Evaluation of an automated luminescent bacteria assay for in situ aquatic toxicity determination

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ABSTRACT

A new system for monitoring toxicity TOXcontrol® (MicroLAN BV, The Netherlands) has been used to assess the toxicity of a selection of priority or emergent compounds in the laboratory. In this study, inhibition curves and EC50 – Effective Concentration causing 50% inhibition – of selected compounds (including pesticides, pharmaceuticals, surfactants and metals commonly detected in surface or drinking waters) were determined. This new technology is based on the measurement of Vibrio fischeri bioluminescence inhibition (ISO 11348). The main advantage of this equipment, compared to other laboratory assays, is the fully automation of the procedure. The instrument can be operated online in a simple, rapid and reproducible way. The variability of the results obtained with the TOXcontrol® biomonitoring system has been studied. A comparison with standardised technology based in V. fischeri (Microtox®) and additional test with Daphnia magna for selected organic compounds is presented. The results show that the methodology based on the TOXcontrol® system being validated is accurate and reproducible enough enabling this system to be used as an on-line automatic alert system to detect abnormal concentrations of toxic compounds.

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1. Introduction

Legislation related to the preservation of quality of water bodies is becoming more stringent both at national and international levels. In the European context, the Water Framework Directive (WFD) (2000/60/EC) is an example of the new risk-based attitude adopted in terms of environmental impacts. The WFD requires both a good chemical and biological status of water bodies; therefore new monitoring assays to detect changes in water quality on short notice are required. Although a list of priority substances to be determined in surface water bodies was established by Daughter Directive (2008/105/EC) according to their harmful potential, other substances exist that are not regulated nowadays, called emerging contaminants, suspicious to cause effects on living organisms but their concentrations are not routinely measured. A big number of studies have been performed with the aim of identifying those contaminants present in water flows (von der Ohe et al., 2011).

The objective of the Drinking Water Directive (DWD) (98/83/EC) is to protect human health in the European Union and to make sure that the water is healthy and clean. For this purpose, DWD sets standards for the most common substances that can be found in drinking water. In the DWD a total of 48 microbiological and chemical parameters must be monitored and tested regularly. Thresholds of these substances (including pesticides, metals, bacteria, etc.) are based on the potential detrimental effects to organisms.

Chemical status of water bodies is determined in most cases by spot sampling campaigns and laboratory determinations. This off-line methodology is slow and in some cases ineffective to respond to sudden quality changes as a result of possible contamination. There is the need to use some alternative monitoring tools to complement traditional ones to provide a comprehensive overview of water quality. Biomonitoring protocols use sentinel species, defined as any living organism to be used as an indicator of the presence of a pollutant or the toxicity of a contaminant (Amiard and Amiard-Triquet, 2008). Biological analysis with the help of different biosensors is considered a highly informative testing system since the knowledge of the chemical characteristics of pollutants does not always provide sufficient information about their toxicity and danger for living organisms (Trybulsik and Sazykina, 2010).

Toxicity tests are one example of a commonly used biomonitoring tool. In this case, a biological response of a test organism is measured as the result of the combined effect, including antagonism and synergism, of the mixture of all potential contaminants contained in water. One of the most common biosensor used for the risk assessment in
aquatic environment is based on the inhibition of luminescence produced by marine bacteria *Vibrio fischeri*. The use of this bioluminescence-based assay has been standardised (ISO, 11348-3) for the regulatory purposes because of its sensitivity and short time required to perform the test (Cox et al., 2007). Toxicity is usually represented as EC50, i.e. effective concentration of the tested chemical at which 50% of luminescence inhibition is observed.

Since the early 80’s, many studies have been performed to determine toxicity of different families of chemicals at laboratory. In one of these studies, the drugs investigated (ibuprofen, ketoprofen, naproxen, diclofenac, salicylic acid and gemfibrozil) showed very similar EC50 values when comparing two techniques using the luminescent bacteria assay (14–36 mg L\(^{-1}\) for Microtox\(^{®}\) and 12–43 mg L\(^{-1}\) for ToxAler\(^{®}\) (Farré et al., 2001a). Other comparison performed with surfactants showed larger variability (0.36–127 mg L\(^{-1}\) for ToxAler\(^{®}\) and 0.40–379 mg L\(^{-1}\) for Microtox\(^{®}\)) (Farré et al., 2001b). Antibiotics showed a moderate toxicity on *V. fischeri* and no significant effects at the maximum concentration tested corresponding to the water solubility were observed, but the compounds atrazine, simazine, glyphosate, deltamethrin and leucomalachite green showed EC50 values greater than 10 mg L\(^{-1}\), and therefore they were classified as harmful, according to the Global Harmonized System of classification (UNECE, 2011; Hernando et al., 2007). EC50 values have also been determined for triclosan (0.28 mg L\(^{-1}\)) and methyl triclosan (0.21 mg L\(^{-1}\)) (Farré et al., 2008).

In other studies, EC50 values for individual metal added in the ionic form were obtained for cadmium, chromium, copper, lead and zinc using *V. fischeri* at exposure time of 30 min. Values ranged from 0.12 to 13.8 mg L\(^{-1}\) (Guedéguen et al., 2004). In another study, the toxicity of the 13 priority pollutant metals and non-metals was evaluated using the Microtox\(^{®}\) chronic toxicity test. Among the metals, beryllium was found to be the most toxic in the test while thallium was the least toxic (Hsieh et al., 2004). The toxicity of arsenic, cadmium, lead, and mercury has been tested individually and as a composite mixture using the Microtox\(^{®}\) biosassay. Among the individual metals and non-metals tested, the ranking of toxicity was mercury in first place, lead, cadmium and arsenic (Ishaque et al., 2006). More tests have been performed in assessing toxicity of metals based on this bacterium (Codina et al., 1993; Cho et al., 2004; Rosen et al., 2008; Tsybulskii and Sazykina, 2010). The response of luminescent bacteria to mercury compounds has also been investigated (JM Ribo et al., 1989).

Further studies have been performed for assessing the performance of different sensors. Ten toxicity sensors utilising enzymes, bacteria, or vertebrate cells were compared to rapidly identify toxicity in water samples containing one of 12 industrial chemicals. Microtox\(^{®}\) was the highest scored at the ranking responding to 6 out 12 compounds (van der Schalie et al., 2006). Another study performed a comparison between *V. fischeri*, *Selenastrum capricornutum* and *Daphnia magna* tests. A selection of pesticides, antifouling agents and pharmaceuticals were tested. *D. magna* resulted as the most sensitive test. *D. magna* and *V. fischeri* showed both to have discriminatory ability to separate compounds in different toxicity categories (Hernando et al., 2005). At a different study, the inhibitory effects of 81 chemicals, after 5 min contact time, were calculated at eight concentrations using three commercial assay systems based on the luminescent bacteria toxicity assay (ToxAler\(^{®}\), Microtox\(^{®}\) and LUMISStox\(^{®}\)). Only five compounds gave EC50s that varied more than three-fold between assays (Jennings et al., 2001). Bioluminescent bacteria have been applied frequently to monitor toxicity in several environmental applications like wastewater, seawater, surface and ground water, tap water, soil and sediments, and air (Girotti et al., 2008; M.C. Riva et al., 2007). However those tests are based on discontinuous samples and provide only a partial response in terms of compliance with WFD and related legislations.

The objective of the study was the test of the automated equipment TOXcontrol\(^{®}\) for measuring toxicity of contaminants that can be found at surface and drinking waters. The response of the equipment at laboratory against a selection of contaminants has been assessed. Response for the organic compounds has been compared against response of toxicity tests performed with Microtox\(^{®}\). As it is a non-specific technique for measuring global toxicity, it’s important to perform tests at laboratory with water spiked with single analytes so specific response to one compound can be obtained. Only one reference has been found concerning validation of on-line toxicology sensors, in which TOXcontrol\(^{®}\), was tested in combination with a spectrophotometer for the monitoring of sodium cyanide, azinphos-methyl, sodium fluoroacetate and difenacoum in surface waters (Appels et al., 2007). Additionally, sensitivity of *V. fischeri* for selected organic compounds has been compared against sensitivity of *D. magna*.

On one side, contaminants were selected taking into account their occurrence in semi-arid basins, like the Llobregat river (SE Spain) where a high contribution of treated wastewaters discharges in the total flow of the river is expected as the low flow makes dilution factor almost negligible. The pollutants can become a potential risk to the receiving bodies and, in addition, to the production of drinking water (Gasperi et al., 2008; Muñoz et al., 2009). On the other side, information on the possible presence of metals in drinking water according to their potential of being transferred from the corrosion of internally unprotected metallic water pipelines were taking into account for metals selection (Imran et al., 2009).

Experiments have been performed to assess the aquatic toxicity of a selection of target compounds. Those substances have been selected in view of their occurrence in surface water, especially in semi-arid regions where the water stress leads to low flow rates and higher concentration of dissolved pollutants, and their potential to show toxicity (González et al., 2012) and the possible presence in drinking water mainly due to migration of pipe material (Veschetti et al., 2010).

### 2. Materials and methods

#### 2.1. Reagents and standard solution preparation

Chemical standards for terbuthylazine, triclosan, dimethoate, sodium dodecylbenzenesulfonate (SDBS), diazinon, sodium diclofenac, nonylphenol, propanil, 2-methyl-4-chlorophenoxyacetic acid (MCPA) and iron (III) sulphate hydrate were purchased to Sigma Aldrich (St. Louis, MO, USA). Standards for chromium (III) nitrate nonahydrate, copper (II) sulphate pentahydrate, lead (II) nitrate and nickel (II) sulphate hexahydrate were purchased from Merck (Darmstadt, Germany). HPLC water and dimethyl sulfoxide (DMSO) were also purchased from Sigma-Aldrich. The freeze dry luminescent bacteria *V. fischeri*, cultivation media, zinc sulphate (2500 mg/l) and 20% sodium chloride were supplied by MicroLAN (Waalwijk, the Netherlands). Stock solutions were obtained by dissolving metal salts in HPLC water and organic compounds in DMSO (0.2% v/v). The pH of solutions was not adjusted but it was monitored and no sudden changes of pH were reported.

Luminescent marine bacteria of the species *V. fischeri* (NRRL B-11177) for the Microtox\(^{®}\) determinations were obtained from SDI (Strategic Diagnostics Inc. Newark, DE USA). Luminescent bacteria for TOXcontrol\(^{®}\) determinations were obtained from Microlan B.V. Waaljik. NL. *D. magna* used as test organisms were obtained from a CRIT-UPC maintained culture.

#### 2.2. On-line toxicity monitoring

The instrument for online monitoring of toxicity of water samples is the TOXcontrol\(^{®}\) Toxicity Monitoring System manufactured by Microlan, B.V.
TOXcontrol® is an advanced automatic on-line water toxicity monitor based on the use of luminescent bacteria (V. fischeri) to give an indication of the toxicity of the contaminants in water as a function of the emitted light. After the mix of the luminescent bacteria and the water sample, the toxic material in the sample would alter the metabolism of the bacteria. The decrease of light intensity is directly proportional to the concentration of toxic substances in the sample.

The equipment works on the same basis as the certified methodology for the analysis of toxicity with V. fischeri (ISO 11348-3) but adapted to automatic equipment. The analyser inside the equipment is being designed to work in two parallel lines. Scheme of the modular parts can be seen in Fig. 1. While one of the lines is preparing the mixture of the bacteria solution with sample water and measuring the effect of that sample on bacteria, a second line is using reference water instead of sample water, as the output data is a relative measurement of the light emitted by the first line compared to the second one. Before performing the measure, dry frozen bacteria need to be rehydrated by adding cultivation media and mixing under controlled temperature conditions during several days (2 days at 2 °C, 20 h at 20 °C, 3–5 days at 4 °C for maintenance until used).

The bacteria module, which keeps 5 °C inside all the time, is filled with luminescent bacteria suspension. The luminescent bacteria cannot suffer constant alterations in temperature. After the luminescent bacteria are being taken out of the cup, they are mixed with sample and a sodium chloride solution (2 g in 100 ml of water) in the mixing module. The temperature is kept constant at 15 °C during incubation time (15 min or 30 min). EC50 values have been calculated according to “dilution series procedure” of TOXcontrol® instrument for each target compound. In this procedure, different concentrations of the same compound were tested automatically to obtain the curve concentration–response that will show information for the EC50 calculation.

According to this procedure, 50 μl of bacteria suspension is mixed with 5 ml of the sodium chloride solution for the preparation of the bacteria solution. This first part of the process takes 5 min. Measurements of luminescence are done for assuring activation of bacteria. Then a volume of standard working solution, in substitution of the real sample, is taken from a vial and diluted in another volume of the sodium chloride solution to constitute a final volume of 5 ml of diluted sample. When both solutions are mixed, a final volume of 10 ml of bacteria in contact with the analyte is being incubated during 15 or 30 min. Variables to be specified in this procedure are: the volume of sample to be taken, the number of dilutions per series (being 0 ml of sample the first point, chosen volume of sample as the second point, and doubling the volume of sample for every successive point), and the number of series (repetitions) to be performed.

Due to the low solubility of some of target compounds, solutions for organic analytes were prepared in DMSO 0.2% v/v. Such a low concentration of DMSO has been proven during the preparation of the experiments to show no response for V. fischeri (Hernando et al., 2007). Series of five concentrations of each test solution were prepared and the measures were performed in triplicate.

A quality control of the performance of the tests was executed. Positive and negative controls of the measurements were done before and after each series of measurement. Negative control was done using reference water. Measurement was accepted if toxicity was low.
value was between -3 and 3%. Positive control used zinc sulphate (2500 mg L⁻¹). If toxicity is over 60%, the series of measurement are accepted. If the negative or positive control is out of range, the series of measurements are excluded and the test repeated.

The sigmoidal inhibition curves were calculated with the help of the Prism 4 software (GraphPad Software Inc.).

2.3. The Microtox® toxicity assay

The experimental procedure to determine the toxicity using the Microtox® Toxicity Analyzer has been described (Kaiser and Ribo, 1988) and it is based on the standardised method for the analysis of toxicity with V. fischeri (ISO 11348-3). The toxic effect of an aqueous sample is determined as the concentration of the sample causing a 50% reduction on the light emitted by the bacteria, after a pre-determined exposure time (5 min, 15 min or 30 min). For regulatory purposes an exposure time of 15 min has been established.

In this procedure the freeze dried-bacteria is reconstituted with water, to provide a stock suspension of test organisms that is kept at 5 °C and used to perform the test. The luminescence of the bacteria after exposure to the toxic sample is compared with that of a control at the test temperature (15 °C). A correction factor has to be applied due to the loss of luminescence of the control (reduction in light emitted without exposure to the toxicant). A 10% of the Microtox Osmotic Adjusting Solution must be added to all samples to provide osmotic protection to test organisms.

2.4. D. magna toxicity assay

In this acute toxicity test, the biological end point is the immobilisation of the test organisms caused by a suspected toxic aqueous sample. The assay has been standardised (OECD, 2004; ISO 6341), 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 concerning aquatic toxicology (Damásió et al., 2007, 2008).

In this assay a group of young crustacean of the species D. magna are exposed to the aqueous sample during a 48 h period. The number of immobile individuals after 24 h and 48 h of exposure is recorded and compared with those in control samples. From these results the concentration causing immobilisation to the 50% of the daphnia population is calculated (EC50). The test organisms used are young D. magna (Cladocera, Crustacea), at least of second generation, obtained from acyclic partenogenesis in laboratory culture conditions and under 24 h old. Daphnias are not fed during the assay. The assay is run at temperature 20 °C (± 2 °C), and under darkness.

In the experimental procedure, a series of at least five concentrations of the test sample and one control are prepared in four replicates. The volume of each test vessel is 10 ml. Five individuals are added to each one of the test vessels, making a total of 20 individuals exposed to each test concentrations. When a solvent must be used to dissolve the sample (i.e. 0.2% DMSO), a series of control vessels containing the dilution medium must be added to the test. At the end of the exposure period (24 h and/or 48 h) the number of mobile Daphnia in each vessel is counted. The Daphnia unable to move after a slight agitation of the container are considered immobile, even if they are able to move their antennae. The relationship between test concentration and percentage of mobility is analysed by appropriate statistical methods (e.g. probit analysis) to calculate the concentration causing immobilisation to 50% of the populations (EC50). The measurements were made in triplicate.

3. Results and discussions

Inhibition curves and EC50 values were obtained for a selection of compounds commonly found in European rivers and drinking waters by the use of TOXcontrol®, an automatic on-line biosensor based on the measurement of the inhibition of luminescence by bacteria. Repeatability of the measurements was evaluated by doing experiments in triplicate (coefficient of variation is reported in EC50 values and deviation is graphically shown when representing inhibition curves).

In the first series of experiments, the luminescence inhibition curves were obtained with TOXcontrol® at 15 min and 30 min. Standard solutions and working solutions (automatically prepared by TOXcontrol®) are presented in Table 1. A selection of resulting inhibition curves is represented in Fig. 2. No results are shown for 30 min exposure of diazinon, propanil and MCPA to the luminescent bacteria. In these cases, problems related to the low stability of standard solutions led to non-reliable results that were discarded for their inclusion in this study.

The same organic compounds were tested in an interlaboratory exercise to compare results obtained with TOXcontrol® and standardised methodologies based on Microtox® and D. magna tests. Table 2 shows EC50 values and coefficient of variation (CV) for the several organic compounds tested with TOXcontrol® and Microtox®. A collection of results from literature is also provided for comparison. In Table 3 the results from D. magna tests are included. EC50 values were in agreement with the ones that have been calculated with standardised methodologies (Microtox® and D. magna tests).

Not many results have been reported at literature for the selected organic compounds and variability can be found between reported values so a comparison is not easy to be established. Values obtained for V. fischeri with TOXcontrol® are in general in good agreement with those obtained with Microtox® and those found in literature (Table 2). Higher differences when comparing the two systems in the framework of this study can be observed specially at nonylphenol EC50 values (0.03 mg L⁻¹ for TOXcontrol® against 54.30 mg L⁻¹ for Microtox®, although literature supports TOXcontrol® values), propanil (1594 mg L⁻¹ for TOXcontrol® against 21.71 mg L⁻¹ for Microtox®) and MCPA (3311 mg L⁻¹ for TOXcontrol® against 26.10 mg L⁻¹ for Microtox®, literature found value placed in the middle).

Additionally, selected compounds have been tested with a different technique based on D. magna. Although based on different organisms, it has been considered interesting to obtain new EC50 values and a comparison has been performed to see correlation between techniques (Table 3). Values presenting higher differences among different technologies correspond to diazinon presenting values for D. magna two orders of magnitude below V. fischeri and nonylphenol where differences could reach 4 orders of magnitude between results obtained with TOXcontrol® and D. magna.
In a new experiment, a second series of tests to determine the toxicity of aqueous solutions of metal compounds with the TOXcontrol® system was performed. The percentage of inhibition was calculated from the results obtained with metal solutions. The EC50 values at 15 min and 30 min for copper (II), nickel (II), chromium (III) and iron (III) were calculated (Table 2). Standard solutions and working solutions (automatically prepared by TOXcontrol®) are presented in Table 1. Inhibition curves for copper, nickel, chromium and iron are presented in Fig. 2. Curve for lead (II) was not obtained due to the high variability of values. Even though the exact EC50 value cannot be represented, the approximate toxicological range can be estimated in 70–110 mg L\(^{-1}\) (15 min incubation time) and 80–130 mg L\(^{-1}\) (30 min incubation time). For analysis of metal compounds, variations found among values reported at literature are remarkable. The methodologies main differences are attributable to the salt used to prepare the solution and the pH adjustment, which is performed in some of the studies (Dutka and Kwan, 1981; Greene et al., 1985).

4. Conclusions

Results obtained are in the same order of magnitude than those reported at the literature in most of the cases. It should be highlighted that 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.).
Table 2

<table>
<thead>
<tr>
<th>Compounds</th>
<th>EC50 (mg L(^{-1}))</th>
<th>Range</th>
<th>References</th>
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<tr>
<td>Nonylphenol</td>
<td>0.03</td>
<td>0.06-0.06</td>
<td>Farré et al. (2001a, 2001b); Farré and Barceló (2003)</td>
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<td>Triclosan</td>
<td>0.13</td>
<td>0.06-0.08</td>
<td>Farré et al. (2008)</td>
</tr>
<tr>
<td>Dimethoate</td>
<td>6.80</td>
<td>2.5-4.0</td>
<td>Trajkovska et al. (2009); Köck et al. (2010)</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>10.26</td>
<td>3.9-5.5</td>
<td>Farré et al. (2001a, 2001b); Ferrari et al. (2003)</td>
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<tr>
<td>SDBS (LAS)</td>
<td>50.04</td>
<td>9.7-26.1</td>
<td>Gutiérrez et al. (2002); Farré and Barceló (2003)</td>
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<tr>
<td>Diazinon</td>
<td>192.70</td>
<td>7.2-13.7</td>
<td>Chang et al. (1981); Curtis et al. (1982); Somasundaram et al. (1990); Ruiz et al. (1997); Köck et al. (2010); Somasundaram et al. (1990); Ruiz et al. (1997); Köck et al. (2010)</td>
</tr>
<tr>
<td>Propanil</td>
<td>1594</td>
<td>9-22</td>
<td>Kaiser et al. (1994); Ruiz et al. (1997)</td>
</tr>
<tr>
<td>Copper (II)</td>
<td>10.61</td>
<td>1.9-2.0</td>
<td>Tarkpea et al. (1986); Codina et al. (1993); Sillanpää and Oikari (1996); Tchounwou and Reed (1999); Guéguen et al. (2004)</td>
</tr>
<tr>
<td>Nickel (II)</td>
<td>317.2</td>
<td>0.8-0.9</td>
<td>Qureshi et al. (1984)</td>
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<tr>
<td>Chromium (III)</td>
<td>190.4</td>
<td>3.6-4.3</td>
<td>Qureshi et al. (1984)</td>
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<tr>
<td>Iron (III)</td>
<td>52.08</td>
<td>5.4-10.4</td>
<td>Qureshi et al. (1984)</td>
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<td>Lead (II)</td>
<td>70</td>
<td>130-268</td>
<td>Qureshi et al. (1984)</td>
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</table>

Table 3

<table>
<thead>
<tr>
<th>Compounds</th>
<th>EC50 (mg L(^{-1}))</th>
<th>Range</th>
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<td>Nonylphenol</td>
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<td>Diclofenac</td>
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<tr>
<td>Diazinon</td>
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<td>Propanil</td>
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<tr>
<td>MCPA</td>
<td>115-163</td>
<td>77</td>
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</table>

et al. 2012).}

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