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Toxicity assays applied to wastewater treatment

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Abstract

The utility and validity of toxicity tests for monitoring of wastewater treatment have been assessed. The evaluated acute toxicity tests have been *Vibrio fischeri*, *Selenastrum capricornotum* and *Daphnia magna* tests. The validation studies indicated that the acute toxicity tests can be considered as high sensitivity analytical tools to detect common environmental concentrations of the pollutants at concentration levels as low as ng l^{-1}. The toxicity tests showed to have discriminatory ability to distinguish between different degrees of toxicity, and the toxic specificity of the compounds on target organisms. Synergistic, additive or antagonistic effects were evaluated indicating the capacity of the toxicity test to assess the combined effects of chemicals in wastewaters. The reproducibility of these tests, calculated as relative standard deviation, is acceptable in the range of 5–22.3%. The application of multivariate date analysis proved that toxicity and chemical measures are complementary analytical tools for monitoring of wastewaters quality. The toxicity tests are useful analytical tools for screening of chemical analysis and as an early warning system to monitor the treatment of WWTPs. The use of single toxicity test or battery of tests is the best approach to evaluate the risk because they are reliable indices of the toxic impact of effluents in the aquatic environment. The toxicity tests were applied in the quality control of different European WWTPs.

Keywords: Toxicity; Wastewater; Validation; *D. magna*; *V. fischeri*; *S. capricornotum*

1. Introduction

The standard chemical quality of effluents, has traditionally been based on the control of global parameters such as biochemical oxygen demand (BOD), chemical oxygen demand (COD) or total suspended solid (TSS) according to the Urban Waste Water Treatment Directive 91/271/EEC [1]. More recently, the current guidelines (Directive 2000/60/EC and Decision 2455/2001/EEC) have been based in the detection of specific pollutants included in a list of priority organic pollutants [2,3]. The use of antifouling agents such as diuron or TBT, which are used for controlling growth of marine organisms, have been regulated because toxicity studies have demonstrated their potential negative impact on aquatic ecosystems. Pesticides, which are created to affect weeds, fungi or invertebrate organisms, are other chemicals included in this list because of their known toxic effects.

For monitoring the priority organic pollutants, several analytical methods such as gas or liquid chromatography coupled to mass spectrometry have been developed to assess and maintain the quality of surface waters, which is directly influenced by the wastewater treatment plants (WWTPs) [4–6].

In addition to priority organic pollutants, in the last few years, reports about residues of pharmaceuticals in surface wasters have increased, however there is still an almost complete lack of data concerning their effects on aquatic fauna. Environmental problems may arise when drugs enter via sewage effluent from domestic dwellings or hospitals because conventional biological treatments of WWTPs are insufficient to removal polar compounds. The occurrence of pharmaceuticals has generated a growing demand for analytical methods to monitor the quality of wastewater [7,8].

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The quality control of wastewater based on global chemical measures of total organic pollution load such as total organic carbon (TOC) or the detection of specific pollutants, is not sufficient to assess the environmental risk. They are not real measurements of the toxicity effects on the aquatic ecosystem because toxicity is a biological response. Therefore, effective tools for the evaluation of the negative effects on living organisms are need. The use of biological assays can provide a direct and appropriate measure of toxicity to complement the physicochemical measures of quality of wastewaters [9–11].

The acceptance of a toxicity test as an effective analytical tool requires guarantees of standardization and validation of the experimental procedure to evaluate its sensitivity, accuracy or precision.

In this sense, the main objectives of this work have been to assess the utility and validity of toxicity tests and to apply the toxicity tests for monitoring of wastewater treatment. The acute toxicity tests subject of study have been Biotox based on bacteria organism, Algaltoxkit based on microalgae and Daphtoxkit based on crustaceans. The utility and validity of these toxicity tests have been evaluated using the groups of chemicals mentioned above, including antifouling agents, pesticides and pharmaceuticals.

2. Experimental

2.1. Chemicals

The following group of chemicals were used for validation studies of acute toxicity tests. Pesticides: formetanate, carbofuran, cyromazine and fenamiphos. Antifouling agents: chlorothalonil, Sea nine 211 (4,5-dichloro-2-*n*-octyl-4-*iso*-thiazolin-3-one), irgarol 1051 (2-methylthio-4-*tert*-butylamino-6-cyclopropylamino-s-triazine), diuron, dichlofluanid, TCMTB (2-thiocyanomethyl-*thio*-benthiazole) and tributlytin (TBT). Pharmaceuticals: fenofibrate, gemfibrocil, clofibric acid, sotalol, betaxolol, metoprolol, bezafibrate and atenolol. Pesticides and antifouling agents were purchased from Ciba–Geigy (Barcelona, Spain), Rhom & Hass (Philadelphia, USA), Chemservice (West Chester, USA) and Riedel-de-Haën (Seelze, Germany). Pharmaceuticals were purchased from Sigma–Aldrich, Spain.

Individual stock solutions of the standard compounds were prepared in the specific culturing medium for the three toxicity tests. Mixtures of antifouling agents were prepared by combining individual stock solutions in a composition ratio 1:1. All working solutions were adjusted to a neutral pH.

2.2. Wastewater samples

Samples were colleted from nine different European wastewater treatment plants (WWTPs) designated in this work as follow: from WWTP1 to WWTP9. A pilot survey study was performed in WWTP1 over 5 months, with monthly sampling. Wastewaters were collected in Pyrex borosilicate amber glass containers previously rinsed with tap water and high-purity water and kept in the dark at 4 °C. Toxicity analyses were performed within 24 h of sampling. Before toxicity evaluations, the water samples were adjusted to a neutral pH.

2.3. Bacterial bioluminescence test

The experimental procedure for conducting the bacterial bioluminescence assay was based on the ISO 11348 standard protocol [12]. The bacterial assay used the commercially available Biotox test (Bio-Orbit Oy, Turku, Finland). The freeze – dried *V. fischeri* 1500 Reagent (*Vibrio fischeri* NRRL-B 11177) was reconstituted with 12.5 ml of 2% NaCl, and incubating at +3 °C for 10 min and at 15 °C for 15 min before use. The concentration of toxicants in the test which caused a 50% reduction in light (Inhibition = 50%) after exposure for 15 or 30 min was designed as the 15 or 30 min EC_{50} (effective concentration) value. Tests were performed at 15 °C. The measurements of light were made using a luminometer.

2.4. Daphnia acute immobilisation test

Daphnia tests were conducted following the European Guideline: "methods for determination of ecotoxicity; Annex V, C.2, *Daphnia* acute immbolisation test" (Commission of the European Communities, 1992) [13]. The *D. magna* bioassay used a commercially available text kit ((Daphtoxkit F^{TM} magna, Creasel, Belgium). Daphnids were bred in culture medium imitating natural fresh water. Test plates with *D. magna* neonates were incubated for 24–48 h at 20 °C in the dark. Acute toxicity was assessed by noting the effects of the test compounds on the mobility of *D. magna*. The neonates were considered immobile, if after 24 or 48 h of incubation with the toxicant they remained settled at the bottom of the test container and did not resume swimming within the 15 s observation period. The toxicity end-point (EC_{50}) was determined as the concentration of the toxicant required to immobilize 50% of the daphnids after exposure time.

2.5. Algae growth inhibition test

Algae tests were conducted following the European Guideline: "methods for determination of ecotoxicity; Annex V, C.3, Algal inhibition test" (Commission of the European Communities, 1993) [14]. The commercially available Algaltoxkit (Creasel, Belgium) was used. In the Algaltoxkit F^{TM}, the inhibition of the growth rate of algae *Selenastrum capricornotum* was measured. The initial algal culture was prepared from the immobilized algal beads as described in the instructions and the immobilized cells were pregrown in the sterile growth medium at 25 °C. The initial number of algal cells was adjusted to 10^6 cells ml^{-1} and the test tubes were

incubated at 25 °C for 3 days under continuous illumination. The activated culture was used as the inoculum for the toxicity experiments. Inhibition of the algal growth relative to controls was determined by measurements of optical density in a spectrophotometer at a wavelength of 670 nm. The 72 h EC_{50} value in this test was calculated as the concentration of the test substance which caused a 50% reduction in growth relative to the control.

3. Results and discussion

3.1. Validation studies

An idea on relative sensitivity of each toxicity test can be obtained as quantitative or qualitative information. The quantitative information has been obtained by calculating the toxicity quantifying capacity in terms of effective concentration (EC_{50}) and the toxicity detection capacity in terms of lowest observed effect concentration (LOEC). For this purpose, pesticide and antifouling compounds, which are necessarily toxic for target organisms as a requirement of their function, were selected to determine both EC_{50} and LOEC values.

The qualitative information has been obtained by evaluating the toxicity discrimination ability in terms of degrees of toxicity or by determining the toxic specificity of the compounds on target organisms.

According to EC_{50} values obtained for the selected compounds, the toxicity quantifying capacity of the three acute toxicity tests, are very heterogeneous showing ability to detect toxic effects in a narrow range of concentrations, from ng l^{-1} or μg l^{-1} to mg l^{-1}, specifically in the cases of *V. fischeri* and *D. magna* tests. The ranges of toxic or effective concentrations (EC_{50}) obtained by each test were 11 μg l^{-1} to 35.1 mg l^{-1}, 1 ng l^{-1} to 10.7 mg l^{-1} and 3 to 433 μg l^{-1} for bacteria, crustacean and microalgae, respectively (Table 1).

The ranges of LOEC values obtained for the same compounds were 2 μg l^{-1} to 10 mg l^{-1}, 0.8 ng l^{-1} to 3.5 mg l^{-1} and 100 ng l^{-1} to 150 μg l^{-1} for bacteria, crustacean and microalgae, respectively. By comparing of the ability to detect the lowest observed effect concentration for the toxicity tests, the most sensitivity test, was *D. magna* detecting toxic response at concentration levels as low as 0.8 ng l^{-1} when this organisms was exposed to TBT (Table 1).

The sensitivity cannot be taken as the only feature of the toxicity tests, other important property of the toxicity tests related to the sensitivity is the ability of the tests to distinguish the samples in different degrees of toxicity. Wastewater samples or standard compounds can be considered as "harmful to aquatic organisms" ($10 < EC_{50} \leq 100$ mg l^{-1}), "toxic" ($1 < EC_{50} \leq 10$ mg l^{-1}), "or very toxic" ($EC_{50} \leq 1$ mg l^{-1}) according to the toxicity categories established by the guideline (Directive 93/67/EEC) [15] and expressed in effective concentrations. In addition, for practical reasons, the toxicity category "not harmful to aquatic organisms" was added and used by us for the compounds with an EC_{50} above 100 mg l^{-1}.

Considering the toxicity ranking, *D. magna* and *V. fischeri* showed to have discriminatory ability and the selected compounds can be separated in toxicity categories. The tested compounds are illustrated in Table 1. For this study, compounds classified as pesticides in Section 2, were not analysed by algae test, but compounds such as chlorotalonil, diuron or dichlofluanid are used as pesticides or antifoulings and therefore the effects of different toxicants were evaluated for the three toxicity tests. Take into account the tested compounds, most of the compounds can be considered as very toxic for the three toxicity tests, especially for microalgae test, where all compounds were classified as "very toxic". This category was assigned to 63.63 and 36.36% of the compounds analysed by crustacean and bacteria organisms, respectively. A 27.27 and 9.1% of the compounds were classified as "toxic" for crustacean and bacteria tests, respectively. And toxicity category "harmful to aquatic organisms" was attributed to 9.1 and 27.27% of the compounds for crustacean and bacteria tests, respectively.

Furthermore, the toxicity discriminatory ability can be evaluated in terms of the capacity to distinguish the toxic specificity of the compounds on target organisms. Compar-

Table 1
Sensitivity of acute toxicity tests

Compounds	*V. fischeri* (15 min)		*D. magna* (48 h)		*S. capricornotum* (72 h)	
	EC_{50} (mg l^{-1})	LOEC (mg l^{-1})	EC_{50} (mg l^{-1})	LOEC (mg l^{-1})	EC_{50} (mg l^{-1})	LOEC (mg l^{-1})
Formetanate	7.4 ± 0.7	1.2 ± 0.15	0.077 ± 10^{-2}	$0.01 \pm 2 \times 10^{-3}$	n.a.	n.a.
Carbofuran	31.2 ± 2.5	1.38 ± 0.3	0.018 ± 10^{-3}	$0.0018 \pm 3 \times 10^{-4}$	n.a.	n.a.
Cyromazine	–	–	10.7 ± 0.8	$1.07 \pm 7 \times 10^{-2}$	n.a.	n.a.
Fenamiphos	35.1 ± 2.3	1.38 ± 0.2	$0.005 \pm 5 \times 10^{-4}$	$9.104 \pm 2 \times 10^{-5}$	n.a.	n.a.
Chlorothalonil	–	–	$0.028 \pm 6 \times 10^{-3}$	$0.014 \pm 2 \times 10^{-3}$	$0.0068 \pm 3 \times 10^{-4}$	$0.001 \pm 2 \times 10^{-4}$
Sea nine 211	0.012 ± 10^{-3}	$0.0017 \pm 8 \times 10^{-5}$	$0.004 \pm 9 \times 10^{-4}$	0.001 ± 10^{-4}	$0.003 \pm 3 \times 10^{-4}$	$0.0003 \pm 2 \times 10^{-5}$
Irgarol 1051	50.8 ± 10.5	10 ± 0.1	7.3 ± 0.8	2.4 ± 0.4	$0.108 \pm 7 \times 10^{-3}$	$0.005 \pm 5 \times 10^{-4}$
Diuron	–	–	8.6 ± 0.6	3.5 ± 0.3	$0.045 \pm 6 \times 10^{-3}$	$0.015 \pm 9 \times 10^{-4}$
Dichlofluanid	0.087 ± 10^{-2}	$0.027 \pm 3 \times 10^{-3}$	$1.05 \pm 7 \times 10^{-2}$	$0.62 \pm 3 \times 10^{-2}$	$0.133 \pm 8 \times 10^{-3}$	$0.05 \pm 8 \times 10^{-3}$
TCMTB	$0.058 \pm 7 \times 10^{-3}$	$0.03 \pm 4 \times 10^{-3}$	$0.046 \pm 8 \times 10^{-3}$	$0.021 \pm 4 \times 10^{-3}$	$0.433 \pm 9 \times 10^{-2}$	$0.15 \pm 7 \times 10^{-3}$
TBT	$0.011 \pm 8 \times 10^{-4}$	$0.002 \pm 3 \times 10^{-3}$	$1 \times 10^{-9} \pm 10^{-10}$	$0.8 \times 10^{-9} \pm 10^{-10}$	$0.003 \pm 6 \times 10^{-4}$	$0.0001 \pm 1 \times 10^{-5}$

n.a.: Not analyzed. Toxicity quantifying capacity in terms effective concentration (EC_{50}) and toxicity detection capacity in terms of lowest observed effect concentration (LOEC). Results are expressed as arithmetical mean (EC_{50}, LOEC) ± standard deviation (S.D.).

ing the effective concentrations of the compounds for different organisms, it is possible to known if a compound has specific toxic effects on a target organism. From the EC_{50} values (Table 1), pesticides such as fenamiphos or carbofuran which are insecticide compounds, can be considered as "very toxic" to invertebrate organisms *D. magna* (EC_{50} = 0.005 and 0.018 mg l^{-1}, respectively), and classified as "harmful" to bacteria *V. fischeri* (EC_{50} = 35.1 and 31.2 mg l^{-1}, respectively). Therefore, fenamiphos and carbofuran have a toxic specificity on target organisms and can be considered as insecticides.

However, when checking the formetanate EC_{50} values, which is also an insecticide (EC_{50} = 0.077 mg l^{-1} on *D. magna*), it can be considered as unspecific compound because it has toxic effects on non-target organisms (EC_{50} = 7.4 mg l^{-1} on *V. fischeri*). Antifouling compounds (or herbicide compounds) are a clear example of unspecific compounds, because all of the analysed compounds showed toxic effects on non-target organisms (*D. magna* and *V. fischeri*). These results reveals the high toxicity and toxic unspecificity of Sea nine 211, TCMTB and TBT. They suggest that the use of Sea nine 211 and TCMTB as additives in antifouling paints could have negative effects on aquatic ecosystems because these compounds have a very similar toxicity with respect to TBT, use of which is restricted by the European Union regulations [16,17].

On the other hand, the standard quality of wastewater is established based on the detection of single pollutants [2,3], and wastewater is a mixture of several compounds that can have different activities (bactericide, herbicide or insecticide activity). In this sense, it is relevant to consider the effect of the mixtures because the combined effects of compounds can have a greater negative impact than the individual constituents of the mixture [18].

The effect of a mixture can be predicted following the addition model and using the toxic units that are defined by the relationship between the concentration of the target compound in the sample and its effective concentration according to the formula defined by Sprague and Ramsay [19]. Following this model the theoretical toxicity of a mixture is the additive contribution of single compounds. But the experimental toxicity can have a different behaviour from this model. In contrast to the basic idea of this model, when the experimental toxicity of a mixture is higher than the theoretical or expected additive effects, the effect of the mixture can be considered as synergism, and on the contrary antagonism.

Antifouling agents were selected to evaluate the mixture effects because they are founded in the aquatic environment usually as mixtures because they are used in combination in the composition of paints [20,21]. To estimate the risk to aquatic life in this complex exposure situation, different antifouling agents were prepared in combination of two compounds and their toxicity were determined experimentally. The theoretical toxic effects were evaluated following the addition model. On the basis of the toxicity results obtained, the interaction between these chemicals and organisms do not lead only to additive effect. A comparison of the theoretical toxicity with the experimental toxicity determines if the toxic response can be additive, synergistic or antagonistic effect (see Table 2). Most of the mixtures produced higher toxic effects than the additive contribution of the compounds, showing synergistic effects. The increment between additive approach and experimental toxicity can be estimated by a factor between 3 and 10 times (Table 2), indicating the increase in the risk of these compounds for the aquatic environment when are used in combination.

Synergism has been seen for a clear majority of the cases, showing this effect in 60% of mixtures. For 13.3% of the mixtures, the toxic effect was antagonism and only 26.6% of the mixtures were consistent with the addition model. These results suggest that a consideration of single components alone

Table 2
Toxicity effects of antifouling mixtures

Binary mixtures	Bioassays					
	V. fischeri (15 min)		*D. magna* (48 h)		*S. capricornotum* (72 h)	
	$T.U._M$[a]	$\Sigma T.U._i$[b]	$T.U._M$	$\Sigma T.U._i$	$T.U._M$	$\Sigma T.U._i$
Irgarol 1501–diuron	26 Synergistic +	5.8	550 Synergistic +	183	0.36 Synergistic ++	0.01
Irgarol 1501–Sea nine 211	7.4 Antagonistic ++	333	550 Additive	581	5 Additive	5
Irgarol 1501–chlorothalonil	5.4 Synergistic +	0.87	1100 Synergistic	414	0.271 Antagonistic +	2
Irgarol 1501–dichlofluanid	9.4 Additive	15.6	132 Additive	160	0.152 Synergistic +	0.046
Irgarol 1501–TCMTB	333 Synergistic ++	25.6	330 Synergistic +	151	10 Synergistic ++	1.08

+: Factor ≥ 3; ++: factor ≥ 10.
[a] $T.U._M$: experimental toxicity.
[b] $\Sigma T.U._i$: theoretical toxicity.

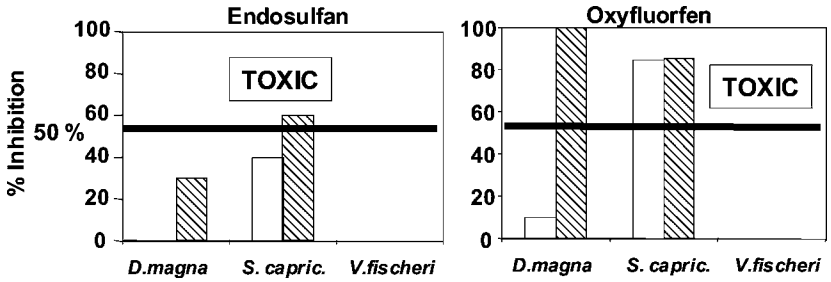

Fig. 1. Combined effects of pesticides in wastewaters. Toxicity effects of endosulfan and oxyfluorfen in culturing medium (opened bars) and wastewater (filled bars) at spiked concentrations levels of 2.5 $\mu g\,l^{-1}$.

is not sufficient for determining the environmental impacts of toxicants.

This consideration has been confirmed for real samples such as wastewater, which are a mixture of compounds and using pesticide compounds such as endosulfan or oxyfluorfen to evaluate the combined effects. For this purpose, both culturing medium and non-toxic wastewaters were spiked at concentration levels of these compounds that usually can be found in the aquatic environment (2.5 $\mu g\,l^{-1}$) [22]. The experiments were performed on the three toxicity tests.

Both compounds were selected for these experiments because endosulfan is a compound included in the list of the priority organic pollutants and therefore it is important to consider its toxicological behaviour in wastewater samples. And on the other hand, oxyfluorfen is not included in this list but it is a halogenated compound, the risk of which is unknown. The toxic effects of both compounds are different when comparing the single effect of these compounds in culturing medium and the combined effect in wastewaters (see Fig. 1). The results indicate an increase in the toxicity for wastewaters as consequence of the presence of several compounds in these samples, especially for microalgae test in the case of endosulfan and for crustacean test in the case of oxyfluorfen.

This approach also has been confirmed for other group of chemicals such as pharmaceuticals. These kinds of drugs have become a noteworthy contamination factor in surface water during recent years because they are not totally removed from wastewater treatment plants and also, actually their toxicological information is limited. Until now the mode of action of pharmaceuticals was not well enough understood to make general statements about their potential environment effects.

The EC_{50} values of the pharmaceuticals on the *D. magna* test, which has been considered as the most sensitivity toxicity test in this work, indicate that the single effect of these compounds in culturing medium can be considered as compounds with a low acute toxicity. The toxicity of most of the tested pharmaceuticals was similar and can be classified as "not harmful to aquatic organisms" with EC_{50} values ranging from 100 to >300 $mg\,l^{-1}$, except fenofibrate with EC_{50} value of 50 $mg\,l^{-1}$ and considered as "harmful". And on the other hand, these EC_{50} values also indicate that high environmentally unrealistic concentrations will be necessary to cause toxicity.

However, the experiments performed on *D. magna* test by spiking non-toxic wastewaters with the pharmaceuticals at concentration level as low as 2 $\mu g\,l^{-1}$, indicate that the combined effect of the single compounds in wastewaters have a greater negative impact (Fig. 2). The concentration level for spiking wastewaters was selected as a real approach to the occurrence of these compounds in the aquatic environment [8,23].

These results show the relevance to consider the ecotoxicological risk of these compounds because these compounds are not totally removed from wastewater treatment plants, as was previously mentioned, and higher negative effects of the mixtures can occur, even if the toxicity of the single compounds is low.

Another step in the validation studies has been to evaluate the reproducibility of the acute toxicity tests. Test reproducibility under consistent laboratory conditions was assessed from the calculated relative standard deviation of the same standard compounds in accordance with generally accepted laboratory statistical procedures. From the analysis of replicates, the reproducibility was found to be acceptable, as indicated by the coefficient of variation ranging from 5 to 22.3% for the three toxicity tests and comparing reported reproducibility of toxicity tests [24,25]. The variability for the toxicity tests was heteroge-

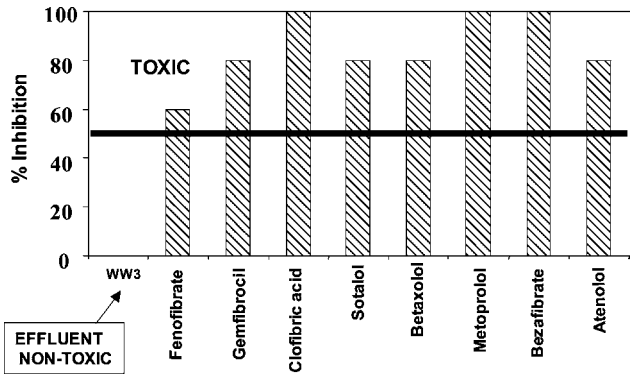

Fig. 2. Combined effects of pharmaceuticals in wastewaters. Toxic effects in spiked (non-toxic) wastewater at concentration levels of 2 $\mu g\,l^{-1}$.

Table 3
Predictive capability of the toxicity tests for the concentration–toxic effect approach

Pesticides	*V. fischeri*		*D. magna*		*S. capricornotum*	
	Correlation coefficient, *r*	Linear range ($mg\,l^{-1}$)	Correlation coefficient, *r*	Linear range ($mg\,l^{-1}$)	Correlation coefficient, *r*	Linear range ($mg\,l^{-1}$)
Formetanate	0.993	1.6–25.6	0.999	0.5–4.12	0.989	0.1–1
Fenamiphos	0.996	6.5–104	0.999	0.5–2.18	0.999	0.096–0.96
Carbofuran	0.991	4.4–70	0.979	0.7–3.07	0.978	0.018–0.18
Cyromazine	–	No toxic	0.999	1.06–4.6	0.986	1.07–10.08

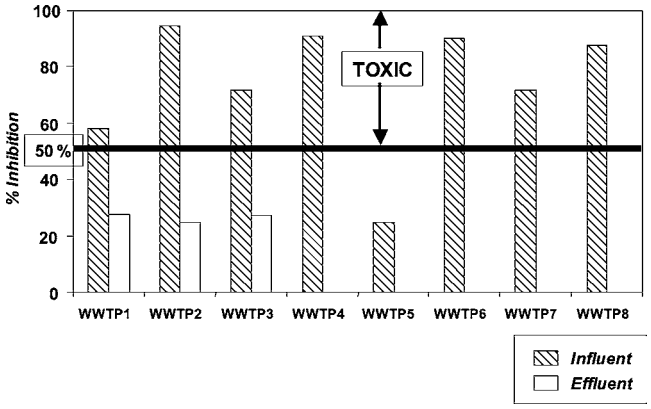

Fig. 3. Application of *V. fischeri* test as early warning system to detect toxic wastewater in the quality control of different European wastewater treatment plants.

neous, thus not indicating a test with better reproducibility.

For evaluating the predictive capability of the toxicity tests for the concentration-toxic effect approach, linearity was evaluated for different standard compounds. The obtained values of r^2 indicate that there is a significant correlation between concentration and toxic effect for the studied compounds, ranging from 0.978 to 0.999. However, in most of cases this correlation is defined in a narrow range of concentrations. Therefore, the predictive capability of the approach was found limited to these ranges (Table 3).

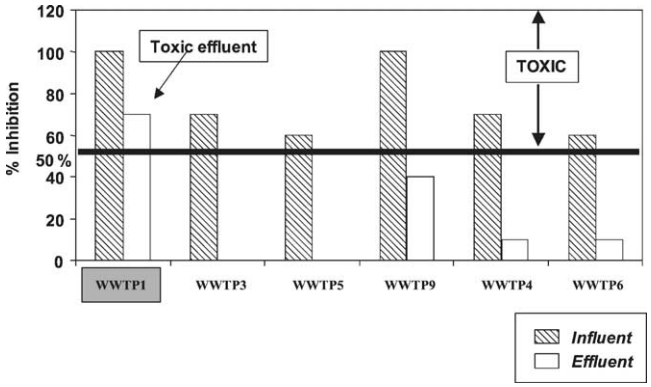

Fig. 4. Application of *D. magna* test as high sensitivity analytical tool to detect toxic wastewater in the quality control of different European wastewater treatment plants.

3.2. Application of toxicity tests for monitoring quality control of wastewaters

Considering the reproducibility, analytical precision of the tests and the wide range of toxicant concentrations that may be detected, the proposed toxicity tests appears to be technically sound. These toxicity tests have been applied as analytical tools for wastewater quality control.

One of the applications of these tests in the quality control of wastewater is as early warning systems. In other words, toxicity tests such as *V. fischeri* can detect toxic response in a short space of time (in 5 or 30 min). This property makes these tests useful analytical tools that allow having quick decisions regarding the convenience and need for treatment before effluent discharge.

V. fischeri was applied in a satisfactory way to the quality control of different European wastewater treatment plants (Fig. 3). The toxicity endpoint expressed as concentration

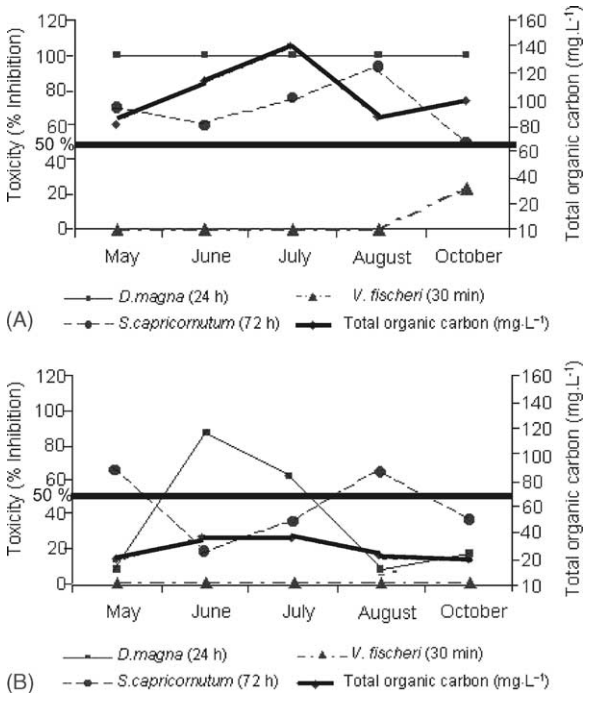

Fig. 5. Application of a battery of toxicity test in the quality control of WWTP1. Toxicity and chemical data (total organic carbon) for influent (A) and effluent (B) samples. A 50% of inhibition is marked in figure for indicating toxic effects.

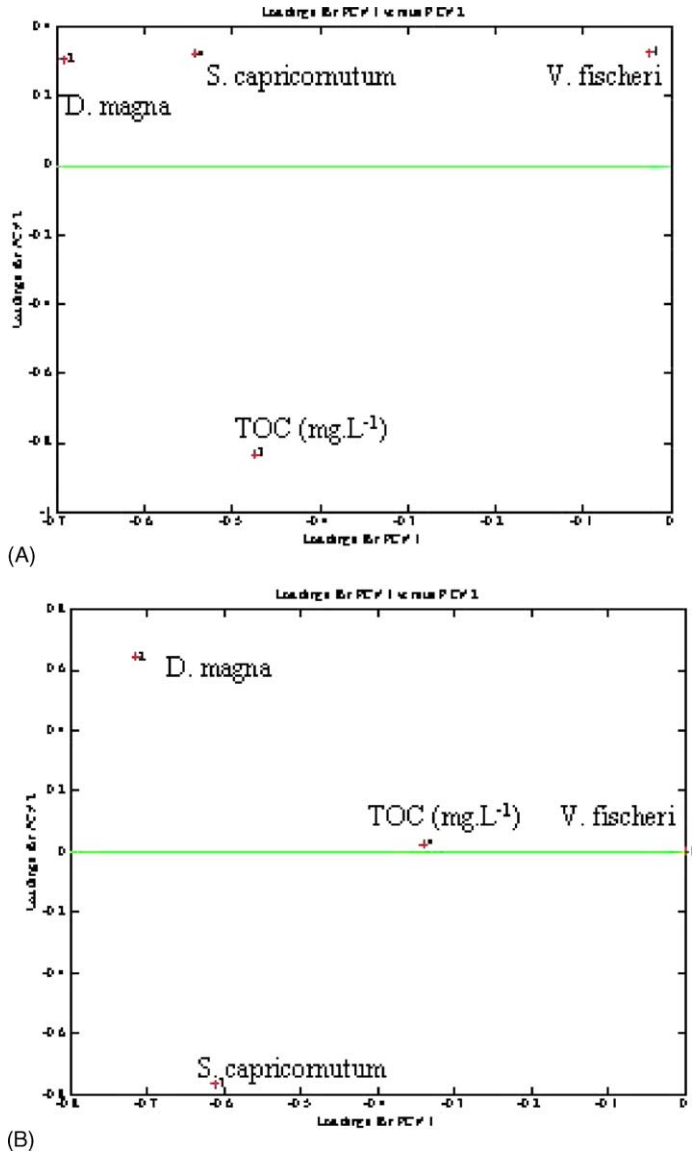

Fig. 6. Multivariate correlations between measured variables (toxicity and TOC results). Loading plot PC1–PC2 corresponding to the principal component analysis (PCA). Location of the three toxicity bioassays and TOC measurement in the two-dimensional space (A) influent and (B) effluent.

of wastewater causing 50% of inhibition showed that most of influents were toxic to *V. fischeri* and none of effluents were toxic to bacteria. Only the effluents corresponding to WWTP1–WWTP3 showed inhibition effects ranging from 20 to 30%, indicating the capacity and utility of this toxicity test to detect this inhibition limit values which are established as preventive measure for the effluent discharge [26]. The toxicity assessment of the wastewaters (influents and effluents) provided a real approach of the effluent risk and allowed to confirm the efficiency of the WWTPs to remove toxic compounds.

Another application of the toxicity tests in the quality control is as a screening analytical tool. This approach, commonly referred to as toxicity identification evaluation (TIE) [27] can help WWTPs or industries to develop cleaner production strategies because the coupling of physical/chemical with toxicity analysis allows for the isolation and identification of toxicants. Thus, the use of toxicity tests allows for the limitation of chemical analysis (i.e. GC-MS, LC-MS) to toxic wastewater samples only. This fact, supposes a significant reduction in the cost of quality control analysis.

The high sensitivity of toxicity tests makes these tests a useful analytical tool in quality control. It means that toxicity tests like *D. magna* are available to detect toxic pollutants at concentration levels as low as ng l^{-1}, which are environmentally realistic concentrations. The toxicity quality control performed in the WWTPs using *D. magna* test permitted the detection of toxic effluent from WWTP1, for which toxicity results for *V. fischeri* were not toxic (Fig. 4).

Therefore, the use of single organisms to evaluate wastewater toxicity cannot provide a real assessment of the risk. An appropriate way to assess the risk of wastewaters is the use

of a battery of toxicity tests with representative organisms of different biological organization.

The application of a test battery appears to be a useful approach because it offers a more complete assessment of the wastewater risk. The different sensitivity of toxicity tests allows the detection of unwanted toxic effluents. According to this proposition, a study for controlling the wastewater quality was performed in WWTP1 by applying a test battery and additionally, chemical analyses were included. This study was performed over 5 months as it is shown in Fig. 5. The results from the toxicity analyses, on the selected organisms, expressed as percentage of inhibition were different. *V. fischeri* test was not available to detect toxic effects and on the contrary, all the influent and some effluent wastewater samples (corresponding to May–August) were toxic to *D. magna* and *S. capricornotum* tests (Fig. 5). Therefore, results from the analyses of effluents indicate that the treatments performed in the WWTP1 are not sufficient to remove toxic compounds.

In order to compare toxicity and chemical analyses, the TOC measurements were evaluated. Considering both toxicity and TOC results, we observed that no correlation was founded. The TOC levels corresponding to toxic influents (for *D. magna* and *S. capricornotum* tests) ranged from 86 to 135 mg l^{-1} and effluents with permissible TOC levels (between 20 and 34 mg l^{-1}) according to the guidelines, were toxic. Therefore, global chemical parameters such as TOC do not offer real information about biological effects or the impact on aquatic ecosystems (Fig. 5).

The combined use of toxicity and chemical measures appears to be necessary in the quality control of wastewaters. In these cases, it may be appropriate to use currently accepted estimation methods [28,29] to assess the complementarity and correlation between chemical and toxicity data. Fig. 6 shows the results obtained by application of principal component analysis (PCA) to toxicity and TOC measurements for both, influent and effluent data, respectively. Two main principal components were obtained in both cases, describing as much as 96.38% of data variance for influent data and 72.22% of data variance for effluent data. Since all variables were measured at similar scales (between 0 and 100), no data pre-treatment was applied before PCA. Fig. 6 (A) gives PC1 (principal component) versus PC2 loadings plot for influent data. First PC is describing toxicological responses, specially of *D. magna* and *S. capricornotum* and TOC (largest negative PC1 loadings), whereas minor second PC describes mostly only TOC values. For influent data there is no clear correlation between the different toxicity bioassays and TOC measurements. *V. fischeri* gave the lowest loading values for both PCs, in agreement with its poorest discriminating toxicity abilities. Effluent data gives a different picture to influent data, showing the WWTP effects. Largest PC1 loadings were obtained in this case for *D. magna* and *S. capricornotum*, which are the best indicators of toxicity. PC1 describes the main source of toxicity measured by the results provided by these two variables (or tests). However, these two variables are clearly distinguished by PC2, giving opposite sign loading values, i.e. they are inversely correlated in PC2, showing that there are samples giving high input values of one of them and low input values of the other. Therefore, PC2 describes a source of toxicity variation detected differently by *D. magna* than by *S. capricornotum*. TOC loading values were intermediate for PC1 and close to zero for PC2. *V. fischeri* loading values were always low. What is clear from all these results is that TOC measurements and *V. fischeri* are not adequate for toxicity analysis, and that *S. capricornotum* and *D. magna* give different information about toxicity levels. Finally, different behaviour of influent and effluent data, shows that the combined use of toxicity and chemical measurements should be used in a complementary way in the control of wastewaters.

4. Conclusions

The acute toxicity tests have high sensitivity to detect common environmentally realistic concentrations of pollutants at concentration levels as low as ng l^{-1} and have toxicity discriminatory ability to distinguish different degrees of toxicity and toxic specificity of the compounds on target organisms. Synergistic, additive or antagonistic effects were evaluated indicating the capacity of the toxicity test to assess the combined effects of chemicals in wastewaters. The reproducibility calculated as relative standard deviation is acceptable with a range between 5 and 22.3%. These tests can be considered as useful analytical tools for screening of chemical analysis and early warning system to monitor the treatment of WWTPs. The application of multivariate date analysis proved that toxicity and chemical measures are complementary analytical tools for monitoring of wastewaters quality. And the use of single toxicity test or battery of tests is the better approach to evaluate the risk because they are reliable indices of the toxic impact of effluents in the aquatic environment.

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