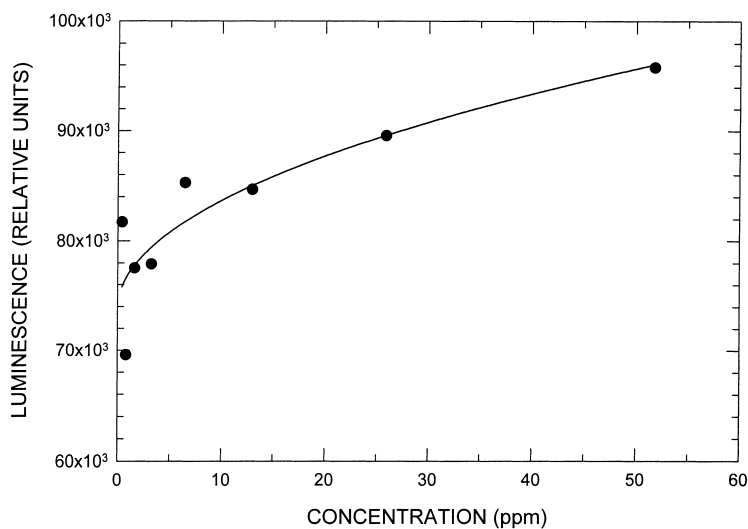


Fig. 4. BioTox™ light emission vs. cyromazine concentration (5 min incubation).

Table 1
BioTox™ toxicity of pure pesticides

Pesticide	EC ₂₀ (mg l ⁻¹)		EC ₅₀ (mg l ⁻¹)		EC ₅₀ (mg l ⁻¹)	
	Incubation time (min)		Incubation time (min)		Incubation time (min)	
	5	15	5	15	5	15
Carbofuran	2.4	2.8	9.8	9.6	41.5	39.7
Cyromazine	None	None	None	None	None	None
Fenamiphos	6.1	3.6	15.8	11.2	41.7	38.9
Formetanate	1.4	0.8	4.5	4.5	49.7	21.3
Propamocarb	3.9	7.8	8.5	8.2	27.0	14.1

generally consistent with similar values in the literature when available. For example, an EC₅₀ (48 h test) value of 9.1 ppm has been previously reported for cyromazine in the *D. magna* assay and this is consistent with 5.1 ppm observed in this work (Table 2).

Because the principle of the BioTox™ system is quite similar to that of the better known Microtox® test, and both assays use luminescent marine bacteria (although different species), the reproducibility and susceptibility to systematic error are likely to be similar for the two test systems. Compared to an EC₅₀ value of about 10 mg l⁻¹ for carbofuran in the BioTox™ test (Table 1), a significantly greater value of 203±43 mg l⁻¹ (15°C, 15 min incubation) has been reported [2] using the Microtox® system, suggesting that for carbofuran at least the BioTox™ method is more sensitive. The Microtox® value was for technical grade carbofuran. Carbofuran was the least toxic of the 10 pesticides tested [2]. For all pesticides, the EC₅₀ value was sensitive to the incubation temperature, declining as the temperature was reduced from 20 to 10°C [2]. The maximum change in EC₅₀ was ~60%. At a fixed temperature, the calculated EC₅₀ depended also on the length of incubation period.

Table 2
Toxicity of pure pesticides using the *Daphnia* assay

Pesticide	EC ₅₀ (mg l ⁻¹)	
	Incubation time	
	24 h test	48 h test
Carbofuran	0.092	0.03
Cyromazine	23.59	5.08
Formetanate	0.685	0.392

The EC₅₀ values were consistently lower for shorter incubations over the range of 5 to 30 min [2]. The latter observation differs with the behavior of the BioTox™ system, as noted earlier.

For carbofuran, EC₅₀ (15°C, 15 min) reported by Ruiz et al. [8] using the Microtox® assay was 24±2 mg l⁻¹, or about 12% of the value measured by Ghosh et al. [2] using the same procedure. The reproducibility of the Microtox® test is better than 25%, therefore the discrepancy is not explained by statistical variation alone. Ruiz et al. [8] used relatively pure (>97% pure) standards of pesticides whereas Ghosh et al. [2] used 'technical grade'. A lower toxicity for the poorer grade is surprising, but impurities can produce both synergistic and antagonistic changes to observed toxicity, as discussed in a later section of this paper. Other similar discrepancies in Microtox® EC₅₀ values (15°C, 5 min) are noted in Table 3 for several pesticides. Obviously, the Microtox® assay and, by analogy, other similar ones are susceptible to a lot of systematic variation. This could arise for example, the way the bacterial culture was prepared and the age of the lyophilized culture prior to use.

Table 3
Discrepancies in Microtox® EC₅₀ values (15°C, 5 min) for some pesticides

Pesticide	EC ₅₀ values (15°C, 5 min) (mg l ⁻¹)		
	Chang et al. [6]	Ruiz et al. [8]	Ghosh et al. [2]
Carbaryl	2	0.6	–
DDT	7	–	33±4
Diazinon	1.7	83.7	–
Malathion	10	33.6	–
Paraquat	780	2322.7	–

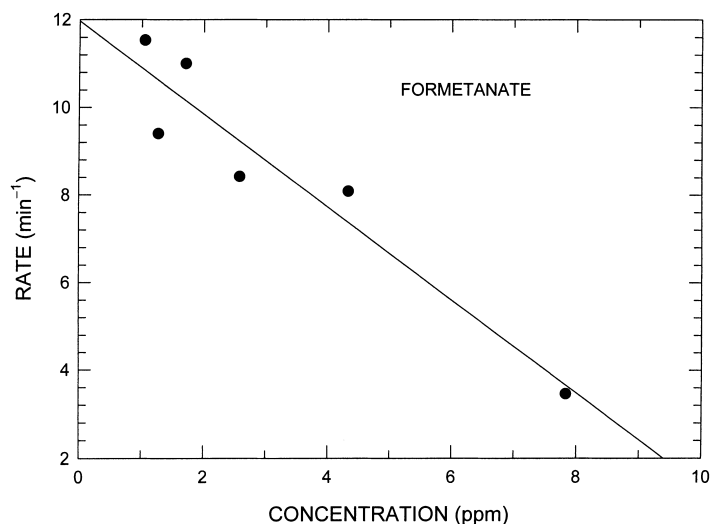


Fig. 5. NADH production rate vs. concentration of formetanate in a representative MitoScanTM assay. The EC₅₀ value was 5.3 ppm.

In contrast to Ghosh et al. [2], Ruiz et al. [8] reported for various pesticides generally higher EC₅₀ (15°C) values for the shorter incubation time (5 min) compared to values at 15 min incubation. This concurs with our observations for the BioToxTM pure compound test. As in our work (Table 1), Ruiz et al. [8] did not see a substantial effect of incubation period on the EC₅₀ value for carbofuran; thus, the values at 5 and 15 min incubation differed at most by about 16% [8].

In the MitoScanTM assay, the rate of appearance of NADH declined as the concentration of the toxicant was increased in separate vials. Consequently, the spectrophotometric absorption (340 nm) was lower in vials with greater amounts of pesticides. Plots of the rate versus concentration were linear, as shown in Fig. 5 for one representative case. From Fig. 5, the EC₅₀ value for formetanate was 5.3 ppm. The pure compound EC₅₀ values obtained with the MitoScanTM assay were generally consistent with those of the BioToxTM system, as noted in Table 4 where results of the three assays are summarized. The MitoScanTM assay was substantially less sensitive than the *D. magna* test system (Table 4).

Unless assays are selected to include biochemical variety and complexity, they may produce mutually consistent toxicity values but such values would be highly biased and could not be considered realistic for practical ecotoxicological purposes. For example,

one study with three bioassays and 10 pesticides noted that all three assays yielded the same relative order of toxicities of the compounds, even though the specific EC₅₀ values depended on the test system [2]. The rather similar results were not surprising: all three assays utilized bacteria; consequently, the biochemical diversity was lower than if widely different assay systems had been used.

3.1. Toxicities of mixtures

The BioToxTM toxicity data for various binary, ternary, and quaternary mixtures of pesticides are noted in Tables 5, 6 and 7, respectively. The ratios of toxicants' concentrations in mixtures listed in Tables 5–7 were based on mass concentrations. A

Table 4
Summary of the pure compound EC₅₀ values for the various test systems

Pesticide	EC ₅₀ (mg l ⁻¹)				
	<i>Daphnia magna</i>		BioTox TM		MitoScan TM
	24 h test	48 h test	5 min	15 min	
Carbofuran	0.092	0.03	9.8	9.6	7.6
Cyromazine	23.59	5.08	None	None	None
Formetanate	0.685	0.392	4.5	4.5	5.3

Table 5
BioTox™ toxicity of binary mixtures of pesticides

Pesticide mixture ^a	EC ₂₀ (mg l ⁻¹)		EC ₅₀ (mg l ⁻¹)		EC ₈₀ (mg l ⁻¹)	
	Incubation time (min)		Incubation time (min)		Incubation time (min)	
	5	15	5	15	5	15
Carb+Fena 1:1	5.8	7.7	12.3	13.5	–	27.7
Fena+Carb 1:3	5.5	3.4	16.7	18.8	–	27.5
Form+Prop 1:1	2.6	2.5	1.8	3.4	20	19.7
Form+Prop 1:3	1.4	1.8	4.9	3.8	49.7	14.5

^a Carb: carbofuran; Fena: fenamiphos; Form: formetanate; Prop: propamocarb.

Table 6
BioTox™ toxicity of ternary mixtures of pesticides at

Pesticide mixture ^a	EC ₂₀ (mg l ⁻¹)		EC ₅₀ (mg l ⁻¹)		EC ₈₀ (mg l ⁻¹)	
	Incubation time (min)		Incubation time (min)		Incubation time (min)	
	5	15	5	15	5	15
Carb+Prop+Form 1:1:1	1.3	0.8	4.3	2.2	13.5	12.0
Carb+Prop+Form 1:2:1	2.6	1.7	6.2	3.5	17.3	16.1
Form+Prop+Cyro 1:2:1	0.6	1.1	3.4	1.9	17.2	16.6
Form+Prop+Cyro 1:2:1	2.0	1.9	7.5	3.8	12.8	16.5

^a Carb: carbofuran; Cyro: cyromazine; Form: formetanate; Prop: propamocarb.

comparison of the EC₅₀ values in Tables 5–7 with data for pure compounds (Table 1) reveals existence of synergistic and antagonistic effects. For example, a 1:1 mix of formetanate and propamocarb (Table 5) is significantly more toxic than either of the pure components (Table 1). On the other hand, the 1:3 mix (Table 5) is about as toxic as the least toxic component present even though the more toxic propamocarb constitutes 75% of the mixture. Interestingly, more complex mixtures, those with three and four components (Tables 6 and 7), were generally at least as toxic as the most toxic pure compound present, even though the latter constituted as little as 1/3 or 1/4 of the mixture.

Toxicities of the mixtures did not have a consistent pattern with respect to the amount of most toxic component; in particular, mixture toxicity did not correlate with the weighted sum of toxicities of individual components present, as has been claimed for mixtures of certain toxicants [11]. Ribo and Rogers [11] showed that for many nonspecific toxicants, the mixture toxicity correlated with that of individual component toxicity in accordance with the equation:

$$EC_{50M} = \frac{C_M}{(C_A/EC_{50A}) + (C_B/EC_{50B}) + (C_C/EC_{50C}) + \dots} \quad (5)$$

Table 7
BioTox™ toxicity of quaternary mixture of pesticides

Pesticide mixture ^a	EC ₂₀ (mg l ⁻¹)		EC ₅₀ (mg l ⁻¹)		EC ₈₀ (mg l ⁻¹)	
	Incubation time (min)		Incubation time (min)		Incubation time (min)	
	5	15	5	15	5	15
Carb+Fena+Form+Prop 1:1:1:1	3.0	1.8	8.8	4.6	18.0	16.4

^a Carb: carbofuran; Fena: fenamiphos; Form: formetanate; Prop: propamocarb.

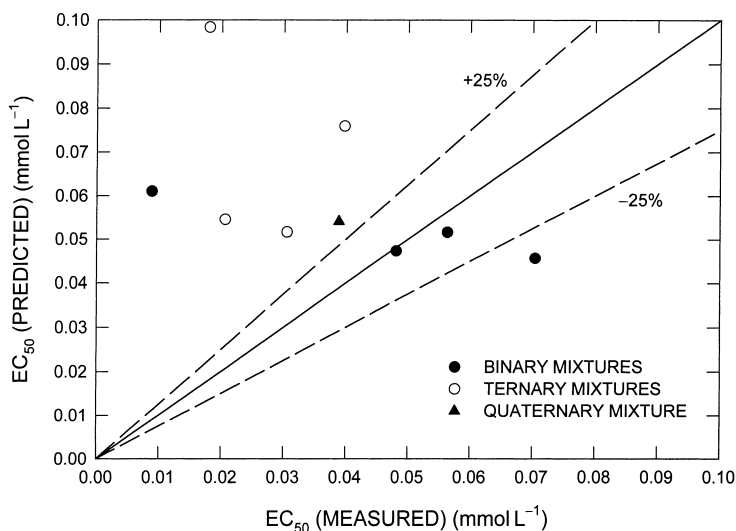


Fig. 6. Parity plot of mixture toxicities (BioTox™ assay) predicted with Eq. (5) vs. measured data. Solid line represents exact agreement.

where EC_{50M} is the toxicity of the mixture, C is concentration ($mmol l^{-1}$), and subscripts A, B, etc., denote individual components. All EC_{50} values in Eq. (5) are molar values ($mmol l^{-1}$). Eq. (5) considers a mixture's toxicity as a weighted sum of toxicities of individual components, with the weighting factor being the mole fraction of the component in the mixture. As shown in Fig. 6, for the pesticides tested, the toxicities were not additive, hence the mixture toxicity predicted with Eq. (5) failed to match exper-

imental data. For the two ternary mixtures with cyromazine (Table 6), the estimations of mixture EC_{50} values according to Eq. (5) disregarded the contribution of cyromazine. This was satisfactory because the concentration of cyromazine in the mixture was small compared to its toxicity ($EC_{50} > 50$ ppm), thus the $(C/EC_{50})_{cyromazine}$ was negligibly small compared to the other terms in the denominator of Eq. (5). As shown in Fig. 6, for most mixtures, the predicted toxicity was substantially greater than actually

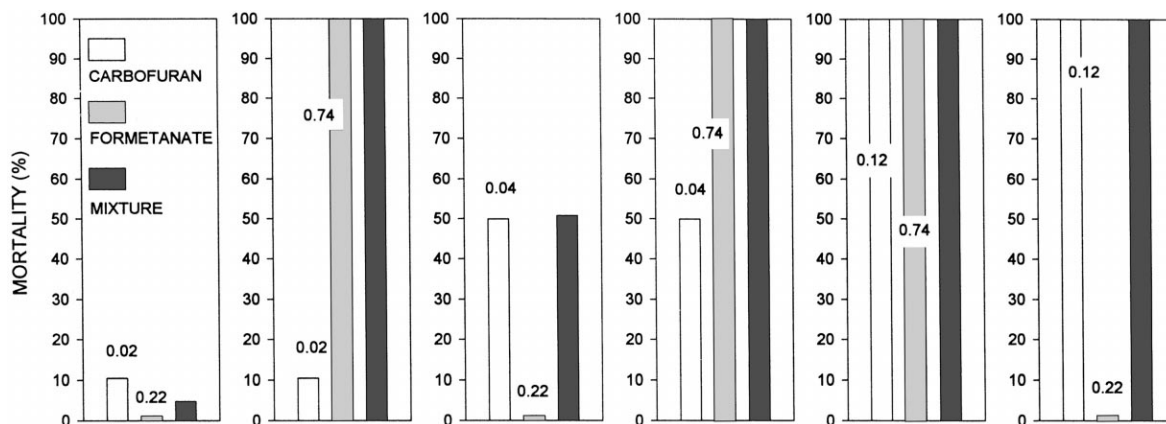


Fig. 7. Effects of various concentrations of pure pesticides and binary mixtures on mortality of *Daphnia magna*. Concentrations ($mg l^{-1}$) of pure pesticides are shown above or below each bar. The concentration in any binary mixture was the sum of the concentrations of the two pure pesticides for a given group of data.

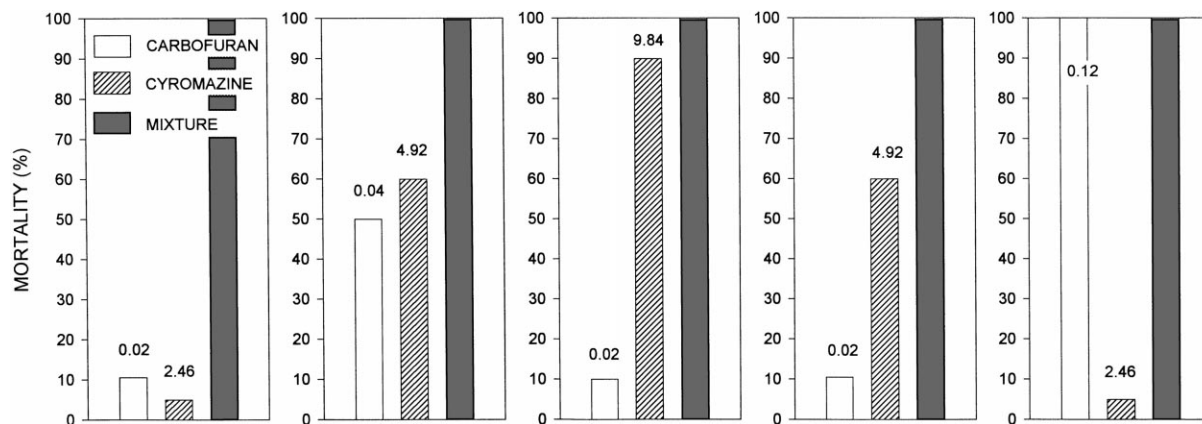


Fig. 8. Effects of various concentrations of pure pesticides and binary mixtures on mortality of *Daphnia magna*. Concentrations (mg l^{-1}) of pure pesticides are shown above or within each bar. The concentration in any binary mixture was the sum of the concentrations of the two pure pesticides in a given group of bars.

measured with the BioToxTM system, suggesting either an antagonistic interaction or a relative inability of the BioToxTM assay to detect synergisms in toxicity. The latter factor appeared to play a role as discussed later with reference to response of the *D. magna* system to pesticide mixtures.

Data on *D. magna* mortality in presence of binary and ternary mixtures of pesticides are shown in Figs. 7–9. Each set of bars in the figures denotes percent mortality when *D. magna* is exposed only to a single pure pesticide component of the mixture,

present in the same concentration as in the mixture. The mortality data for the corresponding mixture are also given. The numbers above or below the bars (Figs. 7–9) represent concentrations (mg l^{-1}) of the individual components present. Fig. 7 for the various mixtures of carbofuran and formetanate does not reveal any synergistic effects, as in each case of 100% mortality in presence of the mixture, at least one of the constituents of the mixture alone achieves the same mortality effect. Only the first set of bars (Fig. 7) suggest an antagonistic effect, but the differ-

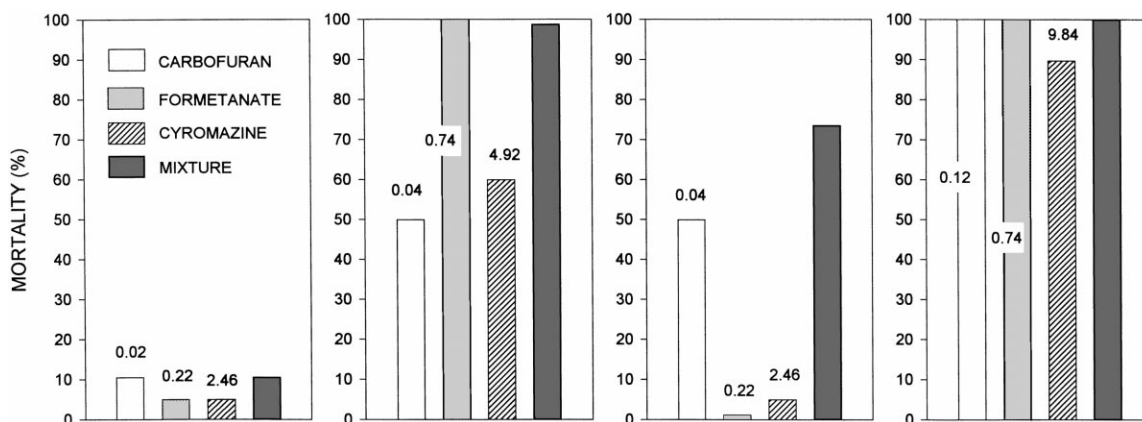


Fig. 9. Effects of various concentrations of pure pesticides and ternary mixtures on mortality of *Daphnia magna*. Pure component concentrations (mg l^{-1}) are shown above or within each bar. The pesticide concentration in any ternary mixture was the sum of the pure component concentrations noted for specific groups of data.

ences in mortalities are small to argue for such an effect. However, the situation is quite different with binary mixtures of carbofuran and cyromazine, as shown in Fig. 8. Certain proportions of carbofuran and cyromazine achieve far greater kill than do the mixtures' constituents singly. A similar substantial enhancement in toxicity is seen in Fig. 9 for the ternary mixture containing carbofuran, formetanate and cyromazine in the concentrations of 0.04, 0.22 and 2.46 mg l^{-1} , respectively.

The MitoScanTM assay also provided mixture toxicity values that could not be accounted for as a weighted sum of toxicities of individual components present. The MitoScanTM EC₅₀ values were (in ppm): 4.2 for 1:1 mix of formetanate and carbofuran; 16.0 for formetanate, cyromazine, and carbofuran (1:1:1); and 7.6 for 1:3:3 mix of formetanate, cyromazine and carbofuran. No toxicity was detected for a 1:1 mix of carbofuran and cyromazine. The pure component EC₅₀ values for the same test are noted in Table 4.

4. Concluding remarks

Toxicity is a biological response and this needs to be taken into account in formulating realistic guidelines on acceptable upper limits on pesticide contamination of wastewater discharges to the environment. Existing discharge limits such as $1\text{--}10 \text{ mg l}^{-1}$ for individual pesticides are arbitrary. As documented here, different pesticides in mixtures interact to produce synergistic and antagonistic effects on toxic response. To assure adequate environmental safeguards, antagonistic effects must be necessarily disregarded in designing discharge limits because such effects cannot be predicted for all possible combinations of components in an effluent. However, synergistic enhancements in toxicity cannot be ignored. Therefore, as a safer discharge practice, an effluent ought to simultaneously satisfy two strength criteria with respect to toxicity: 1. The total concentration of the components present should remain below a threshold value, e.g. $0.1 \sum x_i \text{EC}_{50i}$ where x_i is the mole fraction of the component i in the mixture and EC₅₀ is its toxicity value; and 2. The total concentration of all toxic constituents should not exceed a certain fraction of the EC₅₀ value for the mixture. In cases where the components do not influence toxic response of other constituents and the

toxicity depends linearly on concentration, criteria 1 and 2 converge.

A sound EC₅₀ value to use in establishing the discharge limit is one obtained with the well-known *D. magna* standard test procedure. While the BioToxTM and the MitoScanTM assays are easier to use and faster, the EC₅₀ values they provide are meaningless in the context of assessing a toxicant's impact on the biosphere. EC₅₀ values from a given test are undoubtedly useful in assessing relative toxicities of different samples tested with the same assay. Also, within limits, BioToxTM and MitoScanTM assay results may be correlated with the EC₅₀ values of the *D. magna* test; nevertheless, so far as absolute EC₅₀ values are concerned, the *D. magna* whole organism assay has no substitute. The *D. magna* EC₅₀ values are consistently and considerably lower than ones obtained with the other tests. *D. magna* is sensitive to a great variety of toxicants and because of its substantially more complex biochemistry than the other two test systems, *D. magna* better detects synergisms in toxicity. A combination of the *D. magna* and the BioToxTM assays covers a broad range of possible toxic responses; however, *D. magna* toxicity indices are more directly relevant to toxicity of a compound in higher animals, including humans and other mammals, marine life, and birds. MitoScanTM test provides complimentary information and its broader usefulness needs to be further evaluated. The lower sensitivity of the MitoScanTM and the BioToxTM tests may be compensated for by their ease of use and the potential for implementing as portable field devices. In addition, modified test formats with a minicolumn preconcentration step may improve toxicity detection threshold of these tests.

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