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Acute and Synergistic Toxicity of Drugs in Water by Luminescent Bacteria Assay

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The acute toxicity in water of a series of drugs, including antibiotics, antihistamines, antifungals, steroidal and non-steroidal anti-inflammatories, was evaluated through the measurement of bioluminescence from the bacterium *Vibrio fischeri*. The drugs were spiked in water at concentration in the range 1.0-50.0 μg ml^{-1}, distributed over six concentration levels, and their toxicity evaluated in terms of response rate calculated along 30 min of incubation. The test was also applied to real river samples previously assayed by the HPLC method. The parameters LOEC (lowest observable effect concentration) and EC_{50} (half effective concentration) were calculated. Chlortetracycline, promethazine, betamethasone, ketoconazole and econazole were found to be very toxic. Diclofenac and ketoprofen showed toxicity only at the highest concentrations tested. Clindamycin, neomycin and oxatomide induced a decrease in bioluminescence but below the toxicity limits. In contrast, erythromycin and diphenhydramine showed an increase in bioluminescence, known as hormesis. The toxicity was amplified in samples containing drug mixtures, demonstrating additive or synergistic activity.

Keywords: Drug toxicity, Water pollution, Risk assessment, Synergistic effect, Luminescent bacteria test

INTRODUCTION

The presence of pharmaceutical compounds in environmental waters represents an important health emergency since the amount of drugs found in aquatic systems is considerably increasing [1-3]. Despite these alarming results, the toxicity of most drugs in the water is still unknown. Several studies have shown that the presence of pharmaceutical products in wastewaters is due to different causes. The excretion of drugs wastewater treatment significantly increases the concentration of drugs in the water [4]. Furthermore, when the drugs are used in creams and lotions, the not absorbed amount through the skin may be washed, and then found in the sewage system and in surface waters [5]. Due to the degradability of many drugs with light, monitoring of their photodegradation products is also very important [6-9].

The chronic effects on humans and aquatic ecosystem of the drugs released into the environment have been documented in some papers [10-12]. In these studies, chronic toxicity was reported as the development of adverse effects during long-term exposure to a toxicant or other stressors. *Daphnia magna* is used in most chronic toxicity tests applied to water matrices, in which survival and reproduction are evaluated during 21 days [12]. On the contrary, the acute toxicity of water contaminated by drugs is still little studied, probably because it is caused only by accidental exposure [13-15]. In this case, the adverse effects are investigated as a result of a single exposure or multiple exposures in a short period of time (usually less than 24 h) [15].

In the present study, the acute toxicity of twelve drugs belonging to different therapeutic classes was investigated. The tested compounds included four antibiotics, chlortetracycline (CHL), clindamycin (CLI), erythromycin (ERY), neomycin (NEO); three antihistamines, diphenydramine (DIP), oxatomide (OXA) and promethazine (PRO); two non-steroidal anti-inflammatories, diclofenac (DIC) and ketoprofen (KEP), one steroidal anti-inflammatory, betamethasone (BET); two antifungals,

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econazole (ECO) and ketoconazole (KET). These drugs were selected because already found in water, as detailed below, and for the high sales of pharmaceuticals containing them.

The use of antibiotics in medicine, farming and aquaculture results in a continual supply of these drugs and their breakdown products in the environment [16]. Prolonged exposure to low amounts of antibiotics in aquatic environments can lead to selective proliferation of resistant bacteria, which could transfer the resistance genes to other bacterial species. Azole substances are widely used in antifungal pharmaceuticals for humans and animals, biocides, and agricultural fungicides [17]. The extensive usage of these compounds may lead to substantial amounts of azole substance residues in the environment [18,19]; with potential adverse impact on endocrine systems of aquatic organisms [20]. Moreover, the sewage treatment plants have been pointed out as the major discharge source of anti-inflammatory drugs to the environment [21]. Few works describe the determination of antihistamines in drinking waters [22,23] and the evaluation of their toxicity in environmental waters [13].

In consideration of the numerous and different side effects on humans and animals, the toxicity evaluation of the drugs studied in water systems seemed to be useful and interesting. Several methods are available to test the toxicity profile of drugs in wastewater by means of bacteria, including *Vibrio fischeri*, *Pseudomonas* or *Escherichia coli*. In particular, the *Vibrio fischeri* bioluminescence inhibition bioassay could be applied for monitoring the toxicity on all types of matrices such as organic and inorganic compounds, wastewater, river water or treated wastewater. Compared to other tests, *Vibrio fischeri* shows several advantages due to its shorter duration, high sensitivity, low cost and simple procedure [24].

The Microtox Acute Toxicity Test was selected as a reference method. It is based on the measurement of light from the bioluminescent bacterium *Vibrio fischeri*, frequently used in ecotoxicological bioassays [25-27]. Recent studies have emphasized on the benefits of this procedure for its rapid, reproducible and cost effective features [28-30]. The test also allows to verify the bioluminescence increase, referred as hormesis, induced by drugs showing no toxicity but stimulatory effects [31].

Recent works have also studied the dose-additive combined effects of drug mixtures [32,33]. For this reason, the Acute Toxicity Test was also tested on a series of mixtures of the studied drugs to verify additive or synergistic effects.

EXPERIMENTAL

Chemicals

DIP, KET, DIC and NEO were purchased from Caelo S.p.A. (Italy), PRO, OXA, KEP and ERY from Acef S.p.A. (Italy), BET, ECO, CHL and CLI from Fagron S.p.A. (Italy). Water and ethanol were of instrumental purity grade (J.T. Baker, Holland). The kit of the test includes the freeze-dried organism *Vibrio fischeri*, formerly known as Photobacterium phosphoreum, NRRL No. B-11177, a reconstitution solution, a 2% NaCl diluent and a 22% NaCl osmotic adjusting solution (OAS), supplied by Ecotox LDS (Italy). All other reagents were of the highest purity commercially available.

Instrumentation

The toxicity tests were executed on a Microtox Model 500 Toxicity Analyzer (Ecotox LDS, Italy). The instrument was equipped with a 30 well-temperature-controlled incubator chamber, regulated at 15 °C. A small compartment, held at 5 °C, namely reactivation well, was used to store the bacterial suspension before dilution.

Absorption spectra were registered on the wavelength range of 200-450 nm in a 10 mm quartz cell, by means of a Perkin-Elmer Lambda 40P Spectrophotometer at the following conditions: scan rate 1 nm/s; time response 1 s; spectral band 1 nm. The software UV Winlab 2.79.01 (Perkin-Elmer, USA) was used for spectral acquisition and elaboration. The pH values were carried out by a Crison pH-meter GLP 22 (Levanchimica, Italy).

The HPLC analyses were performed using an HP 1100 Pump fitted with a diode array detector G1315B (Agilent Technologies, USA) and a Rheodyne 7725 manual injector at the following conditions: LC column C18 Gemini (Phenomenex, Italy) 250 × 4.6 mm × 5 μ, mobile phase of acetonitrile (40%)-phosphate buffer pH 5 (60%) for 15 min in isocratic conditions, flow rate of 1 ml min^{-1} at room temperature, UV spectra recorded between 200 and 450 nm

Table 1. Drug Concentration (μg ml^{-1}) of the One-component Samples

Antibiotics	ERY	0.93	4.64	9.28	18.57	27.85	46.42
	CLI	0.92	4.60	9.20	18.40	27.60	46.05
	NEO	0.92	4.59	9.18	18.36	27.54	45.86
	CHL	0.99	4.96	9.92	19.84	29.76	49.60
Antihistamines	DIP	0.97	4.83	9.67	19.32	28.99	48.32
	PRO	0.94	4.69	9.37	18.75	28.12	46.87
	OXA	0.92	4.62	9.25	18.49	27.74	46.23
Anti-inflammatories	DIC	0.97	4.87	9.74	19.48	29.22	48.69
	KEP	0.93	4.66	9.32	18.64	27.96	46.59
	BET	0.93	4.61	9.23	18.46	27.69	46.14
Antifungals	KET	0.99	4.94	9.87	19.74	29.61	49.36
	ECO	1.00	5.01	10.03	20.05	30.08	50.13

registered at 254.4 nm with reference at 360 nm.

Standard Solutions

Stock solutions of all drugs were prepared at the concentration of 2.0 mg ml^{-1} and used to prepare the test solutions in the range 1.0-50.0 μg ml^{-1}. The stock solutions of DIP, KET, DIC, NEO, PRO, CHL and CLI were prepared by directly dissolving the powder in pure water, while ERI, KEP, ECO, BET e OXA samples were prepared in hydroalcoholic solution because of their low solubility in water. In this case, the powder was dissolved in 20% ethanol and then diluting with water. Ethanol concentration was not more than 1%, not toxic for the test microorganism as reported in the standard international procedures [34] . A 1% hydroalcoholic solution was tested as a blank control to demonstrate the absence of toxicity on *Vibrio fischeri*. The drug concentration values of the test solutions are listed in Table 1.

A series of drug mixtures with concentrations in the same range above reported were also prepared to verify additive or synergistic effects. The concentrations of the drugs to be used in these analytical samples were selected by a random experimental design, according to the amount detected in real samples [16-23]. Mixtures with the drugs belonging to the same classes and mixtures with drugs showing different toxicity profiles were prepared and tested to verify potential additive or synergistic effects. The concentration values of the drugs are listed in Table 2.

Sample Solutions

Validation of the method was performed on six surface water samples spiked with the drugs in the same concentration range used to prepare the standard solutions. The samples were collected in different points of the rivers Crati and Busento, both located in Cosenza, a city of the Italian region Calabria. Samples (2 l) were taken at 0.7 m depth and stored in dark glass bottles, according to the guidelines concerning water analysis [35]. Crati is the first river of Calabria with a length of 91 km and an annual water average of about 36 m^3 s^{-1}. Busento river is a left side tributary of the Crati river which flows about 16 km and joins the Crati in Cosenza. These two rivers cross a high number of municipalities and are therefore much more sensitive to pollution from drugs than other rivers in the Calabria region that flow into less populated areas. These samples, suitably pre-treated, were analysed by HPLC

Table 2. Drug Concentration (μg ml^{-1}) of the Drug Mixtures

Mixture	ERY	CLI	NEO	CHL	DIP	PRO	OXA	DIC	KEP	BET	KET	ECO
1	46.42	46.05	45.86	-	-	-	-	-	-	-	-	-
2	46.42	46.05	45.86	0.99	-	-	-	-	-	-	-	-
3	46.42	46.05	45.86	4.96	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	0.99	1.00
5	-	-	-	-	9.67	-	9.25	-	-	-	-	-
6	-	-	-	-	28.99	9.37	-	-	-	-	-	-
7	-	-	-	-	4.83	4.69	4.62	-	-	-	-	-
8	-	-	-	-	9.67	4.69	9.25	-	-	-	-	-
9	-	-	-	-	4.83	28.12	4.62	-	-	-	-	-
10	-	-	-	-	-	4.69	27.74	-	-	-	-	-
11	-	-	-	-	-	-	-	4.87	4.66	-	-	-
12	-	-	-	-	-	-	-	19.48	18.64	-	-	-
13	-	-	-	-	-	-	-	19.48	18.64	0.93	-	-
14	-	-	-	-	-	-	-	4.87	4.66	4.61	-	-
15	-	-	-	-	-	-	-	9.74	9.32	9.23	-	-
16	4.64	-	4.58	-	9.67	-	9.25	-	-	4.61	-	-
17	-	-	-	0.99	-	0.94	-	4.87	4.66	-	0.99	1.00
18	4.64	-	-	-	-	9.37	9.25	-	-	-	0.99	-

[13,35] as described above. The HPLC analysis was repeated on the same real samples spiked with the drugs in the same concentration range used to prepare the standard solutions.

Toxicity Test Procedure

The Microtox reagent is a freeze-dried culture of V*ibrio fischeri* developed by Azur Environmental (formerly Microbics Corporation) in 1979, specially formulated in measuring acute toxicity because of its sensitivity to a broad range of toxicants. The selected protocol is known as Basic Test [36,37], specially used to test analytes slightly soluble in water.

A cuvette containing 1.0 ml of reconstitution solution was maintained in the instrumental reactivation well at 5.5 ± 1 °C for 15 min. This solution was then added into the freeze-dried Microtox reagent vial and shaken for approximately 30 s. The obtained opalescent suspension was left in the reactivation well at 5.5 ± 1 °C for 30 min and then stirred immediately before the test. Six standard solutions of each drug in the range 1.0-50.0 μg ml^{-1} were prepared. In order to ensure the bacterial viability, the pH value of each solution was confirmed between 6-8. 250 μl of OAS was added to 2.5 ml of the standard solutions in order to maintain 2% osmolarity and reducing the drug concentration to 91% of the initial value. After that, the

diluent was added to each drug solution to obtain four samples with a drug content of 45.5, 22.7, 11.38 and 5.69% (samples A). A sixth A sample, containing only the diluent, was used as a blank. All the operations were performed in the incubator wells at 15 ± 0.5 °C.

Bioluminescence evaluation, namely inhibitory percentage (I), was firstly recorded at zero time (I_0) on 0.10 ml of the bacterial suspension placed in six cuvettes (samples B). 0.90 ml of samples A were added to samples B and bioluminescence was measured after 5 (I_5), 15 (I_{15}) and 30 (I_{30}) min. Repeatability and sensitivity of the Microtox cultures were verified by a reference test using phenol 10 μg ml^{-1} as a standard toxicant.

The lowest observable effect concentration (LOEC) and the half effective concentrations (EC50) were selected as significant parameters to compare the toxicity of the drugs and their mixtures. LOEC values were validated by analysis of variance (ANOVA) and Dunnett's test [38] applied on data sets with an equal number of replicates. Dunnett's test compares group means and is specifically designed for situations where all groups are to be pitted against one reference group.

RESULTS

When the basic test was applied to the studied drugs, a mathematical algorithm calculated the toxicity in terms of effect percentage for the samples A. The percentage of response was calculated between I_0 and I_5, I_{15}, I_{30}, by using the following relationship:

$$\%Response = 100 - \{100 \times [(f_k \times I_c) - I_t)]/I_c\}$$

where I_c is the light emission of the control and I_t is the light emission of the samples (I_5, I_{15}, I_{30}). The f_k values (15 or 30 min) were calculated as the ratio I_k/I_0 measured on the control solution (diluent) where I_0 and I_k are the bioluminescence values before and after bacterial incubation, respectively.

The results were calculated as average from six determinations. Most of the compounds showed an inhibitory effect, with a marked decrease of bioluminescence (α-curve). Other drugs pointed out bio-stimulation, with a typical β-curve profile. Typical response curves are reported in Fig. 1.

A drastic decrease of bioluminescence in the first five minutes of incubation was observed for BET, CHL, CLI, DIC, ECO, KET and PRO, followed by a phase of plateau. The response after 30 min of incubation resulted in a wide range between 1 and 84% for the highest concentrations (Fig. 2).

DIP and ERY showed a clear bio-stimulation, with typical β-curves between 99% and 120%, compared to the saline controls. The luminescence increased constantly along the experiment time up to 30 min. This behaviour was shown by both drugs and at all levels of concentration tested. However, the bioluminescence for DIP increased with the increase of the drug concentration but ERY showed an inverted correlation between concentration and bioluminescence. Figure 3 shows the response profiles of DIP and ERY.

The NEO and OXA samples (Fig. 4) exhibited bioluminescence increase at low concentrations up to 23.0 and 14.0 μg ml^{-1}, respectively, and then inhibition at higher concentration values. KEP showed this behaviour too but the bio-stimulation was measured only with concentrations below 2 μg ml^{-1}.

This phenomenon, known as hormesis, represents an adaptive response characterized by a biphasic dose response directly induced or the result of compensatory biological processes [31,39].

Finally, toxicity of the samples containing mixtures of drugs variously combined was tested to assess possible synergy or inhibition effects. The percentage of response values are listed in Table 3.

Table 4 lists the LOEC values experimentally measured on the drugs showing bioluminescence inhibition. According to the guidelines ISO [34], a sample can be considered toxic when an effect greater than 20% is shown. Thus, the LOEC20 values were also measured and added in Table 4. Finally, the half effective concentrations (EC50) were calculated and also reported. EC50 was not calculated for CLI, NEO and OXA because of their low solubility in water.

Application of the Toxicity Test to Surface Waters

The potential interference of real matrices in the absence of the drugs tested was evaluated by applying the toxicity

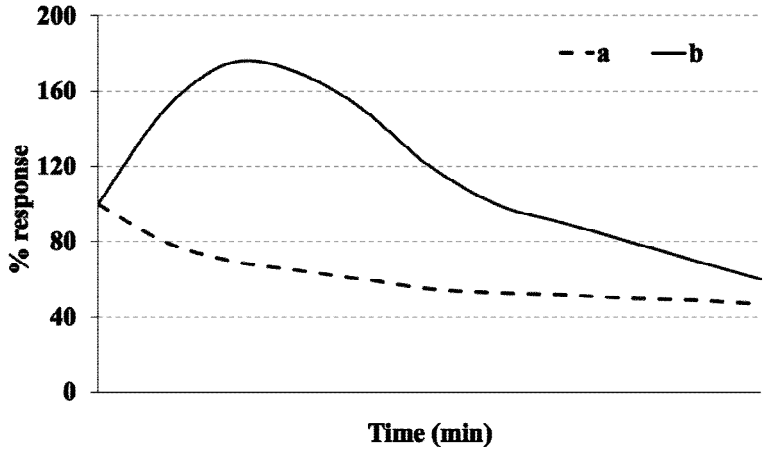

Fig. 1. Response profiles from bioluminescence test.

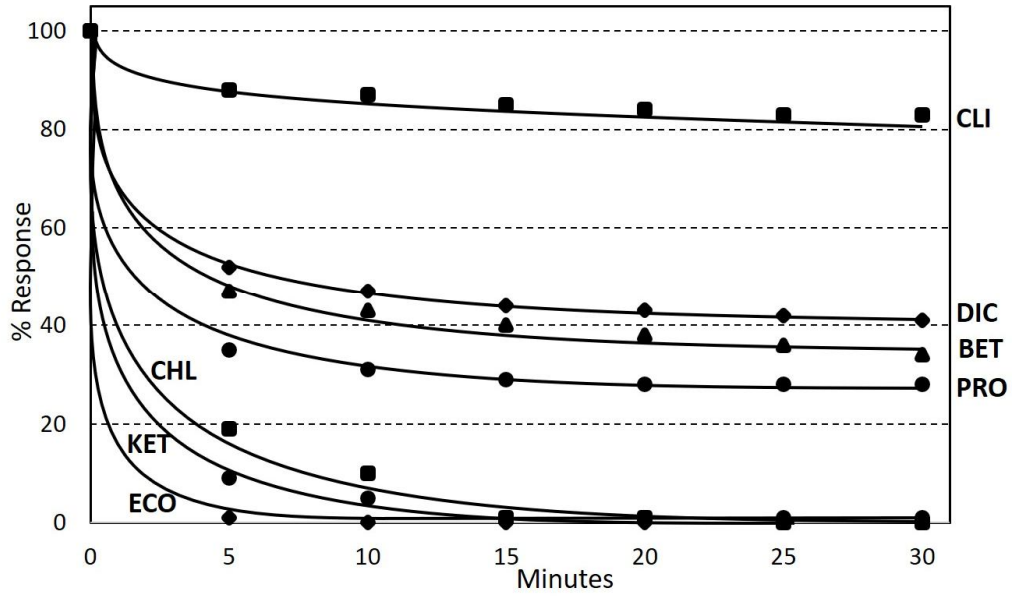

Fig. 2. The α-type response profiles from bioluminescence test on BET, CHL, CLI, DIC, ECO, KET and PRO solutions at the highest tested concentration.

test on six surface water samples collected from two rivers located in the Calabria region, named Crati and Busento (Italy). These samples were analysed by HPLC and subdued to the toxicity test. Four samples showed no toxicity whereas two samples gave low bioluminescence inhibition in the range 18-22%. Actually, in these two samples, HPLC showed the presence of PRO in a concentration of 1.98 and 2.31 μg l^{-1}, respectively.

A second aliquot of the same samples were then spiked with a single drug at a time. DIP, ECO and KEP, at a concentration of 20.0 μg ml^{-1}, were chosen because they had shown a behaviour different in the toxicity tests. Table 5 lists the toxicity values collected for the blank water samples and for the spiked samples. A second series of the

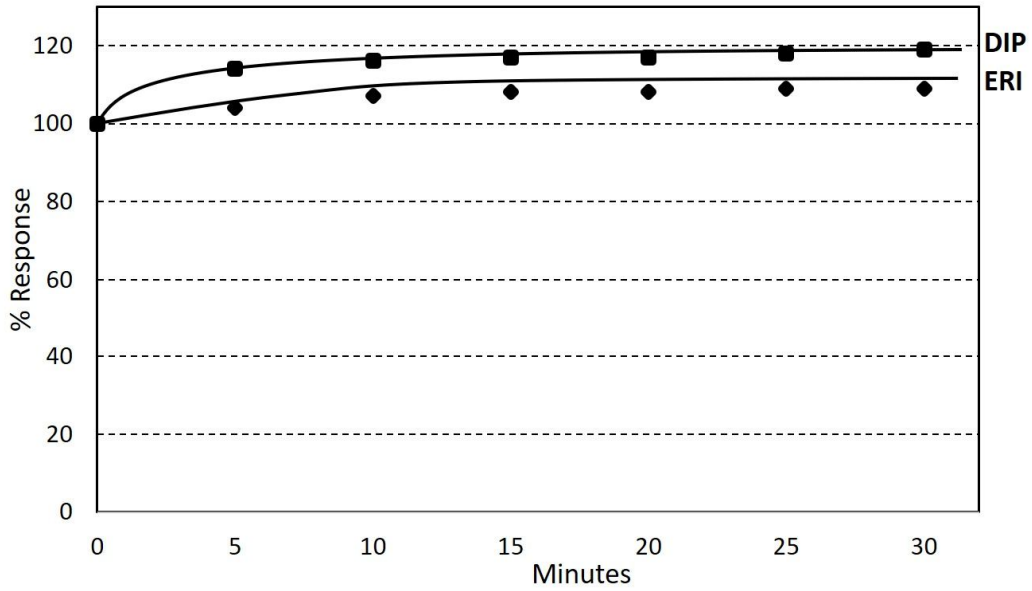

Fig. 3. The β-type response profiles from bioluminescence test on DIP and ERY solutions at the highest tested concentration.

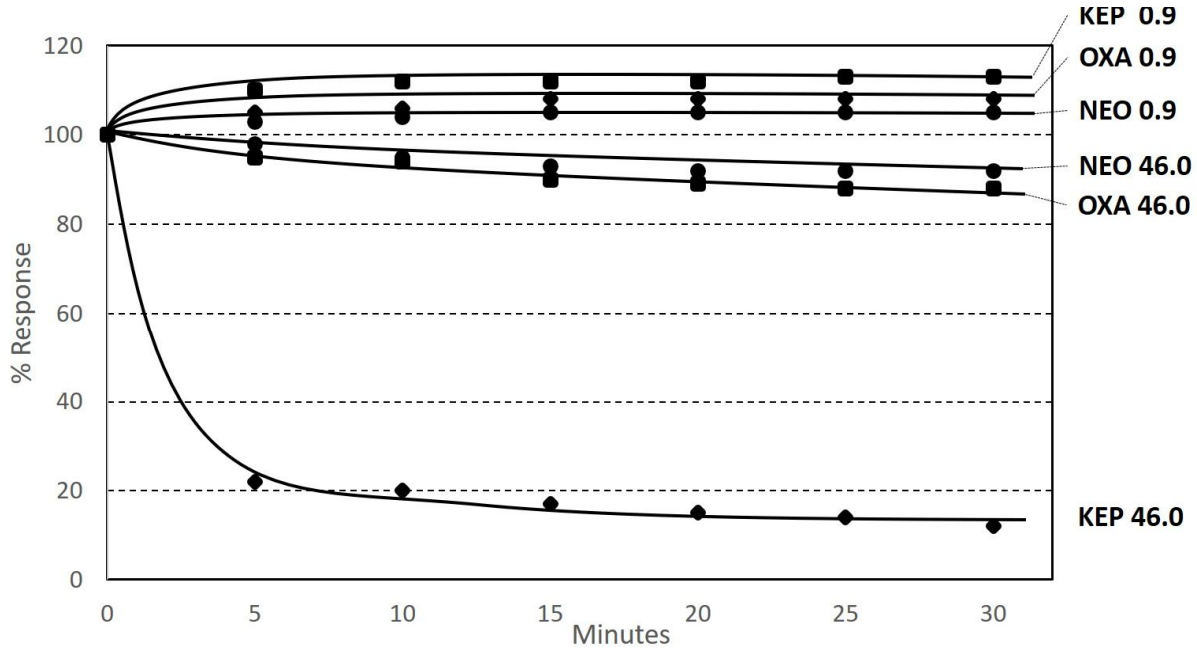

Fig. 4. The α and β-type dose-dependent response profiles from bioluminescence test on KEP, OXA and NEO solutions at the lowest and highest tested concentration.

blank water samples were finally spiked with the drug mixtures 16, 17, 18 (detailed in Table 2) and tested. The results are listed in Table 6. The toxicity values from spiked samples were compared with those from the drug standard solutions to evaluate whatever influence of the matrix on the toxicity results.

Table 3. Percentage Response from Drug Mixtures after 5, 15 and 30 min of Incubation

Drug mixture	%Response (mean of 5 determinations)		
	5 (min)	15 (min)	30 (min)
1	92.40	87.42	84.03
2	89.23	77.41	73.21
3	80.12	65.44	53.46
4	85.02	76.21	76.03
5	95.72	94.88	93.21
6	69.54	63.87	60.14
7	73.27	71.59	70.33
8	71.43	64.12	62.01
9	49.83	45.76	39.23
10	77.35	72.26	69.05
11	97.23	95.44	91.22
12	79.21	77.15	70.48
13	78.41	76.24	70.45
14	91.46	88.58	84.27
15	74.59	67.12	59.88
16	94.66	86.21	85.07
17	78.44	65.23	60.21
18	71.25	62.38	55.46

DISCUSSION

The Microtox system is increasing in popularity for toxicity measurement of environmental pollutants, including drugs, on living organisms. This method uses the measurement of light emission from *Vibrio fischeri* as test organism which depends on several factors affecting its functional metabolism. Usually, the variation of some basic parameters, as metals, oxygen or osmolarity, can influence the luminescence. In order to ensure the accuracy of the bioassay and to guarantee the appropriate experimental conditions, the testing medium used in the Microtox assay has been formulated to contain several ions as potassium, calcium and magnesium and sodium chloride in the diluent. The control of pH and temperature within a recommended range is also guaranteed. Since cell density can also affect bioluminescence, the bacterial suspension is used up to 5 h and the cell density is throughout monitored along the

Table 4. LOEC, $LOEC_{20}$ and EC_{50} (μg ml^{-1}) of the Drugs Showing Bioluminescence Inhibition

Drug class	Drug	LOEC	$LOEC_{20}$	EC_{50}
Antibiotics	CHL	0.05	1.88	9.92
	CLI	1.84	50.65	-
	NEO	27.54	73.40	-
Antihistamines	PRO	1.88	3.76	23.43
	OXA	27.74	64.72	-
Antinflammatories	BET	2.79	5.54	28.62
	DIC	4.87	19.48	38.96
	KEP	3.73	13.98	32.62
Antifungals	ECO	0.20	0.80	10.03
	KET	0.20	0.40	2.47

experiments [40].

The Microtox test provided the percentage responses along the incubation of bioluminescent bacteria with drug up to 30 min. LOEC was adopted as the main criterion to evaluate the toxicity of the drugs. This parameter was identified as the lowest concentration causing a statistically significant adverse effect on the exposed population of *Vibrio fischeri*, compared against a control sample. The LOEC, $LOEC_{20}$ and EC_{50} values experimentally measured are listed in Table 4.

A different response to the Microtox test was recorded between the drug classes and within the individual classes. The group of antifungals had the higher toxicity than individual drug alone. The antibiotics revealed a significant discordance. Actually, ERY showed hormesis whereas NEO and CLI caused bioluminescence inhibition, with a response under 20%. On the contrary, The CHL solutions were very toxic also at low concentrations. The antihistamine drugs have also shown different response profiles. Toxicity of PRO was above the permitted limit of 20%, whereas DIP and OXA resulted not toxic. The anti-inflammatories tested had similar behaviour showing toxicity at high concentration values.

With regard to the response of drug mixtures (Table 3), sample 1, containing the antibiotics ERY, NEO and CLI at the highest concentrations tested, did not show bioluminescence inhibition. These results confirmed the non-toxicity verified on the single-component solutions without additive or synergistic effects. The subsequent addition of the fourth antibiotic CHL to this mixture (samples 2-3) showed a significant increase of the toxicity. This increment was higher than the values measured on the CHL samples containing the same concentrations, showing a clear synergistic effect.

The mixture of the antifungals ECO and KET (sample 4), at the lowest concentrations, showed high toxicity, slightly increased if compared with the single drug solutions. The mixture of the antihistamines DIP and OXA (sample 5) confirmed the absence of toxicity shown by the individual drugs. However, the bioluminescence inhibition was higher than the sum of values from the one-component samples. On the other hand, the toxicity of all the mixtures containing PRO (samples 6-10) increased considerably in comparison with the PRO single-solutions at the same concentrations. The mixtures containing anti-inflammatories (samples 11-15) showed toxicity amplified respect to the solutions of the single components. The bioluminescence decreased with the increase of the drug

Table 5. %Response of Surface Water Samples Spiked with Drugs

Samples	%Response (mean of 5 determinations)		
	5 (min)	15 (min)	30 (min)
Surface water			
1	98.92	98.01	97.05
2	99.21	98.44	98.12
3	97.54	96.24	95.48
4	98.01	96.32	96.01
5	88.56	83.29	82.11
6	86.41	82.47	78.14
Spiked with DIP			
1	109.23	107.42	107.29
2	109.54	109.01	107.26
3	105.24	103.02	103.01
4	105.87	105.14	103.25
5	101.20	99.56	97.44
6	98.47	96.28	85.97
Spiked with ECO			
1	20.56	9.88	7.12
2	20.48	10.02	8.67
3	19.85	9.12	8.02
4	21.05	12.56	10.35
5	15.36	5.44	4.86
6	14.23	5.28	4.03
Spiked with KEP			
1	80.23	74.41	70.51
2	78.59	72.01	69.23
3	80.54	75.12	73.26
4	79.25	73.42	70.26
5	69.52	60.25	54.42
6	67.24	60.01	55.36

Table 6. Percentage Response of Surface Water Samples Spiked with Drug Mixtures

Samples	%Response (mean of 5 determinations)		
	5 (min)	15 (min)	30 (min)
Spiked with mixture sample 16 (ERY, NEO, DIP, OXA, BET)			
1	95.26	85.14	85.36
2	94.41	86.17	86.12
3	93.25	84.17	83.95
4	92.68	82.19	81.97
5	85.29	78.16	76.59
6	83.65	76.41	76.02
Spiked with mixture sample 17 (CHL, PRO, DIC, KEP, KET, ECO)			
1	79.41	65.25	62.30
2	77.42	63.87	59.84
3	79.85	64.58	61.47
4	78.03	65.28	62.71
5	67.29	54.19	51.89
6	65.41	53.16	50.24
Spiked with mixture sample 18 (ERY, PRO, OXA, KET)			
1	70.19	61.84	56.32
2	72.01	62.35	57.14
3	71.96	62.84	57.12
4	69.52	58.36	54.84
5	59.32	49.65	43.28
6	56.49	46.32	40.51

concentrations with a good dose-response correlation.

The toxicity results of the drugs mixtures belonging to different classes were unpredictable with respect to the single substance samples. The mixture 16, containing DIP, OXA, BET, ERY and NEO at low concentration values, showed a toxicity value under the limit permitted by law

[35], in agreement with the same results carried out from samples of the pure compounds. On the contrary, the mixture 17 showed a very high percentage of bioluminescence inhibition, despite the low concentration values of the components PRO, DIC, ECO, KET, KEP and CHL. Moreover, the toxicity was not dependent on the toxicity of the single solutions. The combination of PRO and KET both showing high toxicity when added to two non-toxic compounds, ERY and OXA (mixture 18), showed a clear synergistic effect with high toxicity not predictable from the values of the single solutions.

Our studies have shown the difficulty in predicting the toxicity of mixtures of drugs, even if some works report the prediction of the hormesis response in mixtures of drugs or other pollutants [31,33]. The phenomenon of hormesis due to drugs still does not yet have a certain explanation. However, the response of mixtures of chemicals giving hormesis could be hypothesized when a model of concentration addition is followed. In contrast, the toxicity of mixtures demonstrated to be not dose-additive but often causing synergistic effects. The analysis of blank river samples and spiked samples showed no significant influence of the matrix on the toxicity results. In contrast, when the toxicity was measured on real samples already influencing the bioluminescence, toxicity values suffered a deviation from their actual values in the presence of the drugs, according to potential additive or synergistic effects in a similar way as already demonstrated for the laboratory samples.

Accordingly, the toxicity of environmental matrices containing drugs mixtures is difficult to predict and therefore should in any case be monitored, also because the results can be affected by the presence of other pollutants of various origin.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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