µMAC-ToxScreen: a novel automatic luminous bacteria-based early warning online monitor for water toxicity

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Abstract
µMac-ToxScreen is an innovative automated online water quality monitoring system that uses luminescent bacteria biosensors to detect µg/L concentrations of toxic organic and inorganic chemical pollutants in surface or ground water, as well as raw and treated drinking water. The ToxScreen bioassay uses a renewable suspension of luminescent bacteria. When the bacteria are automatically mixed with a water sample, their light production, which is directly tied to cell respiration and other critical metabolic pathways, is decreased in proportion to the toxicity in the sample. The analytical part of the instrument is an automatic analyzer that uses a patented technology called Loop Flow Analysis (LFA) widely used in a variety of online chemical analyzers for water quality. At 14 day intervals, the instrument is re-supplied with a fresh inventory of liquid assay buffers and a freshly hydrated suspension of the freeze dried luminescent bacteria. Automatic safeguards have been engineered into the system to assure reagent and data quality and appropriate instrument functioning. The instrument is also equipped with auto calibration features to assure reliable instrument performance; microprocessor based system controls provide for data storage, data downloading, real time communication with a remote PC, and user adjustable alarm levels.

Keywords
Automatic alarm analyzer, Biomonitoring, Biosensor, Early warning monitoring system, luminescent bacteria, Water toxicity.

INTRODUCTION
The quality of potable water is one of the most important elements for human health. Intense development of the chemical industry, and the use of pesticides in agriculture can result in contamination of natural water resources. In addition, chlorinating of water (used to inhibit bacterial contamination) may lead to the generation of complex mixtures of toxic and genotoxic chlorinated hydrocarbons in drinking water. Public water supply systems are also vulnerable to intentional contamination of raw and finished water.

The goal of an early warning monitoring system is to reliably identify low probability/high impact contamination events in source water or distribution systems. Monitoring water quality in real time allow an effective local response that minimizes the adverse impacts that may result from the event. Other desirable features of the monitoring system include: affordable cost; low skill and training; coverage of all potential threats; ability to identify source; sensitivity to quality changes at regulatory levels; minimal false positive or negative responses; robustness; reproducible and verifiable results; allow remote operation; function year-round (Brosnan, 1999).

Analytical-chemical methods are usually target oriented; i.e., these methods can detect only a specific compound or a range of compounds having similar properties. Among such methods, screening techniques based on chromatography allow many different organic substances to be
identified in a single run; however, only a fraction of potentially occurring pollutants will be detected, especially at lower concentrations. Furthermore, analytical-chemical identification does not by itself give information about bioavailability and possible toxic effects, especially from mixtures of compounds. On the other hand, changes in the behavior or properties of on-line biological early warning systems may indicate the sudden occurrence of a pollutant not detected in conventional, analytical warning systems. Examples include the dynamic fish test, dynamic mussel test, dynamic water flea tests, delayed algal fluorescence, and aquatic toxicity tests such as Microtox-OS. In these systems, effects of pollutants are detected rather than concentrations (Brosnan, 1999).

The use of intact luminous bacteria for toxicity assessment has some clear advantages that have been scientifically validated (Bulich and Isenberg, 1981; Kaiser, 1998). Bioluminescence-based assays have been widely used for different applications (Bitton and Koopman, 1992), such as monitoring of plant effluents, leachate toxicity, soil contamination and monitoring of remediation processes. Due to their limited sensitivity to many toxicants, however, they have not been widely utilized as early warning systems for drinking water toxicity.

A novel luminous bacteria-based assay developed by CheckLight Ltd (ToxScreen-I) was recently evaluated and was found to enable the detection of sub-mg/L levels of heavy metals, pesticides, PAHs, and chlorinated hydrocarbons within 20-45 minutes (Ulitzur et al., 2002). Toxic agents that affect cell respiration, electron transport systems, ATP generation, the rate of protein or lipid synthesis, alter the level of luminescence. Similarly, agents that affect the cell's integrity and especially membrane function, have a strong effect on in vivo luminescence. Hence, toxicants of different characteristics such as, pesticides, herbicides, chlorinated hydrocarbons, heavy metals etc, exert a dramatic and measurable effect on the bacterial luminescence system. By comparing the luminescence level obtained in a suspected toxic sample with that obtained in a clean water-control sample after a short period of incubation, one can detect very low concentrations of a broad range of toxicants. For most of the toxic agents reported in this study, the new assay was markedly more sensitive than comparative bacterial bioluminescence toxicity data reported in the literature with the Microtox™ Vibrio fischeri assay.

The tests’s performance at detection of potential threats to drinking water (including chemicals, nerve agents and biotoxins) has been recently officially verified by the EPA’s Environmental Technology Verification (ETV) program (http://www.epa.gov/etv/verifications/verification-index.html).

The µMAC-ToxScreen is a novel automated on-line water quality monitoring system that uses luminescent bacteria to detect the presence of a wide variety of organic and inorganic pollutants at very low concentrations within 30-60 minutes. The system is designed to operate on-site, unattended for two weeks providing real time and continuous early warning of chemical pollution in water.

**METHODS**

**LFA technology**

The analytical part of the instrument is based upon Loop Flow Analysis (LFA) technology, widely used in a variety of chemical analyzers for water quality measurements (Sigon, 1998), stand alone use, surface water, ground water (Gunatilaka, 1996), and sea water (Zappalà, 1998-2002). This patented technology ensures maximal mixing of the used reagents in the water sample in a simple and reliable way and allows automatic measurement and recording of the analytical reactions.
To allow the automatization of the bioluminescence-based bioassay, the standard Loop Flow Reactor (Colosimo, 2002) was modified to include a photomultiplier tube (PMT) with internal counting to enable real time evaluation of the changes in bacterial luminescence (Figure 1).

**Figure 1**: LFA analytical reactor for toxicity measurements

The whole injection assembly was given a very strict thermo-regulation capability (using a Peltier cooling system) to enable maintenance of the hydrated bacteria compartment and the assay chamber at defined temperatures (3°C and 26°C, respectively). In addition, a mixing device was incorporated within the bacteria compartment in order to maintain the long-term homogeneity of the suspension.

The modified LFR device includes a new upgraded software to manage the specific new measuring method. A new graphic display was also designed to enable recording changes in luminescence. A programmable software was programmed to provide real time alarms in case of a water toxicity event. Such alarm may be directly provided through a local digital output contacts or through RS-232 output. Each set of data is stored in the analyzer microprocessor and is available for post-kinetic studies and monitoring the pattern of the changes in water quality with time.

**Automatic management of the measuring cycle**

The automatic ToxScreen test starts at defined interval times by external command (originating from digital contact, RS-232, or SMS from GSM), giving out toxicity alarms only when detected and evaluated by its internal quality check procedures. The user may set the alarm to any chosen inhibition level. At the end of the analysis and after proper washing the analyzer remains in standby mode, ready for a new cycle. The system is designed to periodically test negative (clean water) and positive (spiked toxic agent) controls. Automatic flushing and cleaning cycles ensure long-term consistency of measurements and minimization of biofouling build-up.

**ToxScreen II bioassay**

The reagents provided for running the automatic biomonitoring procedure are a modification of the published ToxScreenI assay (Ulitzur *et al.*, 2002). The higher sensitivity of the ToxScreen-II test to diverse group of toxicants with comparison to other bioluminescence-based tests is due to improvement of the test with respect to both strain selection and specific assay conditions. The selection of a highly sensitive variant of *P. leiognathi*, an improvement of its lyophilization procedure, and the selection of special assay conditions resulted in significant increase in sensitivity for most of the important toxic agents (see Table 1 for a few examples).
The ToxScreen-II Test was purchased as a kit from CheckLight Ltd., Israel. It included lyophilized bacteria, Hydration Buffer, Storage Buffer, Pro-Organic buffer (favors the detection of organic pollutants), and Pro-Metal buffer (favors the detection of heavy metals). These buffers were developed to enhance the sensitivity of the test to a wide range of agents with different modes of action. The added advantage of including strong buffers in the assay system lies in minimization of the artefactual toxic effect of extreme pH occasionally encountered when using other bioluminescence-based assays having only NaCl solution as the primary exposure medium.

**Reagents preparation**
A vial containing the lyophilized luminous bacteria was removed from the freezer, re-hydrated with 1.7 ml of Hydration Buffer and incubated at ambient temperature for 5 minutes, after-which it was mixed with 16.3 mL of Storage Buffer and placed in the chilled (~3°-5°C) compartment within the instrument, or in a refrigerator, for 12-24 hours before use. The concentrated Assay Buffers (Pro-Metal and Pro-Organic) were placed in the small upper compartment and connected to the proper tubing. Clean reference water (in this study- double distilled water) was placed in a container within the instrument. A wash solution, 70% ethanol in clean water, was placed in a second container and connected to the proper tubing (see Figure 1).

**Testing cycle sequence**
A testing cycle included the following steps: a cross-flow filter drew pre-filtered water out of a current flow. The sample water was allowed to run through the assay chamber for 1-2 minutes before 4mL were trapped inside. A 1:8 dilution (0.625ml) of the chosen concentrated Assay Buffer (Pro-Metal or Pro-Organic) was dispensed into the assay chamber (set at 26°C) and thoroughly mixed by circulating through the system. Next, 60µl aliquot from the chilled suspended cells was dispensed into the assay chamber and mixed well. Luminescence was recorded at 10 min intervals for 1 hour.

**Toxicity data**
In Table 1 IC50 values for the spiked chemicals were defined and graphically calculated as the inhibitory concentration (in mg/L) at which luminescence is inhibited by 50%, as compared to the clean water control sample. The toxicity threshold (LDL) was defined as the lowest concentration of contaminant to exhibit a percent inhibition significantly greater than the negative control. An inhibition was significantly greater than the negative control if the average inhibition plus or minus the standard deviation did not include zero. Data generated with the ToxScreen toxicity test were compared with Microtox™ luminescence toxicity data reported in the literature (Bulich and Isenberg, 1981; Munkittrick et al., 1991; Kaiser and Palabrica, 1991; Kahru, 1993).

**Table 1 – IC50 and LDL values (mg/l) obtained for common toxicants using ToxScreen-II and Microtox Tests™ (in brackets).**

<table>
<thead>
<tr>
<th>Toxicant</th>
<th>IC50 (mg/l)</th>
<th>LDL (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsenic (V)</td>
<td>0.15 (1.5II)</td>
<td>0.017 (NF)</td>
</tr>
<tr>
<td>Cadmium</td>
<td>0.04 (41.4IV, 106IV)</td>
<td>0.024 (20)</td>
</tr>
<tr>
<td>Lead</td>
<td>0.15 (11I, 0.6II)</td>
<td>0.06 (0.6)</td>
</tr>
<tr>
<td>Selenium</td>
<td>1.2 (NF)</td>
<td>0.3 (33)</td>
</tr>
<tr>
<td>Copper</td>
<td>0.11 (8I)</td>
<td>0.01 (0.8)</td>
</tr>
</tbody>
</table>
Tested in Pro-Organic Buffer:

<table>
<thead>
<tr>
<th>Toxicant</th>
<th>IC50 (mg/l)</th>
<th>LDL (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4,5- T</td>
<td>0.03 (52.2III, 158III)</td>
<td>0.01 (NF)</td>
</tr>
<tr>
<td>2,4,-Dinitrophenol</td>
<td>0.09 (10.6III)</td>
<td>0.025 (NF)</td>
</tr>
<tr>
<td>Arsenic (III)</td>
<td>1.7 (NF)</td>
<td>0.75 (7.8)</td>
</tr>
<tr>
<td>DDT</td>
<td>0.07 (7III, 13.8III)</td>
<td>0.006 (NF)</td>
</tr>
<tr>
<td>Potassium cyanide</td>
<td>0.45 (8.4I)</td>
<td>0.1 (0.73)</td>
</tr>
<tr>
<td>2,4-D</td>
<td>0.03 (5.7III, 100III)</td>
<td>0.01 (NF)</td>
</tr>
<tr>
<td>Chromium (VI)</td>
<td>0.1 (31II, 70I)</td>
<td>0.03 (15.3)</td>
</tr>
<tr>
<td>Flouroacetate</td>
<td>0.5 (NF)</td>
<td>0.05 (50)</td>
</tr>
<tr>
<td>Mercury</td>
<td>0.008 (0.065I)</td>
<td>0.002 (0.07)</td>
</tr>
<tr>
<td>Trinitrotoluene (TNT)</td>
<td>0.4 (NF)</td>
<td>0.03 (19.78)</td>
</tr>
</tbody>
</table>


Test compounds

In order to evaluate the discriminatory ability of the biomonitoring analytical results for various classes and groups of contaminants and to evaluate the reproducibility of test results, individual batches of baseline water and test water (spiked with various chemicals at defined concentrations) were prepared in the necessary volume for each test. All chemicals tested were reagent-grade (purity of >99%). Mercury was tested as HgCl₂; copper as CuCl₂.2H₂O; lead as Pb(II); arsenic (V) as NaAsO₂.7H₂O; cadmium as CdCl₂, chromium (V) as K₂Cr₂O₇, and selenium as Na₂SeO₄.

Organic chemicals were dissolved and kept as 1g/L stock solution in either ethanol (2,4-D (2,4-Dichlorophenoxyacetic acid), 2,4,5-T (2,4,5-Trichlorophenoxyacetic acid), dieldrin, DDT (dichlorodiphenyl-trichloroethane)), acetone (diazinon) or water (trinitrotoluene (TNT), round-up, potassium cyanide, arsenic (III)(tested as NaAsO₂.H₂O), sodium flouroacetate). For each chemical, a series of at least six double dilutions in ultra-pure water was prepared from a 10mg/L working solution and a parallel dilution set of the corresponding diluent, as control. The controls were clean double distilled water or the corresponding concentrations of ethanol and acetone were used as solvents.

RESULTS AND DISCUSSION

A full description of the modified ToxScreen-II test will be published elsewhere (in preparation). Briefly, ToxScreen-II test is based on the effect of toxic agents on the development of luminescence in the natural marine bacterium Photobacterium leiognathi (strain SB) that shows higher temperature tolerance than the Photobacterium leiognathi variant TANI-I used in the ToxScreen-I version. An important advantage of the new test is the extended usability of the suspended reagent: unlike the ToxScreen-I test version, the hydrated luminous bacteria are transferred into Storage Buffer and may be kept at 3°-5°C from which single tests may be drawn for up to 14 days. During the 14 days storage, light output diminishes but sensitivity and reliability of results remains quite stable. The ToxScreen-II exhibited improved sensitivity to a wide range of toxic agents, some of which are known as potential terrorism hazards (arsenic, cyanide, sodium flouroacetate, TNT).

In general, the dual assay buffer system (Pro-Metal and Pro-Organic) could discriminate between heavy metals and organic toxicants. However, there were a few noted exceptions, such as mercury, that was more active in the organic buffer.
Table 1 compares the sensitivity values obtained by the widely used Microtox™ test and ToxScreen-II test for common toxicants. For most of the toxic agents reported in this study, the ToxScreen-II test was markedly more sensitive than comparative bacterial bioluminescence toxicity data reported in the literature.

The µMAC-ToxScreen apparatus has undergone extensive lab testing to confirm the sensitivity and reliability of the data generated from the manual test.

CONCLUSIONS
The µMac-ToxScreen device is currently undergoing extensive field testing to confirm its lab performance. Once successfully validated, the technology developed by CheckLight and Systea is expected to have a major influence on the water testing and monitoring business worldwide, opening new possibilities for frequent monitoring of residential and commercial water systems as well as wastewater and industrial water treatment processes.

REFERENCES