

# Toxicity of Some Pesticides, Heavy Metals and Their Mixtures to *Vibrio fischeri* Bacteria and *Daphnia magna*: Comparative Study

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## Abstract

The present study was conducted to evaluate the sensitivity of the *Vibrio fischeri* bioluminescence inhibition test (Microtox<sup>®</sup> assay), and the standard acute *Daphnia magna* test; using 3 heavy metals, 3 organic pesticides, and their mixtures. In *Daphnia* tests, either at 24h or 30 min exposure times, the pattern of toxicity order for heavy metals was Cu > Cd > Pb. Chlorpyrifos-methyl was the highest toxic at 24h, while Triazophos was the highest toxic at 30 min exposure times. In the Microtox<sup>®</sup> test at 5 min exposure time, the estimated EC50 values were 4.20, 4.53 and 6.60 mg/L for Cu, Cd and Pb, respectively. At the same exposure time, the EC50 values of Triazophos, Chlorpyrifos-Me and Profenofos accounted to 1.76, 3.36 and 4.12 mg/L, respectively. Similar order of toxicity was obtained when tests were

conducted at 15 min exposure time. The paired mixtures of pesticides, as well as the mixtures of Cu + Cd and Pb + Cd, showed potentiation effects, while the mixture of Cu + Pb showed additive effect against *D. magna*. The tertiary mixtures of the pesticides or the heavy metals reacted antagonistically. In the Microtox<sup>®</sup> assay, the heavy metal mixtures reacted antagonistically, while pesticide mixtures showed synergism. It was concluded that both *Daphnia* and Microtox<sup>®</sup> tests showed similar pattern of sensitivity to the single toxicants, but dissimilar pattern to the heavy metal mixtures. On the other side, using shorter exposure time (ca. 30 min) with *Daphnia* bioassay may enable us to held reliable comparisons with Microtox<sup>®</sup> results.

**Keywords:** *Daphnia magna*, *Vibrio fischeri*, Microtox<sup>®</sup>, Heavy metals; Pesticides, Mixtures

## 1. Introduction

Contamination of aquatic environments by various natural and industrial chemical compounds is being considered as a major environmental problem of global concern (Schwarzenbach et al., 2006). Pesticides and heavy metals are among important contaminants of aquatic ecosystems. Monitoring of such contaminants in different environmental components helps to protect the natural environment and human health. Physicochemical monitoring processes have traditionally been used for control and assessment of environmental chemical pollutants. Therefore, the analytical instruments associated with physicochemical measurements have received great advancements in their design and accuracy (Doull et al., 2007). However, the data borne from such procedures doesn't provide information about toxicity interaction between the chemical pollutant(s) and the biological material. Therefore, a realistic interpretation of chemical toxicity to biological systems can only be carried out by use of a system which actually employs living organisms (Pascoe, 1987).

The freshwater cladoceran *Daphnia magna* Straus is one of the oldest and widely used test organisms in aquatic toxicology (Baudo, 1987; Lambolez et al., 1994; Ortiz et al., 1995; Kaneko, 1996; Seco et al., 2003). These water flea organisms are important link in freshwater trophic chains representing the filter-feeding zooplankton (Mark and Solbe, 1998). *Daphnia magna* as standard test species has several advantageous characteristics making it the test organism of choice. Among favorable characteristics of these organisms are their small sizes, easy to culture in the laboratory, short life-span, parthenogenetic reproduction under non-stressed conditions, reproducibility and repeatability of the test results, and relative sensitivity to most chemical compounds (Versteeg et al., 1997; Mark and Solbe, 1998). Therefore, *D. magna* is the most commonly tested freshwater species in acute as well as in chronic tests (Ratte and Hammerswartz, 2003).

It worthy to mention that the *Daphnia* assay has been standardized (OECD, 2004; ISO, 1996), and it is used in routine control of aquatic toxicity assessment of effluents and in environmental safety evaluation of chemical substances (Barata et al., 2006), and in mechanistic studies concerned with aquatic toxicology (Damásio et al., 2007, 2008).

On the other hand, the marine bacteria *Vibrio fischeri*, is one of the most common biosensor

used for the risk assessment in aquatic environment based on the inhibition of luminescence produced by the bacteria in the presence of toxic substances. Kaiser and Ribo (1988) had been previously described the experimental procedure of toxicity determination using the Microtox® Toxicity Analyzer based on the standardized method (ISO, 2009) with *V. fischeri*. Toxicity is usually represented as EC50, i.e. effective concentration of the tested chemical at which 50% of luminescence inhibition is observed after a predetermined exposure time (5 - 30 min). This bioluminescence based assay is sensitive and rapid, and thus has been long recognized for the regulatory purposes (Coz et al., 2007). The Microtox® test is considered relatively inexpensive, provides well-reproducible results, and offers a fast testing procedure. The toxicity data obtained with the Microtox® test correspond well with acute toxicities obtained with standard toxicity tests for many bioassayed samples (Kaiser and Palabrica, 1991; Toussaint et al., 1995; Weideborg et al., 1997). This Microtox® biotest has been widely used recently to investigate the toxicity of various inorganic and organic compounds in water samples (Trang et al., 2005; Gueune et al., 2009; Katritzky et al., 2010).

The present study was undertaken to compare between the *Daphnia magna* acute toxicity assay and the *Vibrio fischeri* Microtox® test; with respect to their sensitivity to some heavy metals, pesticides and their mixtures.

## 2. Materials and Methods

### 2.1 Experimental Animals

A single laboratory colony of *Daphnia magna* Straus cultured in our laboratory, at  $20 \pm 2$  °C and 12:12 h light: dark cycle, was used in this study. Bulk cultures of 15 animals each were maintained in ASTM hard synthetic water (Barata et al., 2000) and the animals were fed daily with *Scenedesmus subspicatus* (corresponding to 2 mg C/L; Boersma, 1995). The culture medium was changed every other day and neonates (<24 h) were removed and transferred to 2-L beakers and reared under the same conditions as their mothers until they reached their 4<sup>th</sup> instars (4-5 days). At this stage, groups of juveniles were collected and used for toxicity studies.

### 2.2 Chemicals and Reagents

#### 2.2.1 Heavy Metals and Insecticides

The substances employed in the toxicity experiments included three heavy metals (Cd, Cu and Pb) and three pesticides (Chlorpyrifos-Methyl, Profenofos and Triazophos). The heavy metals used were purchased as chloride salts of high purity grade from the following sources: PbCl<sub>2</sub> (Panreac Quimica SA, Spain); CdCl<sub>2</sub> and CuCl<sub>2</sub> · 2H<sub>2</sub>O (S.D. Fine-Chem. Ltd. BOISAR, Laboratory Rasayan). The pesticides were procured from the Egyptian Ministry of Agriculture as commercial formulations of specified active ingredient (a.i.) content as follows: Reldan® 22.5% EC (Chlorpyrifos-Methyl), Hostathion® 40% EC (Triazophos), and Selecron® 72% EC (Profenofos).

#### 2.2.2 Microtox® Reagents and Apparatus

The freeze-dried luminescent bacteria, *Vibrio fischeri* (13F4067A), reconstitution solution

(AFZ686016), Osmotic Adjusting Solution (20% NaCl; AFZ686019), and diluent solution (2% NaCl; AFZ686011) were supplied by Modern Water Inc., New Castle, DE 19720, USA. The tests were performed using the Microtox® Model 500 Toxicity Analyzer from Modern Water Inc. The analyzer was equipped with a 30-well temperature-controlled incubator chamber at 15 °C. A small compartment held at 5 °C was used to store the bacteria before dilution. The light output was recorded from a digital display.

### 2.3 *Daphnia* Toxicity Bioassay

#### 2.3.1 Toxicity of Single Toxicants

Acute toxicity tests, using the above mentioned heavy metals and pesticides, against *D. magna* were carried out as described below. Either heavy metals or pesticides were dissolved in deionized tap water to prepare stock solutions which were used to prepare working solutions of different concentrations. Concentrations were expressed in terms of mgL<sup>-1</sup> (ppm) active ingredients (a.i.). About 60 animals were used for each test divided into five replicates of 10 organisms each plus control for each series of concentrations (5-7). The test animals were placed in 250 mL glass beakers containing 200 mL of test solution. Mortality (complete immobilization) was counted 24h after exposure, adjusted by Abbott's formula (Abbott, 1925) and subjected to probit analysis (Finney, 1971) to estimate EC values, 95% confidence limits and slopes of regression lines. The latter's were constructed by the aid of an Ld-P Software program. Test methods were performed in general accordance with respective to standardized protocols (OECD, 2000) and *Daphnia* were not fed during the assay. Similar tests were carried out at 30 minutes exposure time for comparison purposes.

#### 2.3.2 Toxicity of Toxicant's Mixtures

Joint action studies were carried out by mixing heavy metals and pesticides together in paired combinations at a level of their corresponding 24h-EC25 values. Also, a mixture combining the 3 pesticides and another combining the 3 heavy metals were prepared at their respective 24h-EC25 values. Each mixture was tested in 4 replicates alongside a control treatment. The tests were carried out as mentioned above where mortality percentages were determined after 24h exposure time, and the action of each mixture was expressed as a co-toxicity factor according to Sun and Johnson (1960) to differentiate between potentiation, antagonism and additive effects, using the following equation:

$$\text{Co-toxicity factor} = (O - E) / E \times 100;$$

Where O is observed % mortality and E is expected % mortality. This factor differentiates the results into three categories. A positive factor of  $\geq 20$  indicates potentiation, a negative factor of  $\leq -20$  indicates antagonism, and the intermediate values of  $> -20$  to  $< 20$  indicate an additive effect. In routine work, the expected mortality for pairs of toxicants is usually considered as 50%. For more accuracy, mortality was determined for each toxicant at its EC25 value, and the expected mortality of a combined pair was the sum of the mortalities of its single compounds. The observed mortality is the recorded mortality obtained 24 h after using the mixtures.

Another set of experiments was carried out on tertiary mixtures combining either the 3 heavy metals or the 3 pesticides at the corresponding EC<sub>25</sub> values, where the co-toxicity values were calculated by the above mentioned equation but the expected % mortality (E) was considered to equal 75%.

## 2.4 Luminescent Bacteria Bioassay

### 2.4.1 Toxicity of Single Toxicants

Based on active ingredient (a.i.) content either in heavy metal salts or commercial insecticides, the test solutions were prepared in deionized tap water. Preliminary experiments were carried out in order to find out the most suitable concentration range allowing the determination of the EC<sub>50</sub> values for each of the tested toxicants. The concentrations of the finally tested solutions were: 15, 25 and 25 mg/L for Cu, Pb and Cd, respectively and 15, 30 and 30 mg/L for Triazophos, Profenofos and Chlorpyrifos-Methyl, respectively. The solutions were freshly prepared. Solutions were then adjusted to pH6.0 by addition of 0.1N-HCl solutions and used immediately. Each assay was performed at least in triplicate.

EC<sub>50</sub> values, defined as the concentration which provokes a 50% light reduction on *V. fischeri*, were obtained by following the Microtox® basic test protocol (Villaescusa et al., 1996). Practically, the EC<sub>50</sub> values were calculated by regression analysis of the linear relationship between the logarithm of the toxicant concentration against the logarithm of the lost/remaining light intensity ratio “gamma”. The EC<sub>50</sub> values were determined at 5 and 15 min exposure time.

### 2.4.2 Toxicity of Binary Mixtures

Equitoxic binary mixtures were prepared on the basis of concentrations of each toxicant which produced a similar toxic effect when being alone, i.e., the EC<sub>50</sub> values which produce a 50% light reduction. These solutions were used to evaluate the toxicity of the three possible combinations of Cu(II), Cd(II) and Pb(II), as well as the three possible combinations of Triazophos, Profenofos and Chlorpyrifos-Methyl. The toxicity assays of the mixtures were measured at 5 and 15 min exposure time and each assay was performed at least in triplicate.

A simple mathematical model based on the theory of probabilities (Kungolos et al., 1999) was applied using the following formula (Tsiridis et al., 2006):

$$P(E) = P_1 + P_2 - P_1 P_2 / 100.$$

According to this model, if  $P_1$  is the inhibition caused by a certain concentration of chemical  $A_1$  and  $P_2$  the inhibition caused by a certain concentration of chemical  $A_2$ , then, the theoretically expected additive inhibition  $P(E)$ , when those concentrations are applied together will be given by the above mentioned formula. The null hypotheses were that the observed values were higher or lower than the theoretically predicted ones, for synergistic and antagonistic effects, respectively. The result was considered to be antagonistic or synergistic, only if the observed effect was significantly lower or higher respectively than the theoretically predicted one at the 0.05 level of significance.

### 3. Results

#### 3.1 *Daphnia* Bioassay

Toxicity values of the tested metals and pesticides against *D. magna* after 24 h exposure period are shown in Table 1. At the level of EC50 values, Cu was the most toxic (0.0002 mg/L) followed by Cd (0.254 mg/L) and then Pb (0.413 mg/L). On the other hand, Chlorpyrifos-Methyl was the most toxic (0.0027 mg/L) followed by Triazophos (0.0093 mg/L) and then Profenofos (0.014 mg/L). At the level of EC95 values, Cu and Chlorpyrifos-Methyl were also the most toxic.

Table 1. Toxicity values (mg/L) of heavy metals and pesticides against *Daphnia magna* after 24 h-exposure time.

Metal/ Pesticide	EC25 & (95% Fudicial limits)	EC50 & (95% Fudicial limits)	EC95 & (95% Fudicial limits)	Slope
Cd	0.078 (0.035-0.115)	0.254 (0.193-0.336)	2.419 (1.239-10.494)	1.31
Cu	0.0001 (0.0001-0.0001)	0.0002 (0.0002-0.0003)	0.0012 (0.0009-0.0019)	1.70
Pb	0.099 (0.024-0.167)	0.413 (0.303-0.556)	6.261(2.518-83.728)	1.09
Chlorpyrifos-Methyl	0.0011 (0.0007-0.0014)	0.0027 (0.0022-0.0034)	0.0158 (0.0099-0.0371)	1.69
Profenofos	0.003 (0.002-0.005)	0.014 (0.010-0.018)	0.057 (0.014-0.737)	1.09
Triazophos	0.0012 (0.0003-0.002)	0.0093 (0.0052-0.071)	0.433 (0.06161-1.308)	0.77

Toxicity of the tested substances at 30 min exposure time (Table 2) was differed greatly with respect to the toxicity values obtained. For instance, the EC50 of Cu is 0.36 mg/L which equaled 1800 times that obtained after 24 h exposure time (0.0002 mg/L; Table 1). Triazophos showed the highest toxicity among the tested pesticides and its 30 min EC50 (0.213 mg/L; Table 1) equaled 23 times that obtained after 24 h exposure time (Table 1). The insecticide Profenofos showed the lowest toxicity and its 30-min exposure time equaled 0.89 mg/L (Table 2); giving rise to be as 64 times that estimated at 24 h exposure time (Table 1).

Table 2. Toxicity values (mg/L) of heavy metals and pesticides against *Daphnia magna* after 30 min-exposure time.

Metal/ Pesticide	EC25 & (95% Fudicial limits)	EC50 & (95% Fudicial limits)	EC95 & (95% Fudicial limits)	Slope
Cd	4.39 (3.70-4.88)	6.48 (5.95-7.21)	13.6 (10.99-20.17)	3.98
Cu	0.12 (0.07-0.17)	0.36 (0.27-0.46)	2.79 (1.77-5.77)	1.43
Pb	7.11 (5.19-8.37)	14.76 (12.55-20.24)	59.10 (34.85-209.36)	2.13
Chlorpyrifos-Methyl	0.15 (0.07-0.22)	0.64 (0.46-0.99)	10.31 (4.28-60.78)	1.06
Profenofos	0.45 (0.32-0.56)	0.89 (0.76-1.04)	3.19 (2.39-5.18)	2.31
Triazophos	0.030 (0.006-0.069)	0.213 (0.107-0.333)	8.709 (3.644-52.172)	0.79



A total of 15 paired mixtures of pesticides and heavy metals and 2 tertiary mixtures were tested against *D. magna* to investigate their joint action of toxicity; based on estimating the co-toxicity factor for each mixture according to the above described method. The co-toxicity factor for the tested pesticides equaled 33.4, 43.6 and 73.4 for Chlorpyrifos-Me + Profenofos, Triazophos + Profenofos and Chlorpyrifos-Me + Triazophos, respectively; results indicating potentiating effects with greater action for the mixture “Chlorpyrifos-Me + Triazophos” (Table 3). The mixture of Cu + Pb showed additive effect (co-toxicity factor = 6.8), while the mixtures of Cu + Cd and Pb + Cd showed potentiation. The highest potentiating effect has seen for the mixture of Cd + Triazophos (co-toxicity factor = 99.8). Mixtures of Cd + Profenofos, Cu + Chlorpyrifos-Me and Cu + Profenophos reacted additively. Either the mixture of the 3 pesticides or the mixture of the 3 heavy metals showed antagonistic effects; where their co-toxicity factors accounted to (-23.7) and (- 66.4), respectively (Table 3).

Table 3. Joint action analysis for pesticides and heavy metals mixtures against *Daphnia magna*

Mixtures	Observed Mortality (%)	Expected Mortality (%)	Co-toxicity Factor <sup>a)</sup>	Joint Action <sup>b)</sup>
Paired Pesticides				
Chlorpyrifos-Me + Profenofos	66.7	50.0	33.4	Po
Triazophos + Profenofos	76.7	53.4	43.6	Po
Chlorpyrifos-Me + Triazophos	86.7	50.0	73.4	Po
Paired Heavy Metals				
Cu + Cd	86.7	50.0	73.4	Po
Cu + Pb	53.3	49.3	6.8	Ad
Pb + Cd	73.3	53.4	37.3	Po
Paired Pesticides & Heavy Metals				
Cd + Chlorpyrifos-Me	55.3	43.3	27.5	Po
Cd + Triazophos	93.3	46.7	99.8	Po
Cd + Profenophos	56.7	52.7	7.6	Ad
Cu + Chlorpyrifos-Me	46.7	45.9	1.7	Ad
Cu + Triazophos	75.0	49.3	52.1	Po
Cu + Profenophos	53.3	46.9	13.6	Ad
Pb + Chlorpyrifos-Me	63.3	50.0	26.6	Po
Pb + Triazophos	93.3	53.4	74.7	Po
Pb + Profenophos	83.9	51.0	64.5	Po
Tertiary Mixtures				
Chlorpyrifos-Me + Triazophos + Profenophos	56.7	74.3	- 23.7	An
Cd + Cu + Pb	23.3	69.3	- 66.4	An

<sup>a)</sup>Co-toxicity Factor =  $\frac{\% \text{ Observed mortality} - \% \text{ Expected mortality}}{\% \text{ Expected mortality}} \times 100\%$

<sup>b)</sup>Joint Action: Po: Potentiation; An: Antagonism; Ad: Additive

### 3.2 Luminescent Bacteria Bioassay

The results of the Microtox<sup>®</sup> test were given in a report illustrating the relation between “Gamma vs concentration” and “% effect vs concentration” of the tested toxicant at 5 and 15 min exposure times (N.B.: Gamma is the ratio of light lost to light remaining after the bacteria and reagent are challenged by the tested sample). The concentration that produces an EC50 has a gamma value of one. Figure 1 represents a typical chart for copper toxicity determination as example.

Table 4. Estimated EC50 values based on light intensity, after 5 and 15 min-exposure time of *Vibrio fischeri* to single divalent metals and pesticides.

Toxicant/Bioassayed concentration <sup>a</sup> (mg/L)	Light Intensity & (95% confidence limits) %		Equivalent EC50 values (mg/L)		Slope	
	5 min	15 min	5 min	15 min	5 min	15 min
Cu (15)	27.97 (25.38 - 30.82)	18.95 (17.07 - 21.03)	4.20	2.84	0.73	1.42
Cd (25)	18.13 (13.89 - 23.66)	17.87 (nd)	4.53	4.47	0.73	-2.72
Pb (25)	26.41 (7.816 - 89.27)	23.33 (7.68 - 70.84)	6.60	5.83	0.95	0.65
Chlorpyrifos-Me (30)	11.21 (4.99 - 25.20)	9.81 (4.35 - 22.13)	3.36	2.94	1.05	0.88
Profenofos (30)	13.73 (8.41 - 22.40)	13.10 (nd)	4.12	3.93	1.40	1.81
Triazophos (15)	11.75 (1.55 - 89.08)	7.79 (1.28 - 47.49)	1.76	1.17	1.44	0.95

<sup>a</sup>Values between brackets indicate bioassayed concentration for each corresponding toxicant.

As shown from Fig. 1, the EC50 value is reported as percent of concentration at 5 and 15 minutes (e.g., 27.97 % and 18.95%, respectively). These values were modulated to the tested concentration of Cu solution (15 ppm) to calculate the equivalent EC50 values in mg/L (e.g., 4.20 and 2.84 mg/L, respectively). Table 4 includes the estimated EC50 values for the tested metals and pesticides by Microtox<sup>®</sup> basic test. Based on the EC50 values at 5 min exposure time, Cu was the most toxic (4.20 mg/L) followed by Cd (4.53 mg/L) and then Pb (6.60 mg/L). At the same exposure time, Triazophos was the most toxic (EC50 = 1.76 mg/L) followed by Chlorpyrifos-Me (EC50 = 3.36 mg/L) and then Profenofos (EC50 = 4.12 mg/L). Toxicity bioassay at 15 min exposure time resulted in EC50 values which were generally lower than those at the shorter exposure time, but the tested toxicants possessed similar order of toxicity where Cu and Triazophos were the most toxic candidates, while Pb and Profenofos were the lowest (Table 4).

The joint action analyses for the binary mixtures of the tested toxicants as estimated by



Microtox<sup>®</sup> according to the above described method are depicted in Table 5. The 3 pairs of heavy metals, except Cd + Cu at 5 min exposure time, showed antagonistic effects, while the excepted mixture reacted synergistically. Synergistic effects were dominated for the mixtures of pesticides either at 5 or 15 min exposure times (Table 5).

Table 5. Joint action of binary mixtures of heavy metals and pesticides as estimated by luminescent bacteria test using the below formula<sup>a</sup>

Mixture	Exposure Time (min)	% Expected Inhibition; P (E)	% Observed Inhibition <sup>b</sup>	Joint Action <sup>c</sup>
Cd + Cu	5	41.03	48.89	Synergism
	15	33.43	26.00	Antagonism
Cd + Pb	5	39.75	23.90	Antagonism
	15	37.03	29.95	Antagonism
Cu + Pb	5	46.99	23.33	Antagonism
	15	37.86	13.73	Antagonism
Triazophos + Profenofos	5	23.87	74.05	Synergism
	15	19.87	nd	nd
Triazophos + Chlorpyrifos-Me	5	21.64	30.75	Synergism
	15	16.84	26.41	Synergism
Profenofos + Chlorpyrifos-Me	5	23.40	33.37	Synergism
	15	21.62	36.01	Synergism

<sup>a</sup> $P(E) = P_1 + P_2 - P_1 P_2 / 100$  (Tsiridis et al., 2006).

P<sub>1</sub> & P<sub>2</sub> are inhibition caused by individual toxicants of the mixture (refer to Table 4).

P (E) = Expected (theoretical) inhibition given by the above formula.

<sup>b</sup>Observed (practical) inhibition measured by Microtox<sup>®</sup> for the mixture.

<sup>c</sup>Synergism means that Observed Inhibition value is greater than Expected Inhibition value and vice versa for Antagonism.

#### 4. Discussion

To better understanding the toxicological profile of environmental toxicants, the impacts of such toxicants should preferably measured by organisms representing different trophic levels (Choi and Meier, 2001). In the majority of aquatic ecosystems, the most important trophic level in terms of energy flow and nutrient cycling is the bacteria. So, the Microtox<sup>®</sup> assay, based on *Vibrio fischeri*, has been widely applied as a rapid, economical monitoring tool for toxicity of environmental contaminants (McFeters et al., 1983). On the other hand, acute toxicity testing using daphnids, (e.g., *Daphnia magna*), is a common bioassay used internationally for screening toxicity of chemicals and monitoring of effluents and contaminated waters (Persoone et al., 2009). *D. magna* has been recommended as a standard test organism by many international organizations (e.g., ISO, 1996 and OECD, 2004) and has

**MicrotoxOmni Test Report**

**Date: 18/03/2014 02:23 PM ; Test Protocol: Basic Test ; Sample: Cu, 15 ppm ; Toxicant: Copper**

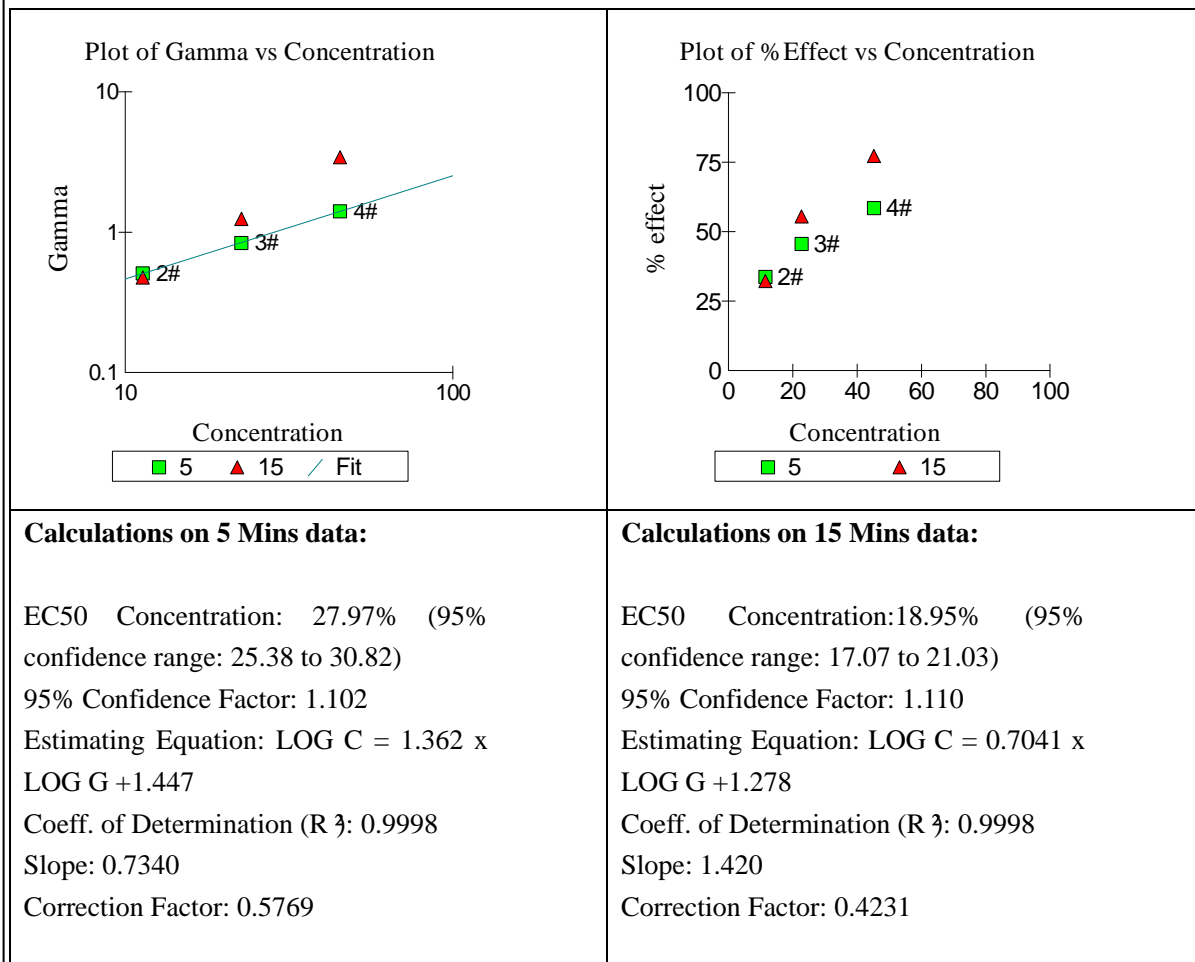


Fig. 1. Typical chart for copper toxicity determination on Microtox<sup>®</sup> at 5 and 15 minutes exposure time to *Vibrio fischeri*.

N.B.: Gamma is the ratio of light lost to light remaining after the bacteria and reagent are challenged by the tested sample.

been used routinely in toxicological studies (Biesinger and Christensen, 1972; Hermens et al., 1984; De Schamphelaere et al., 2004). Hence, it is important to include both *V. fischeri* and *D. magna* in a battery of tests designed for protecting the aquatic ecosystems.

Toxicity results of a given substance to a specific organism are highly affected by the conditions where tests are carried out (e.g., temperature, relative humidity, light/dark cycle, pH of the test media, exposure time, etc.). However, comparisons between toxicity data of different laboratories may give an indication to what extent the data are going in harmony. The data presented in Table 6 show Microtox<sup>®</sup> EC50 values at 15 min exposure time for Cu,

Cd and Pb reported by different investigators, compared with our results. It seems that our result for Cd (4.47 mg/L) was very low as compared with those reported by Codina et al. (1993), Newman and McCloskey (1996), Mowat and Bundy (2002) and Fulladosa et al. (2005); which respectively equaled to 34.70, 21.90, 59.30 and 10.90 mg/L. For Cu and Pb, the opposite was obtained where our EC50 values (2.84 and 5.83 mg/L, respectively) were generally higher than those reported by other investigators (Table 6). However, a very little difference between EC50 value reported for Cu (2.74 mg/L) by Mowat and Bundy (2002), and that obtained in the present study (2.84 mg/L). It has been reported that the pH of test media had a profound effect on the Microtox<sup>®</sup> EC50 values. For example, the 15 min-EC50 values for Cd were reported 10.12 ppm at pH 5.5 (Villaescusa et al. 1996); 16.8 ppm at pH 6.0 (Codina et al., 2000); and 3.0 ppm at pH 5.43 (McCloskey et al., 1996). Also, the 15 min-EC50 values for Pb were reported 0.13 ppm at pH 5.5 (Villaescusa et al., 1996) and 2.24 ppm at pH 5.56 (McCloskey et al., 1996).

Table 6. Comparison of Microtox<sup>®</sup> EC50 values in mg/L after 15 min-exposure for Cu, Cd and Pb with those reported by other investigators.

Metal	This study <sup>a</sup>	Other investigators <sup>b</sup>	Source
Cu	2.84	0.35	Fulladosa et al. (2005)
		0.17	Newman and McCloskey (1996)
		0.457	Codina et al. (2000)
		2.74	Mowat and Bundy (2002)
Cd	4.47	10.90	Fulladosa et al. (2005)
		21.90	Newman and McCloskey (1996)
		59.30	Mowat and Bundy (2002)
		34.70	Codina et al. (1993)
Pb	5.83	0.12	Fulladosa et al. (2005).
		0.177	Newman and McCloskey (1996).
		0.427	Mowat and Bundy (2002)

<sup>a</sup>The present study was performed at pH = 6.0.

<sup>b</sup>Microtox<sup>®</sup> EC50 values in mg/L after 15-min exposure time.

In this respect, it may be convenient to highlight the conclusion reported by Lopez-Roldan et al. (2012): “the results from toxicity experiments are dependent on the conditions in which the test is performed. Potential sources of variability could have its origin in the bacteria (preservation, reconstitution procedure, etc.); in the sample (preparation of standard solutions, pH, etc.) and in the experimental procedure (sample handling, deviations in volume delivery, instrumental error, calculation method, etc.). Due to this fact, values obtained from toxicity test of the same compound can differ depending on the study”.

The time of exposure of animals to toxicants has a profound effect on the percentage of the population responding, and thus it affects the sensitivity and in many cases the practicality of

the method (Frear and Boyd, 1967). However, the time of exposure used in *Daphnia* bioassays varies largely from one method to another. For example, the American Standard Methods (APHA, 1975) recommend an exposure time of 48 h, while the Japanese Standard Methods (Hashimoto and Nishiucbi, 1981) recommend 3 hr only. On the other hand, Frear and Boyd (1967) used a 26 h exposure period, while Parker *et al.* (1970) used 30 min only. In this respect, we have previously suggested that shorter exposure periods (e.g. 1h) may be practically more convenient than longer periods; especially when using *Daphnia* for routine bioassay of pesticides and heavy metals (Mansour *et al.*, 1992; Mansour and Gad, 2010).

Several investigators (e.g., Gälli *et al.*, 1994; Teodorovic *et al.*, 2009; Choi and Meier, 2001; Sponza and Kuscu, 2011; Lopez-Roldan *et al.*, 2012) compared sensitivity of *Daphnia* bioassay with the *V. fischeri* Microtox® test. For example, sensitivity of *V. fischeri* for selected organic compounds has been compared against sensitivity of *D. magna* by Lopez-Roldan *et al.* (2012). Based on Microtox® 15 min-EC50 compared with *D. magna* 24h-EC50 values, the latter investigators found that *V. fischeri* bacterium was more sensitive than *D. magna* for Nonylphenol (54.3 versus 134.0 mg/L); Dimethoate (0.80 versus 2.50 mg/L); Diclofenac (10.50 versus 118.00 mg/L); and MCPA (26.10 versus 136.00 mg/L). While the opposite was found for Triclosan (0.073 versus 0.67 mg/L); Terbutylazine (0.100 versus 2.04 mg/L); Diazinon (2.50 versus 240.80 mg/L); and Propanil (14.00 versus 21.71 mg/L).

The insecticides Dimethoate and Diazinon, which are OP compounds, were reported to have 24h-EC50 values of 2.50 and 2.50 mg/L, respectively against *D. magna* (Lopez-Roldan *et al.*, 2012). The 3 OP insecticides tested in the present study had extremely lower EC50 values (Table 1). On the other side, the Microtox® assay for Dimethoate and Diazinon reported 15min-EC50 values of 0.80 and 240.80 mg/L, respectively (Köck *et al.*, 2010); values which are completely different than those shown in Table 4 for the 3 OP insecticides tested in the present study. It's not strange to find such differences. For instance, disulfoton and thiometon have a similar molecular structure; however they showed different inhibitory effects on *D. magna* and Microtox® (Gälli *et al.*, 1994). The *D. magna* acute test proved to be more sensitive to cadmium (Cd), zinc (Zn) and manganese (Mn) than the *Vibrio fischeri* bacterial assays. Low sensitivity of *V. fischeri* to heavy metals questions its applicability as the first screening method in assessing various environmental samples. Therefore, it is not advisable to replace *D. magna* with bacterial species for metal screening tests. *V. fischeri* and/or other bacterial tests should rather be applied in a complex battery of ecotoxicological tests, as their tolerance to heavy metals can unravel other potentially present toxic substances and mixtures, undetectable by metal-sensitive species (Teodorovic *et al.*, 2009).

Many comparative studies with more widely used biological testing procedures, such as acute daphnid/fish tests, have been conducted to evaluate the applicability of the Microtox® assay in environmental pollution monitoring (Dutka and Kwan, 1981; Lebsack *et al.*, 1981; Curtis *et al.*, 1982; Qureshi *et al.*, 1982; Miller *et al.*, 1985; Toussaint *et al.*, 1995; Wängberg *et al.*, 1995; Sweet *et al.*, 1997; Doherty *et al.*, 1999). Bulich *et al.* (1981) compared the Microtox® assay with acute invertebrate and fish test results derived from several species (i.e., *Daphnia*, mysid shrimp, fathead minnows, rainbow trout, bluegill, and sheepshead minnow) with

varying exposure durations (24, 48, and 96 hr) to various municipal and industrial wastewaters. While the Microtox assay did not correlate well with the *Daphnia* results, a good agreement with fish tests was observed.

Generally, the EC50 values for the tested compounds in the present study against *D. magna* were extremely higher at 24h (Table 1) than at 30 min (Table 2) exposure times. But what about the situation if we compared *D. magna* toxicity results, based on EC50 values, at longer and shorter exposure times with EC50 values of Microtox® at 15 min? Such comparison could be depicted from the data presented in Tables 1&2 (for *Daphnia*) and Table 4 (for Microtox®), and reveal the following:

- a) For Cd & Pb: at longer exposure time, *Daphnia* was more sensitive than Microtox®, but the latter was more sensitive than *Daphnia* at shorter time.
- b) For Cu, Chlorpyrifos-Me, Profenofos & Triazophos: either at longer or shorter exposure times, *Daphnia* was more sensitive than Microtox®.

From the above, we may suggest using shorter exposure time (ca. 30 min) with *Daphnia* bioassay when comparing its sensitivity with Microtox®. This enables us to held comparison test at the same time nearly and gets results of reasonable acceptability. Supporting use of 30 min exposure time with *Daphnia* bioassay, the findings previously reported by Parker et al. (1970) on carbamate pesticides bioassay.

Although the toxicity of individual heavy metals has been assessed in many studies, little effort has been made to understand the environmental impact of these heavy metals in combination, as is usually the case in the natural environment (Soetaert et al., 2007), where the interaction between different compounds can highly influence the overall toxic impact of the heavy metal stressors on the organisms present.

It is uncommon to find an aquatic or other environmental system which is polluted by a single toxicant, and usually several harmful substances are present together in it; leading to possible interactions between such pollutants and between their effects on the tested organisms (van Leeuwen and Hermens, 1988). Therefore, we assessed the joint action resulted from exposure of *D. magna* and *V. fischeri* to mixtures of the tested metals and pesticides. In this respect, a biological response of a test organism is measured as a result of the toxic effect of the combined effect of the mixture of all potential contaminants contained in the analyzed sample (e.g., water), leading to antagonism and synergism. Our results (Table 3) revealed that the majority of paired combinations have induced potentiation (synergistic) effects against *D. magna*. In the Microtox® assay, antagonism dominated the toxicity of heavy metal mixtures, while synergism was obtained for the binary mixtures of pesticides (Table 5). Our results agreed with those reported by Fulladosa et al. (2005) regarding to the antagonistic effect of Cd–Pb and Cu–Pb mixtures against *Vibrio fischeri* bacteria.

Basically, three possible types of interactions between two toxicants can be described as antagonistic, synergistic or simply additive. In the present study, all types of interaction were obtained in the *Daphnia* bioassay, while additive effect was not counted in the Microtox® assay. The extent of deviation from a simple additive effect generally depends on (1) the

measured parameter, (2) the chemical nature of toxicants and (3) the relative contribution of each toxicant to the toxicity of the mixture (Fulladosa et al., 2005). Furthermore, variation in sensitivity of *D. magna* and *V. fischeri* to the tested toxicants should be taken into account. According to Mowat and Bundy (2002), comparison of experimental results obtained from laboratory studies to those obtained using computational calculations based on additivity, as done in this investigation, can provide insight into areas requiring future research using a more mechanistic approach.

Finally, it may be convenient in this respect to mention that in toxicity evaluations one test cannot replace all the other tests, because the organisms' sensitivity varies considerably depending on the type of pollutant (Wängberg *et al.*, 1995). Therefore, the toxicological profile of an environmental toxicant is better understood when its impact is measured by organisms that represent different trophic levels (Choi and Meier, 2001). Several investigators (e.g., Choi and Meier, 2001; Ferrari and Ferard, 2005; Qu et al., 2013; Kokkali and van Delft, 2014) have recommended the user to select a battery of assays and biomonitors for a complete chemical toxicity assessment of an aqueous source considering selection of species from different trophic levels depending on the target matrix.

In light of the results of the present study, both *Daphnia* and Microtox® tests showed similar pattern of sensitivity to the single toxicants, but was dissimilar to the mixtures of heavy metals. On the other side, using shorter exposure time (ca. 30 min) with *Daphnia* bioassay when comparing its sensitivity with Microtox® may enable us to held comparisons at the same time nearly and gets results of reasonable acceptability.

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