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Evaluation of a novel automated water analyzer for continuous monitoring of toxicity and chemical parameters in municipal water supply

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ABSTRACT

A novel tool, the DAMTA analyzer (Device for Analytical Monitoring and Toxicity Assessment), designed for fully automated toxicity measurements based on luminescent bacteria as well as for concomitant determination of chemical parameters, was developed and field-tested. The instrument is a robotic water analyzer equipped with a luminometer and a spectrophotometer, integrated on a thermostated reaction plate which contains a movable carousel with 80 cuvettes. Acute toxicity is measured on-line using a wild type *Photobacterium phosphoreum* strain with measurable bioluminescence and unaltered sensitivity to toxicants lasting up to ten days. The EC50 values of reference compounds tested were consistent with *A. fischeri* and *P. phosphoreum* international standards and comparable to previously published data. Concurrently, a laboratory trial demonstrated the feasibility of use of the analyzer for the determination of nutrients and metals in parallel to the toxicity measurements. In a prolonged test, the system was installed only in toxicity mode at the premises of the World Fair "Expo Milano-2015", a high security site to ensure the quality of the supplied drinking water. The monitoring program lasted for six months during which ca. 2400 toxicity tests were carried out; the results indicated a mean non-toxic outcome of $-5.5 \pm 6.2\%$. In order to warrant the system's robustness in detecting toxic substances, Zn was measured daily with highly reproducible inhibition results, $70.8 \pm 13.6\%$. These results assure that this novel toxicity monitor can be used as an early warning system for protection of drinking water sources from emergencies involving low probability/high impact contamination events in source water or treated water.

1. Introduction

Worldwide environmental laws regulating quality of water bodies are becoming more and more rigorous and demanding in both Western and Asian countries. In Europe, the Water Framework Directive (WFD, 2000) highlights the importance of a novel risk-based approach adopted to assess environmental status including chemical quality, ecological safety and morphological conditions. Thus, the integration of chemical and biological analysis in a single instrument can make this task viable and cost effective.

Toxicity tests are some of the most frequently used tools for the ecological and biochemical assessment of water quality. They are based on the detection of biological signals produced by microorganisms or higher organisms in response to changes in their environment, like the presence of toxic contaminants (Radix et al., 2000). Several instruments for bacteria-, algae-, invertebrate- and fish-based bioassays are now available in the market (review, see Kokkali and van Delft, 2014).

Risk assessment in aquatic environments based on toxicity assays often employs the determination of the inhibition effects produced by pollutants on the light emitted by luminescent microorganisms (Fernández-Piñas et al., 2014). Since the light emission phenomenon requires large quantities of bacterial energy, a difference in photon release is attributed to the effect of sample components which impair the bacterial metabolism. Consequently, the observed decrease of luminescence is regarded as proportional to the biotoxicity of the substances contained in the sample (Kaiser and Palabrica, 1991; Parvez et al., 2006).

Because of their sensitivity, ease and rapidity, the luminescent bacteria toxicity assays have been standardised for regulatory purposes with similar procedures in Europe, US and also in China (EN ISO 11348-3, 2009; ASTM D5660-96, 2009; GB/T 15441, 1995, respectively). The toxicity of the samples is usually expressed by the EC50 value, which is the amount of a pure substance or a sample at which 50% of luminescence inhibition is measured. In contrast to eukaryotic-based tests,

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the responses are quick since acute toxic effects are commonly calculated after 15 or 30 min of bacterial exposure (Kaiser, 1998). Environmental samples are usually analysed offline, with laboratory batch systems and portable devices, exploiting the light emitting properties of *Allivibrio fischeri*, *Photobacterium phosphoreum* and *Photobacterium leiognathi* bacteria (Bulich, 1979; Kuznetsov et al., 1999; Ulitzur et al., 2002; Ma et al., 2014; van de Merwe and Leusch, 2015).

On the other hand, threatened environments or specific situations relevant for public health, such as the water sources intended for human use, require continuous monitoring (van Wezel et al., 2010). Online luminescent bacteria toxicity monitoring via completely automated systems has been achieved by application of the flow-through technology (Kim and Gu, 2005; Pooley et al., 2004) and through batch systems incorporating two parallel lines for the analysis of reference and sample water (Lopez-Roldan et al., 2012). In the former configuration, the use of continuous flow technology poses the risk of cross-contamination and biofouling episodes, whereas, in the latter, dual-channel apparatus suffer from limited analytical frequency since sample analysis is intermittent and give only one test result in typically 15 – 30 min.

To overcome the problems associated with flow technology and significantly increase the analytical through-put, a novel robotic instrument for continuous monitoring of acute toxicity, DAMTA (device for analytical monitoring and toxicity assessment), was designed, realized and tested. The system was based on a direct reading detector incorporating 80 reaction cells in each of which a toxicity test can be run. The sentinel species was a wild type bioluminescent strain of *P. phosphoreum* isolated from the Tyrrhenian sea, characterized by exclusive performances in terms of luminescence, life-time and sensitivity. In a further embodiment of the system, the toxicological tests were combined with the possibility of parallel automated analysis of other relevant chemical parameters required for water quality assessment.

The main aim of this paper was to present the novel instrument DAMTA to scientists involved in toxicological and environmental analysis. The instrument has been tested in laboratory and field for the continuous monitoring of toxicity in combination with nutrient and metal analysis.

In particular, the analyzer was selected by the Authority responsible for the Integrated Water Service for the City of Milano to secure the safety of the water distribution network established for the World Fair "Expo Milano-2015", held in Milano, Italy, from May to October 2015. The system was installed in a high security site, possible target for terror attacks and was used as an early warning system as well as a technological guard to protect the drinking water distribution system.

2. Materials and methods

2.1. Instrument

DAMTA is a multi-parameter monitoring system housed in an industrial cabinet (82 (L) × 55 (W) × 184 (H) cm) and equipped with a LCD colour touch screen control pad. The storage, dispensation, incubation and measuring components were designed, built and assembled by Systea laboratories (Anagni, Italy). A 71 (L) × 40 (W) cm aluminum base plate accommodates the bacteria management device, the liquid handling system and a direct reading reaction tray (Fig. 1). The instrument was conceived to automatically perform in parallel toxicity tests (blue stream) and chemical analysis (red stream).

The groundwork of the acute toxicity assay is the bacteria management device that consists of a vial opener and a refrigerated compartment with three vials containing lyophilized bacteria. The analyzer was programmed to automatically open a new vial, reconstitute fresh bacteria, by adding up to 22 mL of rehydration buffer, discard bacteria in use when exhausted and repeat the same procedure on the next vial.

The liquid handling system is a robotic pipettor that can access samples, bacteria, buffer, blank water and reagent containers, for toxicity tests or chemical analysis. The mechanical arm has three degrees of freedom and is fitted with a stainless-steel needle that allows aspiration, transferring and dispensing of the fluids needed. All of them are added, in the suitable sequence and amount, directly inside 500 micro-litre cuvettes, duly chosen by the software among those available in the carousel. The sampler is equipped with a sensor for liquid level sensing, automated probe washing and sample dilution facilities. Aspiration volume ranges from 2 to 330 µL with 1 µL increment and ± 0.1 µL accuracy.

The thermostated direct reading movable reaction tray is capable to rotate in order to place any of the 80 reaction cells in front of the luminometer, the spectrophotometer or the washing station, according to the requested action. In toxicity tests, a rectangular optic fibre bundle carries out the luminescence signal to a remote and perfectly sealed photomultiplier module (PMT). The spectrophotometer used for chemical analysis is a direct reading, dual channel device supplied with a halogen lamp (6 V / 10 W) as light source and 9 narrow band automatically selectable interferential filters.

The analyzer is managed via dedicated software which provides overall control of both analytical operations and data acquisition functions by a GSM / GPRS device.

2.2. Chemicals and bacterial strain preparation

Reagents were purchased from Merck (Darmstadt, Germany) and they were of the highest purity grade available.

P. phosphoreum wt was isolated from the Tyrrhenian Sea and characterized by 16S ribosomal RNA gene sequencing by the Genomics Platform of Parco Tecnologico Padano (Lodi, Italy). A feature of this novel strain is that luminescence activity is expressed over a wide temperature range from 15 to 25 °C, but optimal sensitivity is observed at 25 °C. This may represent an advantage in case of monitoring under tropical conditions. Bacteria were grown and lyophilized at the Systea laboratories (Anagni, Italy), according to established protocols (Bulich, 1979).

The lyophiles of *P. phosphoreum* T3 mutation (PPT3), used as bioindicator organism in the Chinese standard for acute toxicity testing (GB/T 15441, 1995), were purchased from the Nanjing Institute of Soil Science, Chinese Academy of Science.

Stock solutions for toxicity tests were freshly prepared from > 98% pure substance (3,5-dichlorophenol) or certified 1000 mg L^{-1} standards (Cd (II), Pb (II), CN^-) or by dissolving metal sulfate (Cu (II), Zn (II)) or chloride (Hg (II)) salt in ultrapure water. As (III) and Cr (VI) stock solutions were made from sodium arsenite and potassium dichromate, respectively.

2.3. Automated toxicity tests

The following analytical steps were fully automated: (i) vial opening and bacterial rehydration; (ii) agitation of bacterial suspension for homogenization; (iii) aspiration and dispensing of bacteria, buffer, blank water, reference standards and samples; (iv) sample dilution and conditioning; (v) kinetic luminescence measurements of blanks, reference standards and samples; (vi) cleaning of dispensing needle and incubation cells; (vii) statistical analysis and calculation of EC50.

The analytical sequence was scheduled to run blanks, reference standards, undiluted and diluted samples, all in duplicate. Every analysis started and ended with a blank run such as to minimize the influence of inter-test bacterial variability. Samples or reference standards, water and osmotic buffer were mixed within the sampling arm before addition to the reaction cuvette.

As for the assay with *P. phosphoreum* wt, 0.2 g of freeze-dried bacteria were automatically rehydrated in 22 mL of proprietary rehydration buffer; 10^6 cells mL^{-1} of bacteria were added to blanks and samples and osmotically adjusted with proprietary NaCl-based buffer.

As for the assay with PPT3, 0.5 g of freeze-dried bacteria were

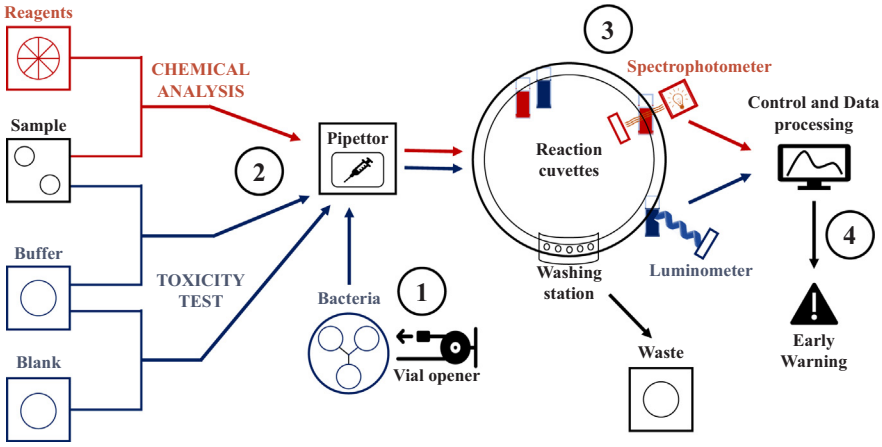

Fig. 1. Scheme of the DAMTA organization: (1) bacterial management device including vial opening, rehydration and housing; (2) liquid handling system, based on automatic pipetting of all fluids requested for chemical and toxicity analysis (red and blue streams, respectively); (3) direct reading movable reaction tray inclusive of 80 cuvettes, spectrophotometer, luminometer and washing station; (4) control and data processing system. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

manually reconstituted with 1 mL of cold 2.5% NaCl solution and thoroughly mixed. After 10 min, bacteria were diluted with 9 mL of cold 3.0% NaCl solution and poured in 50 mL reagent bottle to be used for automated tests. The assay was performed in the DAMTA according to the Chinese standard procedure (GB/T 15441, 1995); briefly, 10^6 cells mL^{-1} of PPT3 were added to blanks and samples and osmotically adjusted to 3.0% NaCl with 20% NaCl.

For both strains, temperature was set at 25 °C. DAMTA was able to measure the emitted light every 40 s during the exposure time and compare it to the luminescence of a blank control (bacteria plus osmotically adjusted Milli Q water). With this short interval measurements, it was possible to follow, in real time, total luminescence kinetics of all toxicity tests. At the end of the analysis, the used cuvettes were automatically emptied, washed with Milli Q water three times and dried out. It was possible to start a new test already after 40 s.

The percent of inhibition at the time t (H_t) was obtained by calculating the average percent decrease for two replicates with respect to blank average luminescence. Required maintenance operations included loading of bacterial vials, refill of cleaning and blank water tanks, replacement of buffer and reference standard solutions.

2.4. Determination of EC50 values

The effective concentrations values (mg L^{-1}) that resulted in 50% inhibition of the bioluminescence of *P. phosphoreum* wt and PPT3 were obtained after 15 or 30 min. They were calculated from linear regression equations of dose / response curves of the form: LOG $y = m$ LOG $x + c$, where y was the value for gamma (gamma = % inhibition / (100 – % inhibition)), x was the dose and m and c were the slope and intercept, respectively. Only percentage inhibitions > 5 and < 95% were used in the regression equations.

2.5. Laboratory tests

A laboratory test program was conducted with the objective to test whether the DAMTA system could be effectively used as a fully autonomous on line toxicity biomonitor for one month. Four main goals characterized the experimental trial: (i) to estimate the bacterial lifetime by using the same suspension for 10 days, (ii) to assess the reproducibility of the inhibition results, (iii) to test the compliance of the automatic biomonitor with guidelines provided by international standards, where Zn^{2+}, 3,5-dichlorophenol (DCP) and Cr^{6+} are used in the EU and the US as reference toxicants for the *A. fischeri* toxicity test (EN ISO 11348-3, 2009) and $HgCl_2$ is the positive control in *P. phosphoreum* Chinese standard (GB/T 15441, 1995) and (iv) to compare the sensitivity of the *P. phosphoreum* wt, isolated in this work, with the commercial strain PPT3 by simultaneously determining on both strains, in the DAMTA, the toxicity of As (III), Cd (II), CN^-, Cr (VI), Cu (II), Hg (II), Pb (II) and Zn (II). This laboratory testing campaign was performed by Systea, Anagni, Italy and by the Water Research Department of Focused Photonics Inc. (FPI) in Hangzhou, China.

2.6. Installation at EXPO 2015

DAMTA was selected by Metropolitana Milanese SpA, the environmental agency in charge of supplying water to EXPO 2015, the World Fair held in Milano from May 1st to October 31st, 2015, as the analytical tool to monitor toxicity on the water distribution network. It was a remarkable opportunity to demonstrate the feasibility of use of the analyzer in a critical field installation.

The analyzer was set in simple toxicity mode with the scope to secure from external danger and detect potential failure to maintain potable water quality intended for human use. The sampling spot was located inside a protected area after chlorination and immediately before distribution. Preliminary tests performed before the beginning of the Exposition showed that *P. phosphoreum* wt luminescence was not affected by the amount of free chlorine contained in the supplied water which was prepared within the range of 0–0.2 mg L^{-1} in accordance with Italian standards for drinking water (D.Lgs. n°. 31/2001, 2001). Free chlorine concentration was monitored daily by Metropolitana Milanese SpA. The temperature of the bacterial stock suspension in the instrument was set to 4 – 8 °C. A 15-min toxicity test conducted with two blank and two sample runs was performed every 90 min. Every day at midnight, a control check tested the bacterial viability using 2.2 mg L^{-1} of Zn^{2+} as reference toxicant solution.

In order to offer early warning protection, the system was programmed to automatically generate an alarm immediately after detecting a light reduction threshold of 50% even before the end of the test. In such case, the analyzer was configured to perform a check on bacterial status and performance by using the reference standard, such as to minimize the occurrence of false negatives or positives. Subsequently, the system confirmed the presumptive toxicity by replicating the analysis and calculating the EC50 value to assess the extent of the pollution threat.

2.7. Chemical analysis

The following standard operation procedures were applied for the determination of chemical parameters. Each method was tested by performing seven replicates of seven different known concentrations (0%, 5%, 10%, 25%, 50%, 75% and 100% of the working range). Method detection limit MDL was calculated by multiplying the correct Student's t-value 3.14 per SD obtained at 5% of the working range. Precision was determined as the relative standard deviation and accuracy as the mean percentage of true value. The coefficient of variation (CoV) was calculated according to ISO 8466–1 (1990), as the ratio of

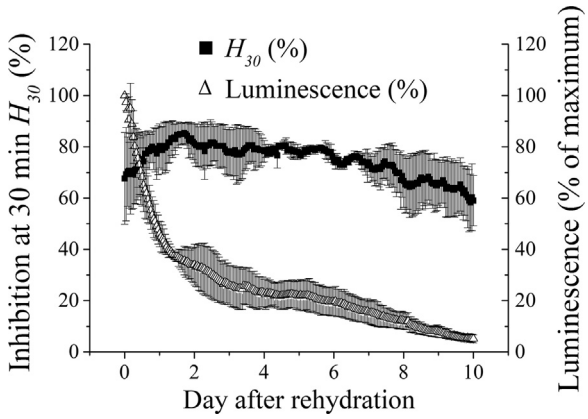

Fig. 2. Continuous one-month monitoring of acute toxicity. 10-day cycles of 24 tests per day were repeated 3 times, for a total of 720 tests over 30 days. The points on the graph show mean ± SD of 30-min *P. phosphoreum* wt sensitivity to 2.2 mg L^{-1} Zn solution (■) and 30-min *P. phosphoreum* wt luminescence (Δ) measured by using three consecutive vials.

the standard deviation of the results obtained for that method used to the mean of the working range of the method.

NO_2, NH_4, NO_3, Cr (VI) and Cu (I + II) were analysed according the Italian drinking water regulation that limits their concentration to 0.5, 0.5, 50, 0.05 and 1 mg L^{-1} respectively (D.Lgs. n°. 31/2001, 2001). PO_4 and Si were measured based on ISO standard methods for on-line analysis of nutrients (ISO 15923-1, 2013). The chemical tests were performed at Systea Laboratories, Anagni, Italy.

3. Results

3.1. Toxicity assay: DAMTA setup and testing

Luminescent emission of rehydrated *P. phosphoreum* wt and the light inhibition produced by 2.2 mg L^{-1} of Zn^{2+} were successfully hourly monitored in laboratory three times for 10 days (Fig. 2). The possibility of using 3 vials of lyophilized bacteria in the instrument, allowed extending the test to one month of unattended activity.

The 720 tests executed in three consecutive cycles of 10 days (3 × 240 tests) resulted in a highly reproducible mean 30 min inhibition value of 74.9 ± 6.3% in agreement with the higher limit of sensitivity to Zn^{2+} recommended by the EU standard (80%, EN ISO 11348-3, 2009) and Zn^{2+} was established as the reference compound for the instrument.

Because acute toxicity testing involves an assessment of a wide range of potential toxic effects, one or more substances producing different effects are commonly required by international standards as positive controls to regulate the acceptance of the validity of toxicity results. The relative trueness of the measured EC50 values was evaluated by comparing the experimental results with data derived from different ring tests reported in international standards (Table 1). As for the reproducibility, CV_R % ranges obtained in this work (11–34%) were comparable with those reported in international standards (10–34%).

To assess the sensitivity of the assay to a wider range of contaminants and the influence of the automation on bacterial sensitivity, six metals commonly found in wastewater, surface water and drinking water and two deadly poisons (cyanide and arsenic) were tested in the DAMTA on *P. phosphoreum* wt and, in parallel on the same samples, on PPT3, and the results are presented in Table 2.

The EC50 values for the compounds analysed automatically were successively compared to PPT3, *Photobacterium* sp. LuB-1 and *A. fischeri* NRRL B-11177 literature values obtained by manual measurements. Due to the vast number of papers using PPT3 and NRRL B-11177 to assess toxicity of different pollutants, this comparison included only the lowest and highest literature EC50 values.

Table 1
Mean ± SD EC50 values (mg L^{-1}) and coefficient of variation of reproducibility (CV_R %) for fifteen replicates of Zn^{2+}, DCP, Cr^{6+} and $HgCl_2$ analysed by the DAMTA on separate days using different vials of reconstituted bacteria and compared to values recommended in different international standards.

Chemical	Time (min)	This work		International standards	
Zn (II)[a]	30	1.21* ± 0.41	± 34.3%	2.17* ± 0.73	± 33.6%
DCP[a]	30	4.41* ± 0.74	± 16.9%	3.36* ± 0.32	± 9.6%
Cr (VI)[a]	30	17.4 ± 5.5	± 31.5%	18.7 ± 6.2	± 32.9%
$HgCl_2$[b]	15	0.11 ± 0.01	± 10.8%	0.10 ± 0.02	± 20.0%

[a] EN ISO 11348-3, 2009.
[b] GB/T 15441, 1995.
* Significantly different ($p \leq 0.05$) based on two-tailed *t*-test (Mean, SD, N of replicates).

Table 2
Mean ± SD EC50 values (mg L^{-1}) at 15 min for pollutants (expressed as ionic concentrations) tested in the DAMTA (run in triplicate on both *P. phosphoreum* wt and PPT3) and compared to PPT3, *Photobacterium* sp. LuB-1 and *A. fischeri* NRRL B-11177 literature values.

Ion	This work		Literature data		
	PP wt	PPT3	PPT3	LuB-1	NRRL B-11177
As (III)	2.66 ± 1.22	1.51 ± 0.58	1.30[a] – 4.40[a]	0.11[b] – 0.17[b]	20.0[c] – 26.0[c]
Cd (II)	0.46 ± 0.07	0.36 ± 0.08	0.14[d] – 19.3[e]	4–43[b] – 10.9[b]	3.03[f] – 14.5[g]
CN^-	0.09 ± 0.04	0.16 ± 0.05			1.91[h]
Cr (VI)	20.5 ± 10.1	17.7 ± 7.8	3.99[d] – 30.7[i]	5.6[b] – 55.0[b]	23[j] – 584[j]
Cu (II)	0.55 ± 0.18	2.89 ± 0.38	0.39[k] – 1.91[l]	0.54[b] – 2.29[b]	0.10[f] – 0.80[m]
Hg (II)	0.08 ± 0.01	0.07 ± 0.02	0.01[n] – 0.07[n]	0.03[b] – 0.04[b]	0.08[g] – 0.18[f]
Pb (II)	1.41 ± 0.48	1.62 ± 0.24	0.90[k] – 1.23[l]	1.06[b] – 1.20[b]	0.13[o] – 5.4[o]
Zn (II)	1.26 ± 0.25	1.13 ± 0.30	0.66[d] – 1.56[k]	1.01[b] – 1.28[b]	2.00[m] – 2.29[f]

[a] He et al. (2016).
[b] Hong et al. (2010).
[c] Fulladosa et al. (2004).
[d] He et al. (2015).
[e] Qu et al. (2013).
[f] McCloskey et al. (1996).
[g] Petala et al. (2005).
[h] Marugán et al. (2012).
[i] Wu et al. (2011).
[j] Villaescusa et al. (1997).
[k] Wang et al. (2016).
[l] Zeb et al. (2016).
[m] Ytreberg et al. (2010).
[n] Wang et al. (2014).
[o] Villaescusa et al. (1998).

3.2. EXPO 2015 (long-term continuous toxicity monitoring test)

The critical acute toxicity threshold of 20% decrease of the bacterial signal compared with the blank luminescence was never crossed during the test period and all toxicity inhibition measurements were found null or negative (non–toxic) (Fig. 3). Despite the stress from a combination of different conflicting effects such as seasonal and diurnal temperature fluctuations and bacterial decay, results fluctuated between −30% and 10%, in a consistent manner as for the requirement of acute toxicity tests.

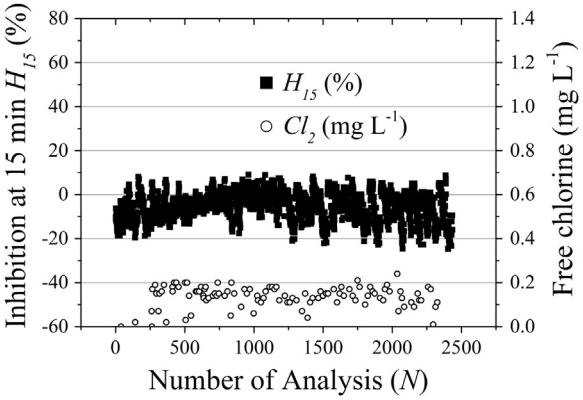

Fig. 3. Trend of acute toxicity (■) and free chlorine (o) data in the EXPO water inlet from April 15th to November 5th, 2015.

The mean observed free chlorine concentration in the water supplied during EXPO was 0.13 ± 0.05 mg L^{-1}. There was no statistically significant relationship between measured light emission and residual free chlorine in the water supply which, at the measured concentrations, did not affect *P. phosphoreum* luminescence. In contrast to *A. fischeri* bioassay (EN ISO 11348-3, 2009), the recommended chlorine reduction with sodium thiosulphate was found not necessary before *P. phosphoreum* wt measurements.

The experimental results of the continuous toxicity tests conducted for a period of six months at the EXPO 2015 drinking water network are summarized in Table 3. Overall mean blank and sample deviations from their test mean were calculated to be well below the deviations of 3.0% recommended by the CEN standard (EN ISO 11348-3, 2009) both for sample and reference runs. Inhibition to the reference substance Zn^{2+} was calculated to be, in the course of the months, highly reproducible.

3.3. Laboratory chemical analysis

A laboratory pilot test was conducted to verify if chemical analysis could be effectively performed with the instrument, in parallel with the toxicity tests. Some of the commonly investigated parameters included in water and wastewater analysis were tested and results are shown in Table 4. Importantly, after seven replicates, all precision and accuracy measurements at 10% and 100% of the detection range produced percentage oscillations lower than 5%, which is the commonly accepted threshold value in on-line monitors.

In addition, an on-line continuous monitoring trial on tap water contaminated with metals and nutrients, was carried out for eight days in the Systea laboratories at Anagni.

These parameters were tested every 3 h at 25 °C in combination with the toxicity tests. The instrument was programmed to perform eighty analytical cycles with daily automatic calibrations. The results for the measured chemical parameters, respectively 45.5 ± 2.4 and 124.4 ± 2.7 μgL^{-1} for Cr (VI) and nitrite, and 10.93 ± 0.22 mgL^{-1} for nitrate, with recovery rates comprised between 99% and 101%, found to be highly accurate and reproducible. Only ammonia concentration, as expected, decreased from 350 to 90 μgL^{-1} during the course of the test. In parallel, samples were checked for bacterial toxicity, but no major acute toxicity events were detected.

Table 3
Statistical evaluation of results from continuous toxicity monitoring tests conducted for a period of six months, at the EXPO 2015 drinking water distribution network. Luminescence inhibition (% Mean ± SD) of (i) real-time water samples of the supplied water taken every 90 min and (ii) 2.2 mg L^{-1} Zn^{2+} solution that was measured daily as a reference toxicant.

	Water samples (real-time)	**Reference runs** [2.2 mg L^{-1} Zn^{2+}]
Mean inhibition (%)	−5.5 ± 6.2	70.8 ± 13.6
Mean blank deviation (%)[a]	2.0 ± 1.6 (78%[c])	1.7 ± 1.6 (84%[c])
Mean sample deviation (%)[b]	1.4 ± 1.2 (89%[d])	0.7 ± 0.6 (99%[d])
Number of tests	2435	184

[a] Mean of blank percentage deviations from luminescence test mean.
[b] Mean of sample percentage deviations from inhibition test mean.
[c] Percentage of blank percentage deviations complying with the CEN standard (lower than 3%).
[d] Percentage of sample percentage deviations complying with the CEN standard (lower than 3%).

4. Discussion

The DAMTA is a fully automated on-line analyzer comprised of a rapid, sensitive and high throughput assay for analysing acute toxicity of water samples, with an option of using spectrophotometric methods for the determination of chemical parameters, if required. In particular, the luminescence detection technique introduced in this work is original and new because, for the first time, a PMT was incorporated in the direct reading reaction plate of a discrete analyzer. The luminescence signal is directly acquired from one full side of the rectangular-sided reaction cuvette, which is positioned to face a rectangular window with the same area (5 × 15 mm). This allows for reading the maximum amount of luminescence generated by the bacteria inside the test suspension.

The toxicity assay uses freeze dried bacteria stored in closed vials and the instrument is able to open a vial and reconstitute the bacteria automatically. The rehydrated bacteria can be used as batch reagents, without any further operation. This simplifies the bacteria management as compared to previous systems for toxicity tests in which the microorganisms were grown in a fermenter (Kim and Gu, 2005; Pooley et al., 2004) for the following reasons: (i) a bacterial culture requires the strict control of nutrient addition, volume, aeration, temperature and turbidity, (ii) due to the presence of nutrients, the risk of culture contamination is increased and (iii) the reactor needs regular maintenance for cleaning and autoclave sterilization. Besides, in the robotic system, the absence of tubing allows for limiting the problem of the development of fungal, algal and bacterial contamination and biofilm formation on the stainless-steel needle and the reaction cells; as a preventive measure they are rinsed regularly by repeated washing and sterilization cycles.

The laboratory testing showed that the bacterial suspensions suffered nearly 90% loss of luminescence in a week, but the light emitted was still sufficient for effective test execution. Importantly, control measurements with Zn^{2+} as a standard reference did not reveal any change in quality and responsiveness of the luminescent bacteria over ten days of use (Fig. 2). Due to the presence of three vials of freeze-dried bacteria, the instrument was thus able to operate in full autonomy for 30 days.

To exclude false positives, DAMTA is able to immediately trigger an emergency alert as soon as toxicity is detected and, in parallel with the ongoing analysis, perform an EC50 sample measurement. Significantly, this allows for quantifying the toxicity threat. The prevention of false negatives requires timely control of assay performance through the execution of reference quality control runs at fixed intervals.

In toxicity monitoring using bacterial luminescence assays, the microorganisms may be exposed to samples containing a wide variety of potential pollutants. Therefore, it was crucial to assess the sensitivity of the DAMTA assay using individual compounds, according to international norms. A dedicated experiment performed with reference standards (Table 1) showed that EC50 values for the three positive controls suggested by the EU standard (EN ISO 11348-3, 2009) were in the recommended order of magnitude. Interestingly, the EC50 value for $HgCl_2$ was perfectly superimposable with the strict range requested by Chinese standard (GB/T 15441, 1995); this may be explained by the

Table 4
Analytical performance of the analyzer for metals and nutrients commonly determined in environmental assessments.

Target	Range (mg L^{-1})	MDL (µg L^{-1})	CoV (%)	RSD at 100%	Accuracy at 100%-	RSD at 10%	Accuracy at 10%
Cr (VI)	0 – 0.2	1.2	1.03	± 1.2%	0.1%	± 2.8%	± 0.6%
Cu (I+II)	0 – 0.5	5.2	1.10	± 1.3%	± 0.5%	± 1.6%	± 0.7%
N – NO_2^-	0 – 0.2	1.6	1.74	± 1.8%	± 0.9%	± 3.3%	± 3.1%
N – NO_3^-	0 – 13.6	180.2	3.69	± 3.2%	± 2.0%	± 3.6%	± 0.2%
N – NH_3	0 – 0.4	6.0	0.71	± 1.0%	± 0.2%	± 4.2%	± 5.3%
P – PO_4^{3-}	0 – 1.0	2.8	3.10	± 2.0%	± 4.0%	± 1.8%	± 3.6%
Si – SiO_2	0 – 5.0	16.8	0.56	± 0.4%	± 0.4%	± 0.8%	± 1.7%

fact that this standard is based on the same bacterial species.

Subsequently, a battery of potential pollutants was tested with the DAMTA using simultaneously *P. phosphoreum* wt and PPT3 (Table 2). This allowed to compare the wild type strain isolated in this work with a standard indicator belonging to the same species. The EC50 values for the compounds analysed with *P. phosphoreum* wt were all in the same range of magnitude as those obtained with PPT3. Among the slight differences, *P. phosphoreum* wt was particularly sensitive to Cu^{2+} and CN^- whereas PPT3 was moderately more sensitive to As^{3+} and Cr^{6+}. There is a good agreement between automated DAMTA data and manual test results described in the literature, as proved by the fact that the EC50 results obtained in this study with both *P. phosphoreum* wt and PPT3 were within the intervals defined by the previously published PPT3 values.

Direct comparison with *Photobacterium* sp. LuB-1, a bacterial strain with measurable bioluminescence in a wide range of NaCl concentration (Hong et al., 2010), highlighted that *P. phosphoreum* wt showed similar sensitivity to LuB-1 for most pollutants, except for As^{3+} and Cd^{2+} which were observed to produce slightly higher and lower EC50 values, respectively.

On the other hand, the most popular commercial systems measuring acute toxicity based on bioluminescent bacteria, among which the laboratory batch systems Microtox® (Bulich, 1979), ToxAlert® (Farré et al., 2001), BioTox® (Juvonen et al., 2000) and LUMIStox® (Guzzella and Galassi, 1993), rely on the use of *A. fischeri* (strain 11177), rehydrated from freeze-dried or liquid dried sources. A study conducted by performing toxicity tests at eight different concentrations on a representative range of 81 contaminants demonstrated that, when tested under identical, controlled conditions, these assays produce results in the same order of magnitude (Jennings et al., 2001).

Consequently, a comparison with *A. fischeri*-based literature data significantly helped to evaluate the performance of the DAMTA assay. However, sensitivity comparisons between different bacterial assays should carefully consider the physico-chemical characteristics during the tests, as demonstrated by the wide range of EC50 values for metals reported for PPT3 and *A. fischeri* in published literature. Despite both strains are able to preserve their internal pH with external pH changes (Fulladosa et al., 2004), the influence of pH, different counterions, medium composition and salinity were shown to be important as these factors modify the chemical speciation that, in turn, significantly affects the toxicity assay. It was reported that sensitivity of luminescent bacteria to metals, and especially Cd (II) and Cr (VI), is strongly pH- and counterion-dependent due to speciation (Qu et al., 2013; Villaescusa et al., 1997). Similarly, the 15 min EC50 values for metals were observed by Villaescusa et al. (1998), to oscillate remarkably when changing chloride concentration. Taking into consideration these factors, the EC50 values measured for *P. phosphoreum* wt are consistent with the literature range reported for *A. fischeri* (Table 2), although *P. phosphoreum* wt was slightly more sensitive to As^{3+}, Cd^{2+} and CN^-. As the assay met sensitivity and reproducibility requirements, the instrument can be considered as technically suitable for field application.

Compared to waste and surface water, drinking water is particularly exposed to both deliberate and unintentional occasional contamination (van Wezel et al., 2010). With this in mind, a challenging field testing was carried out at the water supply network of EXPO 2015. The overall standing time of the instrument was tested over 6 months to show the suitability to real time monitoring over an extended period of water distribution. An excellent reproducibility of test results was demonstrated (Table 3) in agreement with the validity criteria of EN ISO 11348-3 (2009) which specify a maximum replicate variability of 3%. The execution of 2435 analysis resulted in zero false positives while false negatives were prevented by daily execution of the reference runs, of which 92% provided inhibition values comprised between 50% and 90%, and the residual 8% higher than 20%, still in agreement with EN ISO 11348-3 (2009). Accurate and reproducible measurements combined with instrument robustness in long-term monitoring allowed for validating the DAMTA as a reliable on-line automated toxicity alert system.

High concentration of inorganic N species in water can make it unsuitable for human consumption and detrimental to the environment (Gajaraj et al., 2013) and heavy metals are toxic to living organisms above threshold concentrations and highly persistent in the environment (Li and Zhang, 2010). Based on this, the range of chemical parameters initially selected for on-line drinking water monitoring included both nutrients and metals: nitrate and nitrite that may infiltrate from agro-industrial and livestock activities, ammonium, penetrating from septic systems, chromium, widely used in plating industry, and copper that can be released by corrosion of interior plumbing. Two more nutrients, phosphate and silica were added in the second place, with the aim of extending the use of the system to the monitoring of surface water endangered with eutrophication and wastewater effluents.

All variables, including reagent concentrations, sample volumes and incubation times, affecting the analytical performance of the spectrophotometric methods, were carefully studied and optimised by using regression analysis. The final testing of the chemical methods gave sensitive, precise and accurate results (Table 4).

The last part of this study was devoted to proving the full capabilities of the DAMTA in the simultaneous toxicity and chemical monitoring of drinking water samples. Under the instructions of Metropolitana Milanese SpA, the selected parameters to be successfully measured in conjunction with acute toxicity, in artificially contaminated tap water, were Cr (VI), nitrate, nitrite and ammonia.

The importance of supporting the standard chemical analyses for drinking water with toxicity assessment is becoming increasingly evident (Kostich et al., 2017). There is a need for a currently missing analytical instrument capable of simultaneously performing both types of tests. This experiment demonstrated that the developed system is very flexible and able to fill this technological gap, as the toxicity assay can be combined simultaneously with a range of other chemical analytical parameters according to User's needs. The fields of applications comprise water distribution networks, wastewater plants and marine and fresh water environments.

5. Conclusions

An automated stand-alone robotic system for on line toxicity analysis, the DAMTA, was introduced in this paper. Acute toxicity was

measured using a *P. phosphoreum* wt strain and the calculated EC50 values were in agreement with published PPT3, LuB-1 and *A. fischeri* results. The integration of standard chemical analyses of relevant parameters provided an added advantage not found in other available monitoring tools. The field trial was performed in a securely guarded site for the supply of drinking water during EXPO 2015. Key performance indicators of test repeatability and reproducibility and analyzer robustness were satisfied.

Further improvements will focus on adding available refrigerated positions for the bacterial vials, such to extend the unattended time of operation, and increasing the sampling frequency to 30 toxicity tests per hour allowing constant monitoring of critical sites.

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References

ASTM D5660-96, 2009. Standard Test Method for Assessing the Microbial Detoxification of Chemically Contaminated Water and Soil Using a Toxicity Test with a Luminescent Marine Bacterium. American Society for Testing and Materials, West Conshohocken, Pennsylvania, USA.

Bulich, A.A., 1979. In: Marking, L.L., Kimerle, R.A. (Eds.), Use of Luminescent Bacteria for Determining Toxicity in Aquatic Environments, Aquatic Toxicology, ASTM STP 667. American Society for Testing and Materials, pp. 98–106.

D.Lgs. n°. 31/2001, 2001. Repubblica Italiana, Acque destinate al consumo umano (Italian).

EN ISO 11348-3, 2009. Water Quality. Determination of the Inhibitory Effect of Water Samples on the Light Emission of *Vibrio fischer*i (luminescent bacteria test), Part 3: Method Using Freeze-Dried Bacteria. European Committee forStandardization, Brussels, Belgium.

Farré, M.L., García, M.–J., Tirapu, L., Ginebreda, A., Barceló, D., 2001. Wastewater toxicity screening of non-ionic surfactants by Toxalert® and Microtox® bioluminescence inhibition assays. Anal. Chim. Acta 427, 181–189. http://dx.doi.org/10.1016/S0003-2670(00)01022-9.

Fernández-Piñas, F., Rodea-Palomares, I., Leganés, F., González-Pleiter, M., Angeles Muñoz-Martín, M., 2014. Evaluation of the ecotoxicity of pollutants with bioluminescent microorganisms. In: Thouand, G., Marks, R. (Eds.), Bioluminescence: Fundamentals and Applications in Biotechnology - Volume 2, Volume 145 of the series Advances in Biochemical Engineering/Biotechnology. Springer-Verlag, Berlin Heidelberg, pp. 65–135. http://dx.doi.org/10.1007/978-3-662-43619-6_3.

Fulladosa, E., Murat, J.C., Martínez, M., Villaescusa, I., 2004. Effect of pH on Arsenate and Arsenite toxicity to luminescent bacteria (*Vibrio fischeri*). Arch. Environ. Contam. Toxicol. 46, 176–182. http://dx.doi.org/10.1007/s00244-003-2291-7.

Gajaraj, S., Fan, C., Lin, M., Hu, Z., 2013. Quantitative detection of nitrate in water and wastewater by surface-enhanced Raman spectroscopy. Environ. Monit. Assess. 185, 5673–5681. http://dx.doi.org/10.1007/s10661-012-2975-4.

GB/T 15441, 1995. Ministry of Environmental Protection of the People's Republic of China, Water Quality – Determination of the Acute Toxicity – Luminescent Bacteria Test.

Guzzella, L., Galassi, S., 1993. Chemical and toxicological characterization of river water extracts with the *Vibrio fischeri photobacterium*. Sci. Total Environ. 134 (Suppl. 2), S1217–S1226. http://dx.doi.org/10.1016/S0048-9697(05)80127-5.

He, R., Tian, B.-H., Zhang, Q.-Q., Zhang, H.-T., 2015. Effect of fenton oxidation on biodegradability, biotoxicity and dissolved organic matter distribution of concentrated landfill leachate derived from a membrane. Waste Manag. 38, 232–239. http://dx.doi.org/10.1016/j.wasman.2015.01.006.

He, Q., Qu, R., Wang, X., Wie, Z., Sun, P., Wang, Z., 2016. Toxicity of arsenic to *Photobacterium phosphoreum*, *Daphnia magna*, and *Danio rerio* at different pH levels. Clean. Soil Air Water 44, 72–77. http://dx.doi.org/10.1002/clen.201400124.

Hong, Y., Chen, Z., Zhang, B., Zhai, Q., 2010. Isolation of *Photobacterium* sp. LuB-1 and its application in rapid assays for chemical toxicants in water. Lett. Appl. Microbiol. 51, 308–312. http://dx.doi.org/10.1111/j.1472-765X.2010.02896.x.

ISO 15923-1, 2013. Water Quality. Determination of Selected Parameters by Discrete Analysis Systems, Part 1: Ammonium, Nitrate, Nitrite, Chloride, Orthophosphate, Sulfate and Silicate with Photometric Detection. International Organization for Standardization, Geneva, Switzerland.

ISO 8466–1, 1990. Water Quality. Calibration and Evaluation of Analytical Methods and Estimation of Performance Characteristics. Part 1: Statistical Evaluation of the Linear Calibration Function. International Organization for Standardization, Geneva, Switzerland.

Jennings, V.K., Rayner-Brandes, M.H., Bird, D.J., 2001. Assessing chemical toxicity with the bioluminescent Photobacterium (*Vibrio fischeri*): a comparison of three commercial systems. Water Res. 35, 3448–3456. http://dx.doi.org/10.1016/S0043-1354(01)00067-7.

Juvonen, R., Martikainen, E., Schultz, E., Joutti, A., Ahtiainen, J., Lehtokari, M., 2000. A battery of toxicity tests as indicators of decontamination in composting oily waste. Ecotoxicol. Environ. Saf. 47, 156–166. http://dx.doi.org/10.1006/eesa.2000.1943.

Kaiser, K.L.E., Palabrica, V.S., 1991. *Photobacterium phosphoreum* toxicity data index. Water Poll. Res. J. Can. 26, 361–431.

Kaiser, K.L.E., 1998. Correlations of *Vibrio fischeri* bacteria test data with bioassay data for other organisms. Environ. Health Perspect. 106 (Suppl. 2), S583–S591. 〈https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1533390/〉.

Kim, B.C., Gu, M.B., 2005. A multi-channel continuous water toxicity monitoring system: its evaluation and application to water discharged from a power plant. Environ. Monit. Assess. 109, 123–133. http://dx.doi.org/10.1007/s10661-005-5843-7.

Kokkali, V., van Delft, W., 2014. Overview of commercially available bioassays for assessing chemical toxicity in aqueous samples. Trends Anal. Chem. 61, 133–155. http://dx.doi.org/10.1016/j.trac.2014.08.001.

Kostich, M.S., Flick, R.W., Batt, A.L., Mash, H.E., Boone, J.S., Furlong, E.T., Kolpin, D.W., Glassmeyer, S.T., 2017. Aquatic concentrations of chemical analytes compared to ecotoxicity estimates. Sci. Total Environ. 579, 1649–1657. http://dx.doi.org/10.1016/j.scitotenv.2016.06.234.

Kuznetsov, A.M., Rodicheva, E.K., Medvedeva, S.E., 1999. Analysis of river water by bioluminescent biotests. Luminescence 14, 263–265 (doi:10.1002/(SICI)(1522–7243)(199909/10)(14:5 < 263::AID-BIO544 > 3.0.CO;2-Y).

Li, S.Y., Zhang, Q.F., 2010. Risk assessment and seasonal variations of dissolved trace elements and heavy metals in the Upper Han River, China. J. Hazard. Mater. 181, 1051–1058. http://dx.doi.org/10.1016/j.jhazmat.2010.05.120.

Lopez-Roldan, R., Kazlauskaite, L., Ribo, J., Riva, M.C., Gonzalez, S., Cortina, J.L., 2012. Evaluation of an automated luminescent bacteria assay for in situ aquatic toxicity determination. Sci. Total Environ. 440, 307–313. http://dx.doi.org/10.1016/j.scitotenv.2012.05.043.

Ma, X.Y., Wang, X.C., Ngo, H.H., Guo, W., Wu, M.N., Wang, N., 2014. Bioassay based luminescent bacteria: interferences, improvements, and applications. Sci. Total Environ. 468–469, 1–11. http://dx.doi.org/10.1016/j.scitotenv.2013.08.028.

Marugán, J., Bru, D., Pablos, C., Catalá, M., 2012. Comparative evaluation of acute toxicity by *Vibrio fischeri* and fern spore based bioassays in the follow-up of toxic chemicals degradation by photocatalysis. J. Hazard. Mater. 213–214, 117–122. http://dx.doi.org/10.1016/j.jhazmat.2012.01.075.

McCloskey, J.T., Newman, M.C., Clark, S.B., 1996. Predicting the relative toxicity of metal ions using ion characteristics: microtox® bioluminescence assay. Environ. Toxicol. Chem. 15, 1730–1737. http://dx.doi.org/10.1002/etc.5620151011.

Qu, R.J., Wang, X.H., Feng, M.B., Li, Y., Liu, H.X., Wang, L.S., Wang, Z.Y., 2013. The toxicity of cadmium to three aquatic organisms (*Photobacterium phosphoreum*, *Daphnia magna* and *Carassius auratus*) under different pH levels. Ecotoxicol. Environ. Saf. 95, 83–90. http://dx.doi.org/10.1016/j.ecoenv.2013.05.020.

Parvez, S., Venkataraman, C., Mukherji, S., 2006. A review on advantages of implementing luminescence inhibition test (*Vibrio fischeri*) for acute toxicity prediction of chemicals. Environ. Int. 32, 265–268. http://dx.doi.org/10.1016/j.envint.2005.08.022.

Petala, M., Tsiridis, V., Kyriazis, S., Samaras, P., Kungolos, A., Sakellaropoulos, G., 2005. Evaluation of toxic response of heavy metals and organic pollutants using Microtox acute toxicity test. In: Proceedings of the 9th International Conference on environmental science and technology Rhodes island, Greece, 1-3 September 2005.

Pooley, D.T., Larsson, J., Jones, G., Rayner-Brandes, M.H., Lloyd, D., Gibson, C., Stewart, W.R., 2004. Continuous culture of Photobacterium. Biosens. Bioelectron. 19, 1457–1463. http://dx.doi.org/10.1016/j.bios.2003.09.003.

Radix, P., Leonard, M., Papantoniou, C., Roman, G., Saouter, E., Gallotti-Schmitt, S., Thiebaud, H., Vasseur, P., 2000. Comparison of four chronic toxicity tests using algae, bacteria, and invertebrates assessed with sixteen chemicals. Ecotoxicol. Environ. Saf. 47, 186–194. http://dx.doi.org/10.1006/eesa.2000.1966.

Ulitzur, S., Lahav, T., Ulitzur, N., 2002. A novel and sensitive test for rapid determination of water toxicity. Environ. Toxicol. 17, 291–296. http://dx.doi.org/10.1002/tox.10060.

van de Merwe, J.P., Leusch, F.D.L., 2015. A sensitive and high throughput bacterial luminescence assay for assessing aquatic toxicity – the BLT-Screen. Environ. Sci. Process Impacts 17, 947–955. http://dx.doi.org/10.1039/C5EM00012B.

van Wezel, A., Mons, M., van Delft, W., 2010. New methods to monitor emerging chemicals in the drinking water production chain. J. Environ. Monit. 12, 80–89. http://dx.doi.org/10.1039/b912979k.

Villaescusa, I., Marti, S., Matas, C., Martinez, M., Ribo, J.M., 1997. Chromium (VI) toxicity to luminescent bacteria. Environ. Toxicol. Chem. 16, 871–874. http://dx.doi.org/10.1002/etc.5620160506.

Villaescusa, I., Matas, C., Hosta, C., Martinez, M., Murat, J.C., 1998. Evaluation of lead(II) and nickel(II) toxicity in NaCl and $NaClO_4$ solutions by using Microtox® bioassay. Fresen J. Anal. Chem. 361, 355–358. http://dx.doi.org/10.1007/s002160050903.

Wang, X., Qu, R., Wei, Z., Yang, X., Wang, Z., 2014. Effect of water quality on mercury toxicity to *Photobacterium phosphoreum*: model development and its application in natural waters. Ecotoxicol. Environ. Saf. 104, 231–238. http://dx.doi.org/10.1016/j.ecoenv.2014.03.029.

Wang, W., Shi, C., Yan, Y., Yang, Y., Zhou, B., 2016. Eco-toxicological bioassay of atmospheric fine particulate matter ($PM_{2.5}$) with *Photobacterium phosphoreum* T3. Ecotoxicol. Environ. Saf. 133, 226–234. http://dx.doi.org/10.1016/j.ecoenv.2016.07.024.

WFD (Water Framework Directive), 2000. Directive 2000/60/EC of the European Parliament and of the Council of 23rd October 2000 establishing a framework for

community action in the field of water policy, L 327/1-82. EC, Brussels, Belgium.
Wu, S., Zhou, D., Xu, Y., Chu, C., Fan, J., 2011. The biotoxicity of heavy metals mercury, cadmium and chromium to luminous bacteria. J. Agro-Environ. Sci. 12, 2446–2450 (Chinese).
Ytreberg, E., Karlsson, J., Eklund, B., 2010. Comparison of toxicity and release rates of Cu and Zn from anti-fouling paints leached in natural and artificial brackish seawater. Sci. Total Environ. 408, 2459–2466. http://dx.doi.org/10.1016/j.scitotenv.2010.02.036.
Zeb, B., Ping, Z., Mahmood, Q., Lin, Q., Pervez, A., Irshad, M., Bilal, M., Bhatti, Z.A., Shaheen, S., 2016. Assessment of combined toxicity of heavy metals from industrial wastewaters on *Photobacterium phosphoreum* T3S. Appl. Water Sci. 7, 2043–2050. http://dx.doi.org/10.1007/s13201-016-0385-4.