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Fiber optic RecA *lux* sensor and genotoxicity biosensing of samples collected at HMDC sites

Final report for 2001-2002

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The Institute for Applied Biosciences and
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EXECUTIVE SUMMARY

The present report summarizes the results of our one-year study on the genotoxicity biosensing of samples collected at the HMDC (Hackensack Meadowlands Development Commission) and MERI (Meadowlands Environmental Research Institute) sites.

The environmental monitoring system used in this research utilizes whole-cell bacterial bioreporter sensors (genetically engineered to react to target toxicants by the induction of a selected promoter and the consequent emission of bioluminescent light through a recombinant *lux* reporter). We have designed a self-contained, disposable single-step assay, which is based on an optical fiber sensor module that integrates bioreporter microorganisms and a customized photodetector system. The optical fiber tip cores, which are covered with adlayer films consisting of calcium alginate containing the bioluminescent bioreporter, were exposed to environmental samples obtained from HMDC and MERI.

Two instruments were constructed, i.e., a multiple fiber maker, which facilitates simultaneous preparation of a number of fibers (22 fibers in a few min), and a portable photodetector for field measurements.

Most samples were tested by two bioassays, i.e., our optical fiber method and the commercial Vitotox™ kit. We found very good correlations between the results obtained with the two methods, with the Vitotox™ test showing slightly better sensitivity.

Highly genotoxic samples (induction factors higher than 4-5) were not found, but five samples gave positive results for the genotoxicity tests. The most genotoxic samples were those from the river sources (75661 HR3, River; 75662 HR5, River). Four samples were found to be cytotoxic, with the most cytotoxic sample being the 74948 1E-Manhole, Leachate.

The bioassay results did not correlate well with the chemical analyses, probably because not all possible pesticides were subjected to chemical analysis or because other substances that were not recognized by the chemical analyses may have been present.

The salinity and pH of the sample were found to influence the test. We therefore recommend that pH and TDS (total dissolved solids) measurements for samples showing a cytotoxic behavior be taken into account when attempting to delineate the source of the cytotoxic effect.

Preliminary storage experiments showed the ability of our sensors to maintain their functionality for at least the 10 days when stored in a rich organic medium (LB) at 4°C, but at the cost of a loss of activity of 50%.

In general, our method showed itself to be a fairly reliable tool for biomonitoring of genotoxic effects in bacteria, the results being consistent with those with commercial bioassays. Our bioassay facilitates rapid and reliable determination of toxic effects in environmental samples.

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1. PURPOSE

The overall aim of this study is to determine the feasibility of monitoring — with a field-operable fiber-optic biosensor system — water samples collected at HMDC (Hackensack Meadowlands Development Commission) and MERI (Meadowlands Environmental Research Institute) sites. The current report summarizes the results of our one-year study on the genotoxicity biosensing of samples collected at the above-mentioned sites. The work was performed at the HMDC Institute, NJ, USA and at BGU (Ben-Gurion University of the Negev), Beer-Sheva, Israel.

2. THE PROBLEM

The Hackensack Meadowlands is presently being reclaimed after almost a century of ongoing pollution. To objectively measure the impact of public policies and regulations on water, air and sediment quality over time, an environmental decision support system has been set up to aid decision makers and planners to better manage the District's development (Francisco Artigas: internal MERI document).

In discussions with various HMDC members (Mr. Alan Steinberg, Mr. Bob Ceberio, Mr. Irfan Bora, and Mr. Thomas Marturano) and CIMIC/MERI members at Rutgers, Newark (Dr. Nabil Adam, Dr. Francisco Artigas and Dr. Kirk Barett), it came to light that there is a definite need for a rapid, hand-held, field-operable monitoring device to make periodic tests of Hackensack Meadowlands water and sediment samples. Presently, the HMDC makes costly and slow, albeit thorough, chemical analyses (360/sample), including analyses of toxic inorganic and organic chemicals. There is, therefore, a need to simplify the monitoring procedure by providing an early rapid alert system.

In the international arena, a number of projects are presently being funded to help alleviate the monitoring issues encountered on a regular basis by city, regional, state, national and international water bodies. The contribution to this effort of our biosensors laboratory at the Institute for Applied Biosciences, BGU, which specializes in the development of novel biosensor technologies, is presently the development of its novel fiber-optic probe technologies for the monitoring of contaminated environmental water samples.

3. SPECIFIC OBJECTIVES

The need for early detection in the environment of toxic chemicals has lead to the development of two complementary approaches for detection and monitoring: 1) chemical analyses of the sample (as practiced by the HMDC), and 2) bioassays, which determine the negative effects of toxic chemicals on living biological systems. The latter approach comprises rapid and cost-effective microbial-based assays. Such assays may be based on genetically engineered microorganisms that emit a readily detectable luminescent signal in the presence of toxicants [1-9]. There are several ways in which the potential of such microorganisms for environmental monitoring can be translated into a routine assay format; these include the use of fresh [10-13], freeze-

dried [14] or immobilized cells [3, 15-18]. Adopting the latter approach, the laboratory of Dr. Marks at BGU has designed an assay based on the entrapment of bioluminescent bacterial layers in an optical fiber tip [21]. For this assay, *Escherichia* construct DPD1718, [3, 22], harboring a chromosomal integration of its *recA* promoter to *Photorhabdus luminescens luxCDABE* genes, was immobilized into a calcium alginate matrix on the tip of an optical fiber. The luminescent signal induced in the immobilized bacteria by the presence of a genotoxicant is collected by the fiber and amplified electronically [21,34].

Our aim in the current study was to apply this recently developed technology towards solving some of the problems at the HMDC. Our specific aims were:

- to construct a field-operable fiber-optic photo-detector device;
- to study at BGU various water [*Kearny surface freshwater marsh, old landfill well water, leachate chamber, wells around landfill, Berry's Creek contaminated brackish surface water, far up river uncontaminated brackish surface water and tap water (control)*] and sediment (*Kearny marsh, Berry's Creek and uncontaminated river-bend*) samples provided by HMDC personnel; and
- to examine *in situ* various HMDC test sites for their genotoxic potential.

During the course of this research project, we set out to build a first generation field-operable device to monitor wells at the HMDC. Such a development would provide the HMDC with an additional testing device that would bring a new monitoring dimension to its arsenal of testing practices. This study enabled us to test our monitoring device in the field under real-life conditions, with particular emphasis on the particular needs of the HMDC. This study is expected to be instrumental in enabling us to further our research prospects in the field of environmental monitoring.

4. OUR STRATEGY

Our approach to this problem is unique in that we are the first group to have made fiber-optic probes using bioluminescent bio-reporter bacteria for testing environmental water samples. The end-product of this approach is a disposable single-step assay kit that will provide toxicity information in real-time (120 min) and that can be used by nonskilled personnel. In short, we have created fiber optic probes containing bioluminescent bacteria that are sensitive to genotoxicants. The assay kit was used to evaluate measurable toxicity levels in real-life HMDC samples. At this stage, the limitation of the proposed system is that it is restricted to genotoxicity monitoring. In the light of the success of the current system, the ability of other bio-reporter bacteria to monitor other toxicants, such as heavy metals, will be determined. In addition, we foresee that the system can be modified to include GPS capability and radio-transmission to a mother station for immediate interaction or decisive action, when so required.

5. DESCRIPTION OF THE SYSTEM

5.1. Bioluminescent bioreporter organisms used in the present study

The bioassay is based on the use of the genetically engineered bacteria *E. coli* DPD1718 and DPD2794.

Strain	Host	SOS promoter	Reporter	Location of fusion
DPD1718	*E. coli*	*recA*	*Photorhabdus luminescens luxCDABD*	Chromosomal integration
DPD2794	*E. coli*	*recA*	*Vibrio fischeri luxCDABD*	Multi-copy plasmid

In the bioassay bacteria used in this research, bioluminescence (*lux*) genes are fused to promoters of genes that are involved in defense against DNA damage, i.e., the SOS response system and the adaptive response circuits. In the presence of DNA-damaging agents, these genes are induced, leading to the production of luciferase and its substrate, a long-chain aldehyde. During the enzymatic reaction of luciferase that oxidizes the respective long-chain aldehyde, the bacteria emit light in a dose-dependent manner (Fig. 1). In this way, the bacteria rapidly signal the presence of genotoxic compounds.

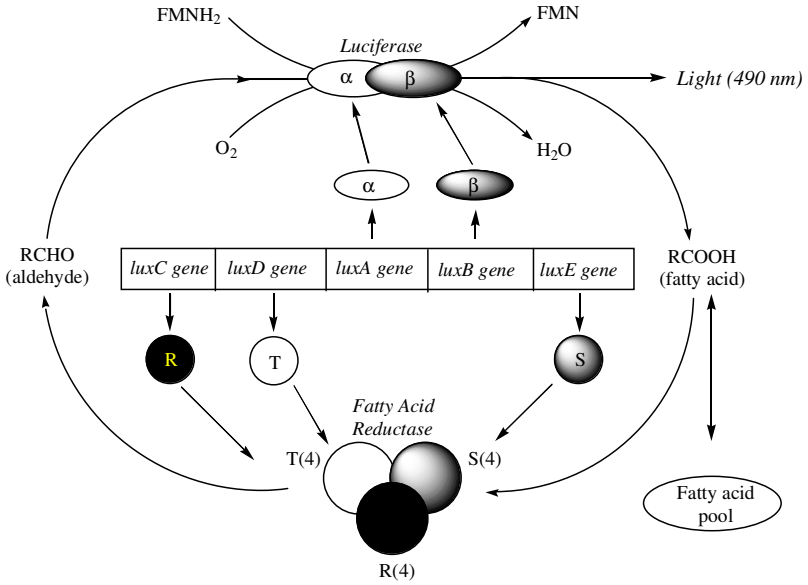

Fig. 1. Relationship between *lux* genes and the corresponding proteins in the bacterial bioluminescence reaction. The fatty acid reductase complex comprises four polypeptide subunits of reductase (R), four subunits of synthetase (S), and four subunits of transpherase (T). Luciferase is a heterodimer comprising an α subunit and a β subunit.

The genetic control that occurs in the bacteria is classified as negative regulation. In a noninduced cell, the product of the *lexA* gene acts as the repressor of both the SOS response genes (including the *recA* and *luxA* genes) and the bioluminescent (*lux*) genes. It does so by binding to similar operator sequences located upstream of each gene or operon. In response to SOS-inducing conditions, a signal that leads to the expression of the SOS regulon is generated. A considerable body of evidence suggests that this signal consists of regions of single-stranded DNA (ssDNA). The latter may be generated when a cell attempts to replicate damaged DNA or under a variety of other circumstances. The binding of the RecA protein to these regions of ssDNA in the presence of nucleoside triphosphate leads to the formation of a nucleoprotein filament and converts RecA to the activated form RecA*. The interaction of the activated RecA* protein with the LexA repressor results in the proteolytic cleavage of LexA and the continued expression of both the SOS response and the conjugated bioluminescence (*lux*) genes (Fig. 2).

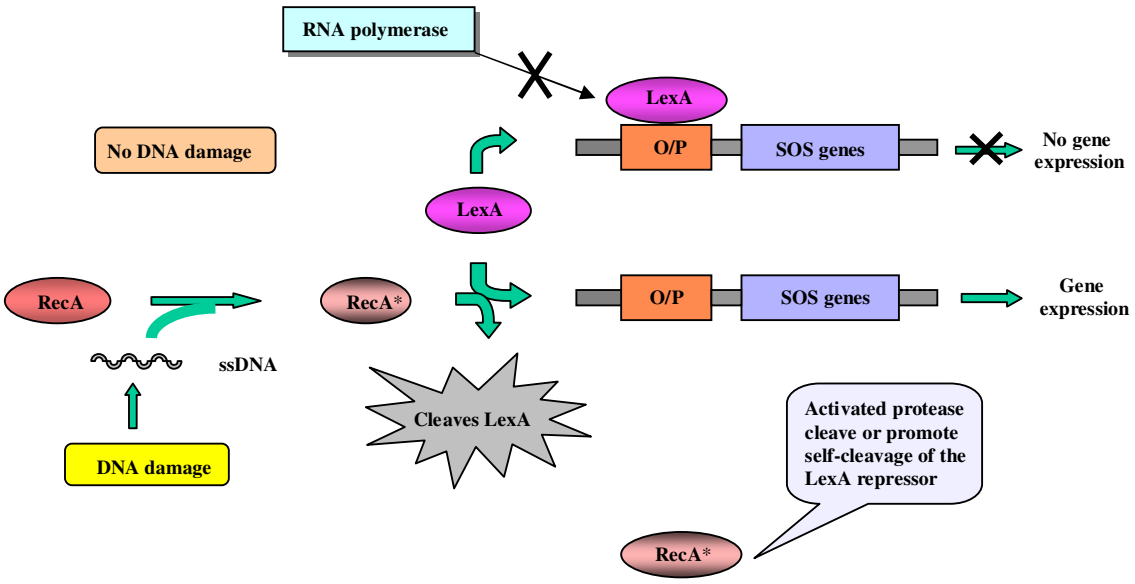

Fig. 2. Model of the SOS regulatory network

5.2. Type of stress monitored by the bacteria

The type of stress that the bioassay bacteria will detect is that imposed by chemicals that cause damage to primary DNA by alkylating DNA bases. This may lead to inhibition of DNA synthesis and of the induction of DNA cross-linking to an extent proportional to its content of guanine and cytosine.

In the bioassay, genotoxic effects are evaluated in terms of activation of the SOS response in the bacteria through the detection of the luciferase activity from an integrated bioreporter system. The fiber-optic biosensor is an innovative format that incorporates bioreporter bacteria in the tip of an optical fiber. Bioreporter cells are immobilized within calcium alginate adlayers (Fig. 3A) by physical adsorption on the 1-cm tip of the optical fiber transducer (Fig. 3B).

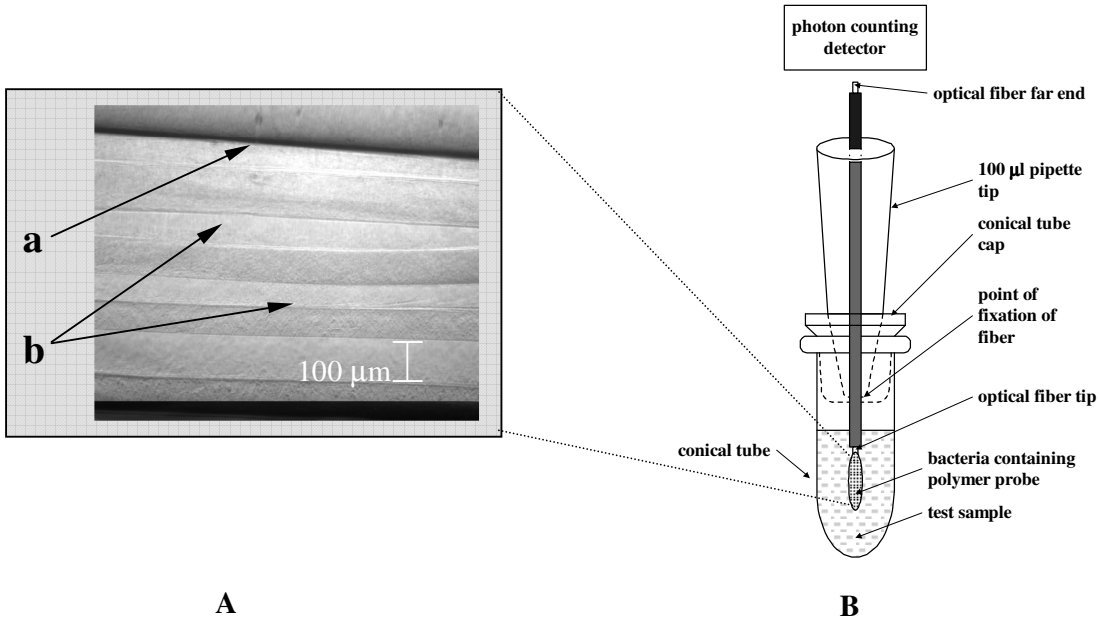

Fig. 3. (**A**) Micrograph of probe adlayers set onto the optical fiber core. (a) The fiber-probe interface. (b) Polymer layers with an approximate thickness of 80-100 µm. (**B**). Optrode set-up.

The far end of the optical fiber is integrated with a photodetector system, which is connected through a specific driver to a laptop computer. The light signal created by the induced bacteria is collected and transferred through the optical fiber to a sensitive photon counting detector and analyzed (Fig. 4). The measurements may be made at a given point in time or in a continuous manner, depending on the kind of information required.

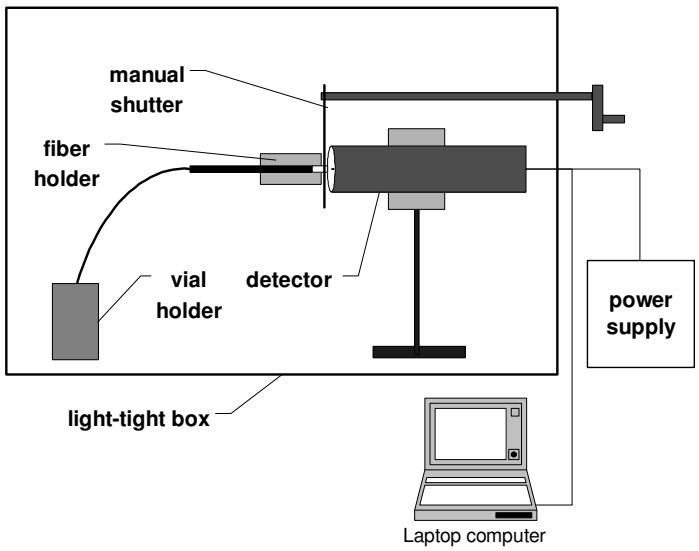

Fig. 4. Photodetector system set-up designed to be used at HMDC

5.3. New field-type photodetector instrument

To measure the low light response produced by the bioluminescent bacteria, a photon counting system was designed and built in our laboratory. The output signal, in analog measurements, is the mean value of the pulses generated after multianode magnification in the photomultiplier tube. Bioluminescence measurements are obtained with a Hamamatsu HC135-01 PMT Sensor Module, comprising a sensitive photomultiplier tube and a microcontroller. The detector is optimized to the blue light region and includes a 21-mm diameter active area, which is convenient for gathering light radiation without any optical focusing elements (Fig. 5).

The instrument set-up is placed in an aluminum light-tight box. The far end of the optical fiber is held in a fiber holder (FPH-DJ, Newport), which is placed in an adjustable single-fiber mount (77837, Oriel). To prevent damage to the photon-counting unit by environmental light, a manual shutter (71430, Oriel) is placed in front of the detector. To move the slide shutter, a custom lever is placed outside the box. To receive and treat data, a specific driver was developed using LabView (version 3.1, National Instruments Corporation), which allowed monitoring of the bioluminescent signal and data handling in real time.

During the bioassay, an optical fiber with its adsorbed biological probe is placed in a 0.5-ml conical tube (Jonplast, Italy) within the test sample solution. The optical fiber is held at the epicenter of the light-proof conical tube by means of the pipette tip (Fig. 3B).

Fig. 5. Field-type portable photodetector device used at HMDC

5.4. New multiple fiber preparation device

To improve and simplify preparation of the optical fiber probe, we constructed an instrument for one-step multiple fiber preparation, as shown in Fig. 6. This device consists of a matrix holder for 22 fibers that can move up and down along the Z axis. Solutions with the alginate/bacteria mixture and cross linker ($CaCl_2$) move in the perpendicular direction (Y). Alternate dipping of all 22 fibers into the bacterial/alginate and cross linker solutions facilitates the creation of polymer layers containing bacteria on the tips of optical fibers. After probe preparation, the matrix holder with its 22 fibers is inserted into the 22-position rack with 22 Eppendorf tubes containing the solutions to be tested (Fig. 6).

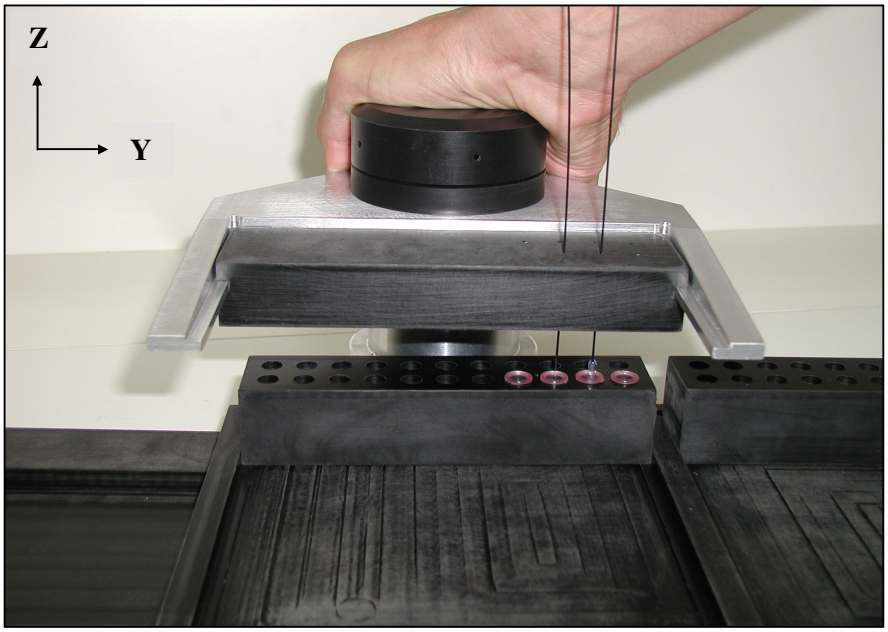

Fig. 6. Multiple probe preparation instrument. Twenty two fibers can be prepared simultaneously within a few minutes

5.5. Commercially available genotoxicity test kits

The most commonly used genotoxicity bioassay kits are summarized in Table 1. The pioneering Ames test (1975) has been superceded by an improved version, known as the Mutatox test (1985). Both tests use mutant strains whose normal biological properties are restored as a result of a back mutation caused by the genotoxic material. The rate of back mutation is proportional to the genotoxic effect. The more recently developed Vitotox™ test (1995) is a more sophisticated test kit, based on recombinant *Salmonella typhimurium*. The mechanism of induction of these cells is similar to that used in our method for *E. coli* (DPD1718 and DPD2497). The difference between the inductive Vitotox strain and the *E. coli* strains lies in the SOS promoters that control the reporter

genes (the *Rec*N promoter in Vitotox and the *Rec*A promoter in DPD1718, 2794). Because the reporter genes are controlled by different promoters, it is expected that the response of the Vitotox strain to a particular activating material may sometimes differ from that of the *E. coli* strains.

Due to the similarity between the Vitotox strain and the strains used in our method, the Vitotox kit was chosen as the validation bioassay for our method. All samples were screened with this kit, which was purchased from the ThermoLabsystem, Finland.

Table 1. Commercially available microbial tests

Test	Organism	Principle and description
Ames	*Salmonella typhimurium*	Measurement of the rate of reversion of His^- to His^+ mutants that have the ability to grow on histidine-deficient medium Rate of mutation proportional to the concentration of mutagens
Mutatox™	*Vibrio fisheri*	Dark variant of the luminous bacteria (*V. fisheri)* employed to determine the ability to restore luminescence Similar to Ames test, the difference being the reporter
Vitotox™	*S. typhimurium* genetically engineered *(lux CDABE* from *V. fisheri)*	Uses two different recombinant *S. typhimurium* strains carrying a luciferase operon to determine: • *Rec-N::luxCDABE*-genotoxicity (inductive strain, in the presence of genotoxicants the *Rec*N promoter is activated and downstream *lux* genes are expressed) Measurement of induction factors. • *Pr1::luxCDABE-cytotoxicity* (strain that produces luminescence constitutively; in the presence of toxic substances, the cytotoxic effect causes the degree of luminescence to decrease) Measurement of inhibition factors

5.6. Metabolic activation of toxic samples

Many substances act as mutagens only after having been metabolized appropriately. Metabolic enzyme systems such as those found in eucaryotic cells are not present in bacteria. Therefore, an exogenic source of metabolic enzymes—in this case a rat liver homogenate (S9-fraction)—has to be added. In addition, an NADPH regenerating system, consisting of nicotinamide adenine dinucleotide phosphate (NADP) and glucose-6-phosphate, must also be added. In the validation experiments with Vitotox, the samples were tested with and without metabolic activation.

6. EXPERIMENTAL METHODS AND PROTOCOLS USED IN THE PRESENT RESEARCH

6.1. Protocol for tests performed at HMDC and BGU with our optical-fiber bioassay kit

Day 1	▪ Prior to immobilization, the transformant cells were grown overnight at 37ºC with shaking (rotary thermoshaker, 140 rpm) in Luria-Bertani (LB) medium [25] containing Bacto Tryptone, 10 g/l; yeast extract, 5 g/l; and NaCl, 10 g/l. For positive selection of the *lux* transposon the medium was supplemented with chloramphenicol (30 mg/l) for strain DPD1718 or ampicillin (100 mg/l) for strain DPD2794.
Day 2	▪ Cultures were diluted to approximately 10^7 cells/ml and then cultured to an early exponential growth phase under the same conditions, but without antibiotics. Cells were harvested when the optical density of the culture reached 0.37-0.38 OD units at 660 nm, which corresponded to a concentration of about $1.5\text{-}2.0 \times 10^8$ cells/ml (1.5-2 h). ▪ Disposable probes were prepared as follows (1-1.5 h): The harvested cells were mixed in a 1:1 ratio with a filter-sterilized 2% (w/v) low-viscosity sodium alginate solution. The 1-cm optical fiber tip was first exposed (for a few seconds) to the bacterial alginate suspension, and then dipped (for a few seconds) into a sterile 0.5 M calcium chloride solution, thus entrapping the bacteria onto the fiber within a hardened calcium alginate matrix. Repeating these steps five times (to give six layers) increased the number of bacterial sensor cells attached to the optical fiber transducer. The optical fibers, with their immobilized bioluminescent bacteria at their end face tip, were then ready for the experimental monitoring of the collected samples. Fibers were used immediately after preparation. ▪ Luciferase activity was then determined as follows (3-5 h): the measurement was started 3-3.5 h after beginning preparation of the assay set up on day 2.

Comments

* Assay-volume: 200 µl.
* Dilutions may be performed. Number of dilution steps depends on toxicity of the sample.
* Measurements are performed at pH 7, with no added salt requirement.
* Metabolic activator was not used, but may be added to test solution if necessary.
* Solvent requirements: It is preferable to dissolve the sample in distilled water. In the case of poor solubility of the sample in water, DMSO may be used as a co-solvent solvent (in a final concentration of no more than 1%).
* Our test cannot be used for the analysis of all categories of compounds, because of matrix limitations. Some materials may react with the matrix material and therefore not reach the bacteria (sensing element) or they may dissolve the matrix, resulting in disintegration of the probe.
* Bacterial cells were routinely maintained at 4°C on LB agar [1.5% (w/v)] plates supplemented with chloramphenicol (30 mg/l) or ampicillin (100 mg/l) for strains DPD1718 and DPD2794, respectively.

6.2. Protocol for tests performed at BGU of cells in suspension (luminometer measurements)

Day 1	▪ The transformant cells were grown with shaking overnight at 37ºC in a rotary thermoshaker at 140 rpm on LB medium [25] ▪ For positive selection of the *lux* transposon, the medium was supplemented with chloramphenicol (30 mg/l) for strain DPD1718 or ampicillin (100 mg/l) for strain DPD2794.
Day 2	▪ Cultures were diluted 1:100 to approximately 10^7 cells/ml and then cultured to an early exponential growth phase under the same conditions, but without antibiotics. The cells were harvested when the optical density of the culture reached 0.37-0.38 OD units at 660 nm, which corresponded to a concentration of about 1.5-2.0 x 10^8 cells/ml (1.5-2 h). ▪ 80 µl of the cell suspension was added to 10 µl of a solution of a standard chemical or 10 µl of the environmental sample plus 10 µl of water (without S9 fraction). ▪ When S9 was used, instead of the 10 µl of water, 10 µl of the S9 (10 %) was added. ▪ Measurement of luciferase activity (3-5 h): the measurement was started 2 h after the beginning of the assay on day 2.

6.3. Protocol for Vitotox

The protocol supplied with the bioassay was followed.

6.4. Standard materials used as positive controls

Materials that do not require a metabolic activation

Note: All these materials are well known genotoxicants.

1. Mitomycin C (M 0503, Sigma)
2. N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG), (Aldrich, 129941)
3. Nalidixic acid, sodium salt (N 4382, Sigma)

Materials that require a metabolic activation

Note: All these materials are well-known precursors of genotoxicants.

1. 2-Aminofluorene (2-AF), (Aldrich, A5,550-0)
2. Benzo(α)pyrene [B(α)P], (Aldrich, 41,062-4)

6.5. Protocol for metabolic activation and preparations of the standards

Stock solution of 2-aminofluorene (2-AF)
The stock solution was prepared in the following way:
8 mg (2-AF)/4 ml DMSO = 2000 µg/ml = 2000 ppm in DMSO.
4 ml (2000 ppm) + 36 ml of water = 200 ppm in 10% DMSO.
10 µl (200 ppm, 10% DMSO) was then taken for the test + 80 µl of cells + 10 µl of S9, so the final concentration will be 20 ppm, 1% DMSO.

The 200 ppm 10% DMSO solution was diluted serially 1:2 with 10% DMSO to give 100, 50, 25, and 12.5 ppm of 2-AF in 10 % DMSO. An aliquot of 10 µl of each of these serial dilutions (in 10 % DMSO) was added to the wells of a microtiter plate, and 80 µl of cells and 10 µl of S9 were added to a final volume of 100 µl. The final concentrations of 2-AF were 20, 10, 5, 2.5, and 1.25 ppm (in 1% DMSO).

Stock solution of benzo-α-pyrene [B(α)P]
The stock solution was prepared in the following way:
A solution of 8 mg of B(α)P/1ml DMSO = 8000 ppm was divided into aliquots stored at –20 ^{o}C for 6 months. A 1/10 dilution of the stock solution (100 µl of stock to 900 µl of water) gives a 800 ppm solution (10% DMSO).

Serial dilutions of this 800 ppm solution (1:2) with 10 % DMSO gave 400, 200, 100 and 50 ppm solutions (in 10% DMSO). Aliquots of 10 µl from each of the serial dilutions were added to the wells of microtiter plate, and each was made up to a final volume of 100 µl with 80 µl of cells and 10 µl of S9. The final concentrations of B(α)P were 80, 40, 20, 10 and 5 ppm (in 1% DMSO).

Test mixture composition for suspension experiments with and without S9

Without S9	Blank 10 µl solvent (10% DMSO) 10 µl water 80 µl cells	Standard or sample dilutions 10 µl standard in (10%DMSO) or sample 10 µl water 80 µl cells
With S9	Blank 10 µl solvent (10% DMSO) 10 µl S-9 (4 or 10 %) 80 µl cells	Standard or sample dilutions 10 µl standard in (10%DMSO) or sample 10 µl S-9 (4 or 10 %) 80 µl cells

S9 preparation
Lyophilized S9 (catalog number 11-01L, Molecular Toxicology, Inc., USA) was rehydrated with 2.1 ml of ice-cold water, divided into aliquots, and stored at –20^{o}C. Different materials require different concentrations of S9 for optimal metabolic activation. The most commonly used concentrations of S9 were 4 and 10 % (v/v). We

found that 4% of S9 was not sufficient for our standards and samples, and therefore a 10 % (v/v) S9 mixture was used in these studies.

10% (v/v) S9 mixture preparation

100 µl from rehydrated S9 + 900 µl Regenesis solution "A" (total 1,000 µl).

Regenesis solution "A" (catalog number 60-200-3, Molecular Toxicology Inc., USA) consists of:

Component	Supplier	Catalog number	Concentration (g/lit) in final solution	Concentration
NaH_2PO_4 xH_2O	Sigma	S-9638	3.1	0.1 M } pH 7.4
Na_2HPO_4	EM Science	SX0720-1	12.0	0.1 M
KCl	Fisher	P217-3	2.7	33 mM
$MgCl_2$	Fisher	M33-500	1.8	8 mM
Glucose-6-phosphate	Roche	153079	1.6	5 mM

The final concentration of NADP-coenzyme should be 4 mM (3 mg NADP/1000 µl of the final S9 mixture). However, for our experiments we found the optimal concentration of NADP to be higher than that recommended in the literature and by the manufacturers, i.e., 7-8 mM (5-6 mg NADP/1,000 µl of the final S9 mixture). NADP was weighed and added to Regenesis solution "A" before treatment and mixed with S9 to give final 10 % (v/v) concentration of S9 as described earlier. Standards and samples were pretreated with the final S9 mixture for 1 h at 4°C before adding cells to facilitate the enzymatic digestion. After addition of cells, the measurements were started immediately.

7. FACTORS AFFECTING TESTS

7.1. Effect of salinity of the sample on the measurements

To determine the effect of the salinity of the sample on the measurements, salt solutions of different concentrations were prepared. The concentrations of these solutions were expressed as a dilutions of the physiological saline concentration (0.9 % w/v NaCl). The following salt concentrations were tested: 0.1× , 0.25×, 0.5×, 1×, 2.5×, 5×, 10× of the normal saline concentration. In all the samples, a constant concentration of 320 ppb of the standard inducer material, mitomycin C, was used for induction of cells.

7.2. STORAGE STUDIES

Microtiter assays were performed at intervals of one or two days over a two-week period on the same batch of alginate beads containing bioreporter cells. A sterile alginate solution (4% w/w) was mixed with an equal volume of suspended cells. The gel beads were formed by dripping the alginate-cell suspension into a 0.1 M $CaCl_2$ solution. Beads were stored in different media (rich organic medium (LB), minimal inorganic

medium AB [35], physiological saline, and Tris buffer, pH 7.4) at 4-6ºC. Mitomycin C solution (3.2 mg/l) was used for the induction of the cells. A luminometer (BMG-labtechnologies, Germany) was used for luminescence measurements in a 96-well format. Cell viability was followed by count plating serial dilutions after bead dissolution on each consecutive day. The average bead diameter was in 3-4 mm.

8. DESCRIPTION OF THE ANALYZED SAMPLES

8.1. Samples collected at HMDC sites on 19 January 2001 and sent to BGU for analysis

Table 2. Samples collected for genotoxicity testing at the sites of HMDC (19 January 2001)

Sample #	Location	Description	pH
46215	Kearny Marsh	Fresh water	7.81
46216	Golf Course	Groundwater	8.14
46217	Meter chamber	Leachate	8.77
46218	1A-1	Groundwater	8.01
46219	1A-4	Groundwater	8.54
46220	HMDC Lab	Tap Water	7.91
46221	UBC	Brackish	7.85
46222	HR1	Brackish	7.97
46223	Berry's Creek 1	Sediment	-
46224	Kearny Marsh	Sediment	-
46225	Kingsland	Sediment	-

8.2. Samples collected and analyzed at HMDC sites during the visit of Boris Polyak

Table 3. Samples collected for genotoxicity testing at the sites of HMDC (5 September 2001)

Sample #	Location	Description	pH
74946	Meter Chamber	Grab	7.13
74947	Meter Chamber	Composite	7.23
74948	1E-Manhole	Leachate	7.26
74951	Peach Island Creek	Brackish	6.86
74951*	Peach Island Creek	Sediment	-
74952	Kingsland	Brackish	6.58
74952*	Kingsland	Sediment	-
74953	Kingsland	Brackish	7.55
74955	Berry's Creek 1	Tidal Creek	6.64
74955*	Berry's Creek 1	Sediment	-
74956	Berry's Creek 2	Tidal Creek	6.65
74956*	Berry's Creek 2	Sediment	-

(*) soil samples were extracted with water for 1 h at room temperature, and filtered through a 0.45-µm pore size filter into a sterile beaker. The filtrates were analyzed for the presence of genotoxicants.

8.3. Samples collected on 31 November 2001 at HMDC sites and sent to BGU for analysis

Table 4. Samples collected for genotoxicity testing at the sites of HMDC (31 November 2001)

Sample #	Location	Description	pH
75657	Berry's Creek	Tidal Creek	7.37
75658	HR 1	River	7.17
75659	Kearny Marsh	Freshwater Marsh	8.02
75660	Penhorn Creek	Tidal Creek	7.98
75661	HR 3	River	7.40
75662	HR 5	River	7.39
75673	Chamber	Grab	8.49

Table 5. Key table of samples analyzed chemically at HMDC

Site	Code numbers			
	Genotoxicity Numbers	**Pesticide Numbers**	**VOA Numbers**	**Semi-VOA Numbers**
Leachate Meter Grab	74946		75670	
Leachate Meter Composite	74947			
1E-Manhole, Leachate	74948			
Sports Complex, Laggon 4	74949			
Sports Complex, Dredge	74950			
Peach Island, Brackish	74951			
Kingsland, Brackish	74952			
Kingsland, Brackish	74953			
Berry's Creek 1, Tidal	74955	75663	75598	75602
Berry's Creek 2, Tidal	74956			
Berry's Creek 3, Tidal	74957			
Berry's Creek, Tidal	75657			
HR1, River	75658	75664	75654	75656
Kearny Marsh, Freshwater	75659	75666	75601	75604
Penhorn Creek, Tidal	75660	75667	75600	75606
HR3, River	75661	75665	74648	75650
HR5, River	75662	75652	75651	75668
Meter Chamber	75673	75674		75672

Note:

Entries in blue: those for which both chemical analyses and bioassay results are given.

Genotoxicity numbers: codes for samples tested by bioassays.

Pesticide numbers: codes for samples tested for the presence of target pesticides.

VOA numbers: codes for samples tested for the presence of volatile organics.

Semi-VOA numbers: codes for samples tested for the presence of volatile and semi-volatile organics.

9. RESULTS

9.1. General

During the present research period, we tested a total of 26 samples. A chemical analysis was provided for eight of these samples. The results obtained were divided into three categories:

1. ***Not geno/cytotoxic***
2. ***Genotoxic or potentially genotoxic or***
3. ***Cytotoxic***

A ***genotoxic*** material is defined as a substance that affects the DNA of the living organism causing DNA mutation; in this case, we will expect to get the induction of luminescence from the tested bacteria.

A ***cytotoxic*** material is defined a substance that causes cell death and therefore no luminescence or a significantly reduced level of luminescence

9.2. EVALUATION CRITERIA

Each bioassay defines its particular criteria for genotoxicity. For Vitotox™ and the "umu" assays, a test compound will be considered genotoxic when the induction factor F_i (luminescence of the sample/luminescence of the control) is > 1.5. The "SOS chromotest" defines this evaluation criteria as $F_i > 2$. Since we compared our results with Vitotox™, we used the same evaluation criteria in our method as those for Vitotox, i.e., for gentoxicity $F_i > 1.5$, and for cytotoxicity $F_i < 0.67$ (0.67 = 1/1.5).

9.3. Optical fiber genotoxicity testing

Fig. 7 (a,b) presents measurements for MNNG (N-Methyl-N-nitro-N-nitrosoguanidine), as a standard chemical. Concentrations >20 µg/ml were cytotoxic, as shown by the dramatic decrease in luminescence in Fig. 7(a). From the calibration curve for this standard chemical, the experimentally obtained lowest observed effective concentration (LOEC) ($F_i \geq 2$) was 0.625 µg/ml (ppm), while the calculated value (dotted line) was 0.48 µg/ml [Fig. 7(b)]. Similar responses were obtained for mitomycin C (detection limit 25 µg/lit ppb, Fig. 8) and nalidixic acid (detection limit 1.5 µg/ml ppm).

The results for the 26 samples tested by the optical fiber method are presented in Fig. 9 (non-geno/cytotoxic samples) and Fig. 10 (genotoxic and cytotoxic).

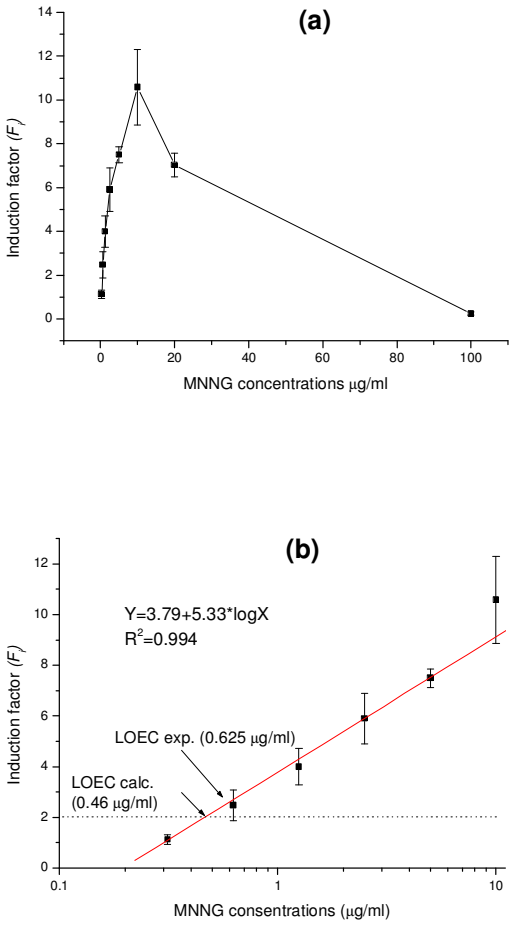

Fig. 7. Bioluminescence induction of *E. coli* DPD1718 presented as a signal to noise ratio (induction factor) vs. concentration of NMMG (0.313-100 µg/ml), (positive control, does not require metabolic activation)

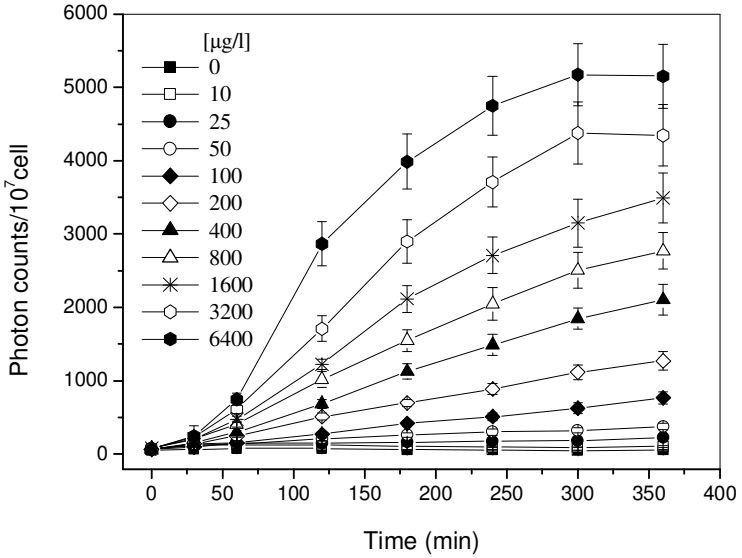

Fig. 8. Light kinetics for mitomycin C at various concentrations (positive control, does not require metabolic activation).

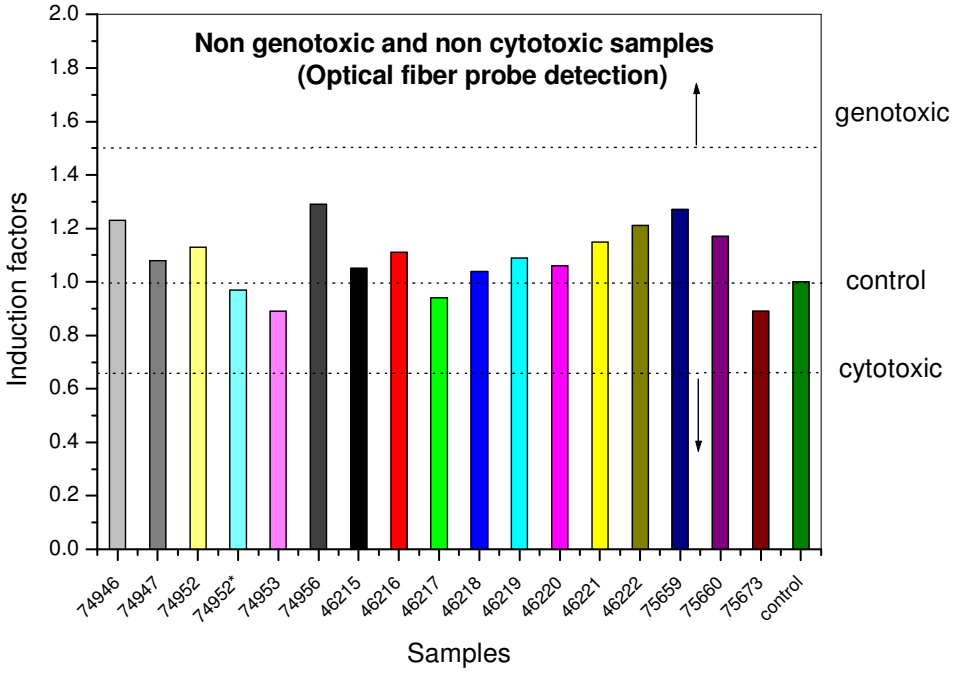

Fig. 9. Samples shown to be neither genotoxic nor cytotoxic by the optical fiber probe detection method

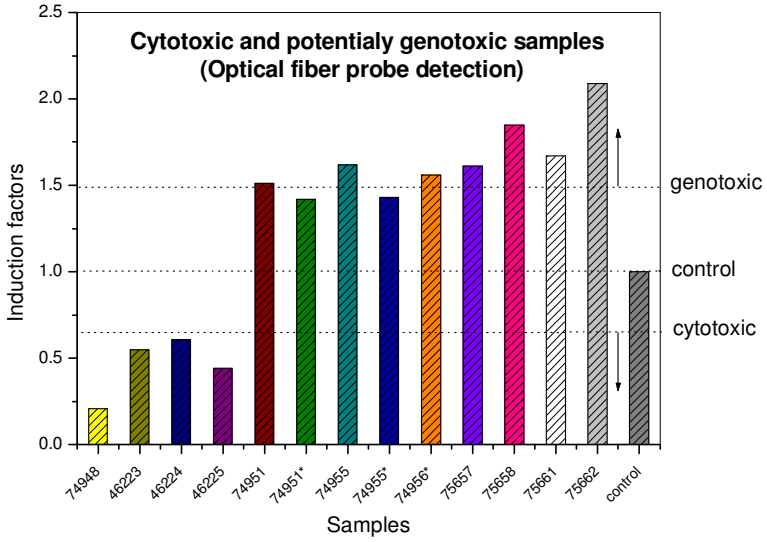

Fig. 10. Samples shown to be cytotoxic and genotoxic by the optical fiber probe detection method

The results presented above do not include the metabolic activation. We still need to optimize the procedure for use of S9 with our method. The metabolic activation was tested only with the Vitotox™ assay.

9.4. VITOTOX™ BIOASSAY

The Vitotox bioassay was used as a validation test for our method. Eighteen samples were tested by Vitotox with and without metabolic activation. The results of these tests are presented in Fig. 11 and 12 (standards that not require the metabolic activation), Fig. 13 and 14 (standards requiring metabolic activation), Fig. 15 (non-geno/cytotoxic samples), Fig. 16 (cytotoxic samples) and Fig. 17 (genotoxic samples).

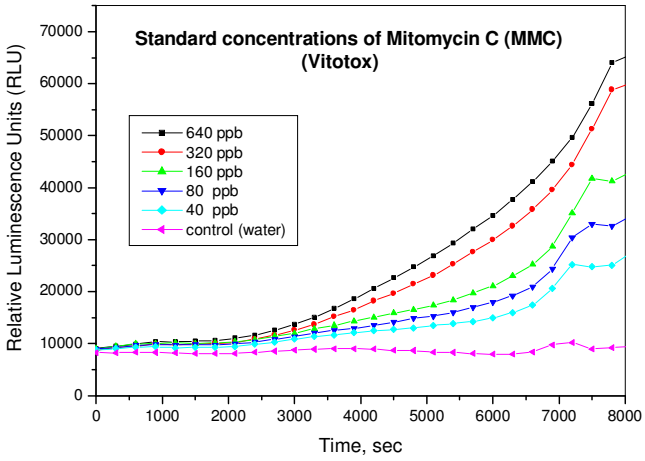

Fig. 11. Light kinetics for mitomycin C (MMC) at various concentrations (positive control, not requires a metabolic activation).

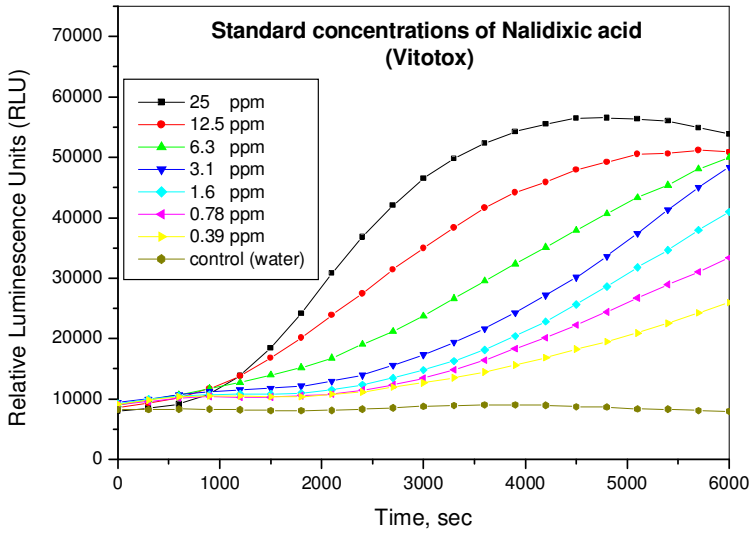

Fig. 12. Light kinetics for nalidixic acid at various concentrations (positive control, does not require metabolic activation)

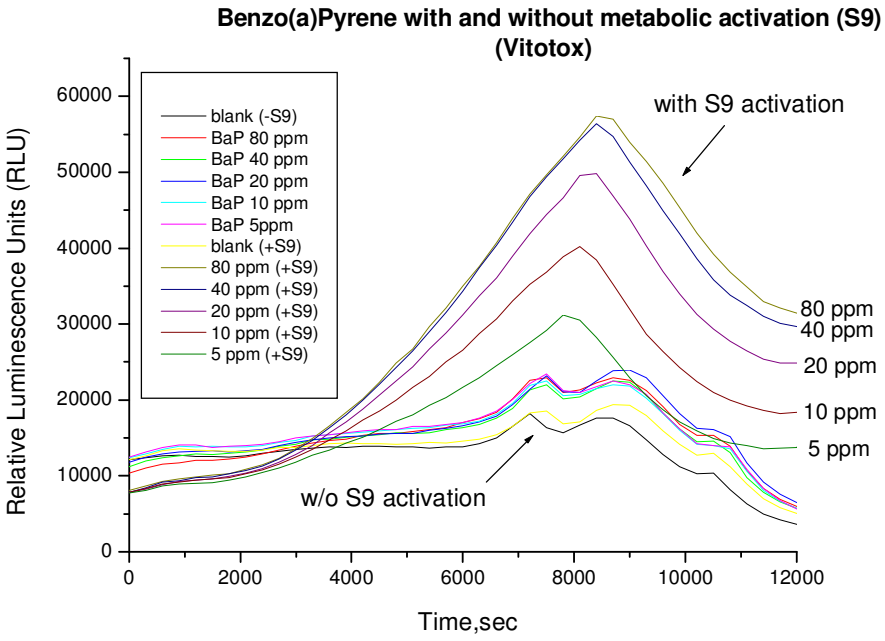

Fig. 13. Light kinetics for benzo(α)pyrene at various concentrations (positive control, requires metabolic activation)

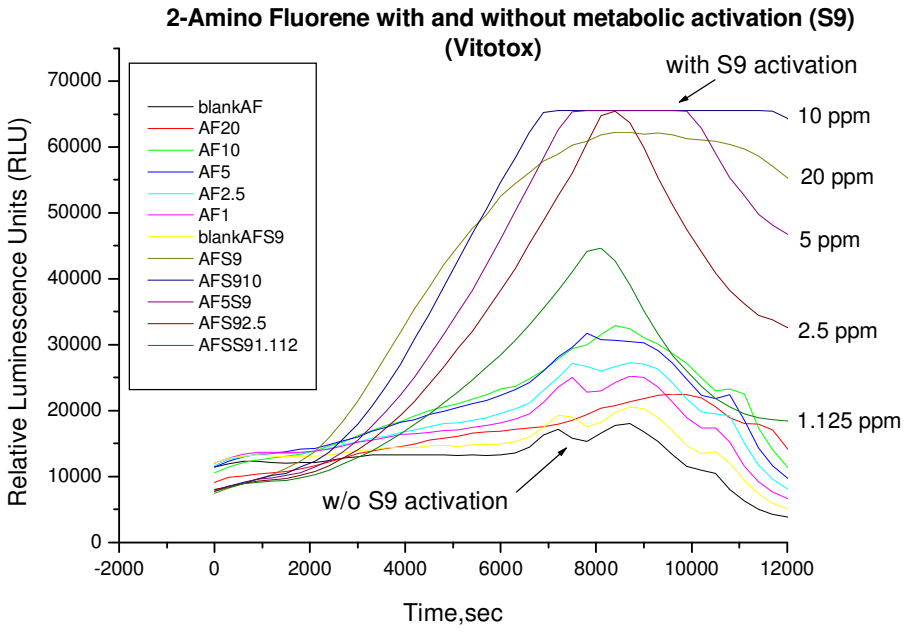

Fig. 14. Light kinetics for 2-amino fluorene at various concentrations (positive control, requires metabolic activation)

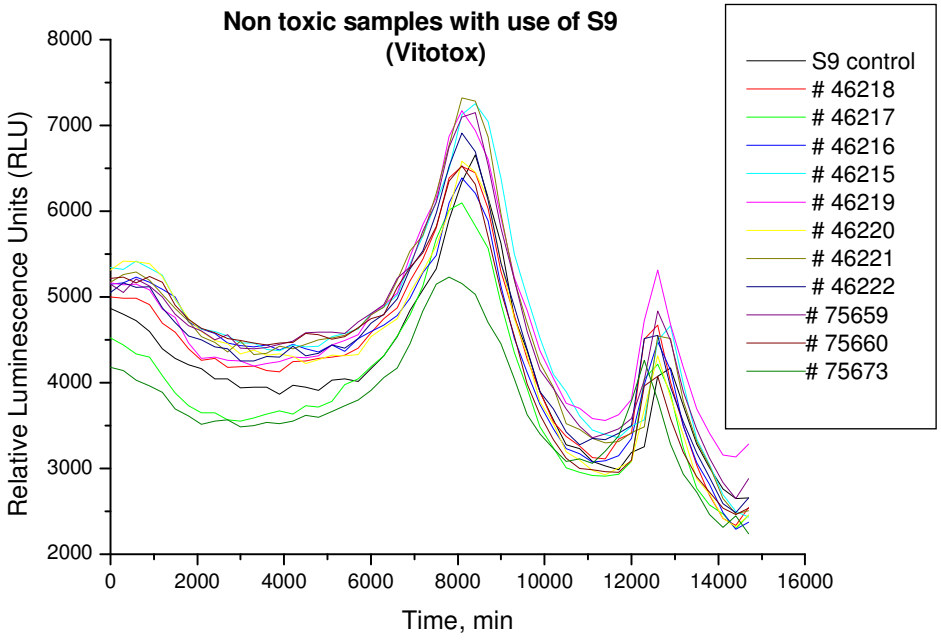

Fig. 15. Samples shown to be neither genotoxic nor cytotoxic by Vitotox™

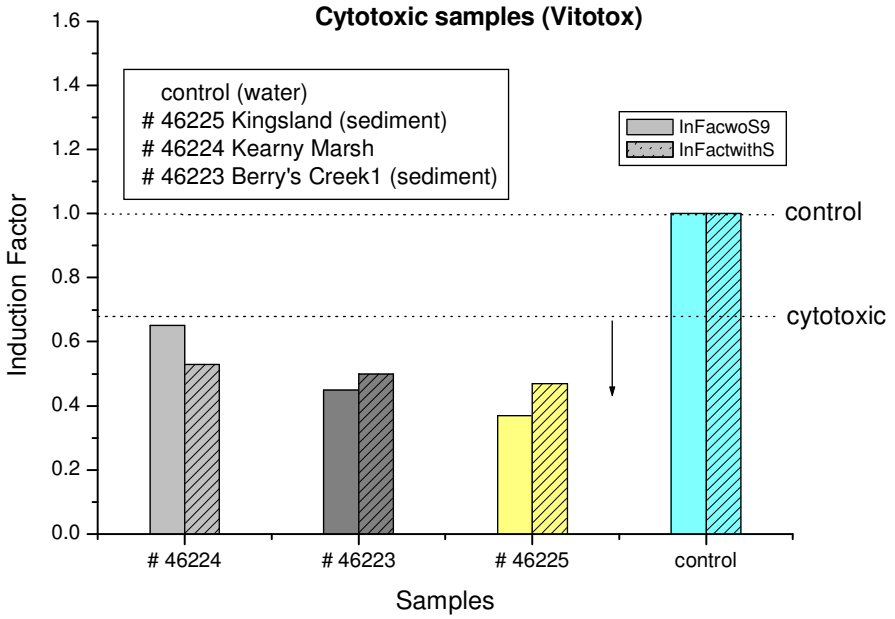

Fig. 16. Samples shown to be cytotoxic using the Vitotox™ bioassay

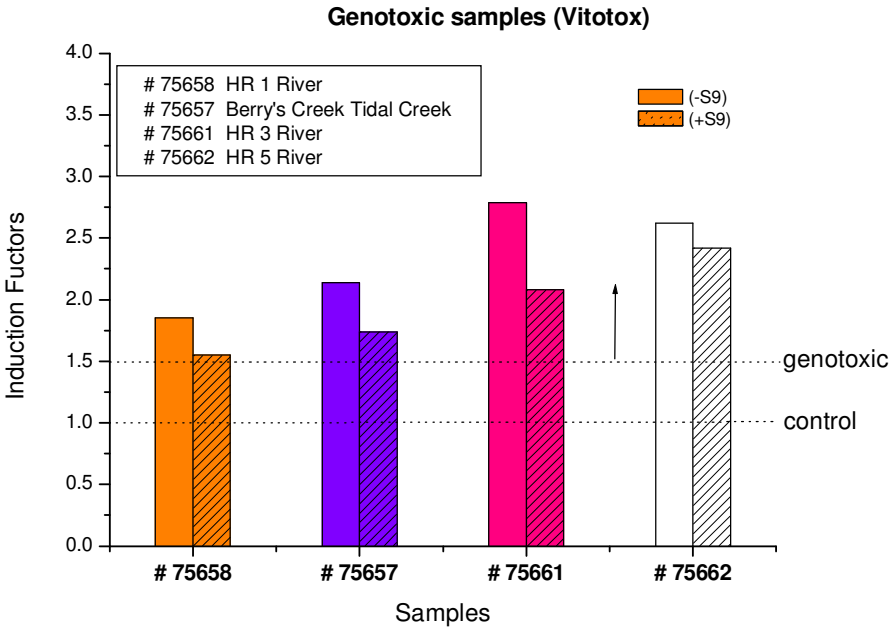

Fig. 17. Samples shown to be genotoxic using the Vitotox™ bioassay

9.5. Chemical analyses results

The chemical analyses provided by HMDC are presented in Table. 6.

Table. 6. Chemical analyses of 8 samples. The analyses were performed for three categories of materials: pesticides and PCBs; volatile and semivolatile organics; and semivolatile organics.

Site	Genotoxicity number	Pesticide		VOA number	Semi-VOA number
		# & name	ppm		
Leachate Meter Grab	74946			75670	
				-	
				(all U)	
Berrys Creek 1, Tidal	74955	75663		75598	75602
		Dieldrin	0.0034	-	-
		4,4-DDE	0.0052	(all U)	(all U)
		4,4-DDD	0.0130		
		Endosulfan sulfate	0.0172		
HR 1, River	75658	75664		75654	75656
		Aldrin	0.0021	-	-
		4,4-DDE	0.0061	(all U)	(all U)
		4,4-DDD	0.0116		
		Endosulfan Sulfate	0.015		
HR3, River	75661	75665		74648	75650
		-		-	-
			(all U)	(all U)	(all U)
HR5, River	75662	75652		75651	75668
		4,4-DDE	0.0046	-	-
		4,4-DDD	0.013	(all U)	(all U)
		Endosulfan sulfate	0.018		
Kearny Marsh, Freshwater	75659	75666		75601	75604
		4,4-DDE	0.0040	-	-
		4,4-DDD	0.0118	(all U)	(all U)
		Endosulfan sulfate	0.015		
Penhorn Creek, Tidal	75660	75667		75600	75606
		4,4-DDE	0.0054	-	-
		4,4-DDD	0.0146	(all U)	(all U)
		Endosulfan sulfate	0.019		
Meter Chamber	75673	75674			75672
		Dieldrin	0.0034		-
		4,4-DDE	0.0069		(all U)
		4,4-DDD	0.0134		
		Endosulfan sulfate	0.017		

Note : U – no compounds detected from the expected list of chemicals, detection limit value.

9.6. Overall summary of the results

Category of samples	Optical fiber induction factor	Vitotox™ induction factor-S9/+S9	Chemical analysis	
			Compound	ppm
Nongenotoxic and noncytotoxic				
74946 Meter Chamber, Grab	1.23	-	all U	
74947 Meter Chamber, Composite	1.08	-		-
74952 Kingsland, Brackish	1.13	-		-
74952* Kingsland, Sediment	0.97	-		-
74953 Kingsland, Brackish	0.89	-		-
74956 Berry's Creek 2, Tidal Creek	1.29	-		-
46215 Kearny Marsh, Fresh water	1.05	1.14/1.12		-
46216 Golf Course, Groundwater	1.11	1.21/1.00		-
46217 Meter chamber, Leachate	0.94	1.12/0.96		-
46218 1A-1, Groundwater	1.04	1.16/1.03		-
46219 1A-4, Groundwater	1.09	1.27/1.13		-
46220 HMDC Lab, Tap Water	1.06	1.18/1.04		-
46221 UBC, Brackish	1.15	1.26/1.15		-
46222 HR1, Brackish	1.21	1.34/1.09		-
75659 Kearny Marsh, Freshwater Marsh	1.27	1.4/1.12	4,4-DDE	0.0040
			4,4-DDD	0.0118
			Endosulfan Sulfate	0.015
75660 Penhorn Creek, Tidal Creek	1.17	1.35/1.03	4,4-DDE	0.0054
			4,4-DDD	0.0146
			Endosulfan Sulfate	0.019
75673 Chamber, Grab	0.89	1.03/0.82	Dieldrin	0.0034
			4,4-DDE	0.0069
			4,4-DDD	0.0134
			Endosulfan sulfate	0.017
Cytotoxic				
74948 1E-Manhole, Leachate	0.21	-		-
46223 Berry's Creek 1, Sediment	0.55	0.45/0.5		-
46224 Kearny Marsh, Sediment	0.61	0.65/0.53		-
46225 Kingsland, Sediment	0.44	0.37/0.47		-
Genotoxic/potentially genotoxic				
75658 HR 1, River	1.85	1.85/1.55	Aldrin	0.0021
			4,4-DDE	0.0061
			4,4-DDD	0.0116
			Endosulfan sulfate	0.01
75657 Berry's Creek, Tidal Creek	1.61	2.14/1.74		-
75661 HR3, River	1.67	2.79/2.08		all U
75662 HR5, River	2.09	2.62/2.42	4,4-DDE	0.0046
			4,4-DDD	0.013
			Endosulfan Sulfate	0.018
75955 Berry's Creek 1, Tidal Creek	1.62	-	Dieldrin	0.0034
			4,4-DDE	0.0052
			4,4-DDD	0.0130
			Endosulfan sulfate	0.0172

10. DISCUSSION

10.1 Toxicity of samples

The bioassays (optical fiber bioreporter probes and Vitotox) gave very similar results for all samples. Since Vitotox is a suspension bioassay, it was shown to be more sensitive than the fiber probes, as seen from the higher induction factors obtained with Vitotox. Four samples [74948 1E-Manhole, Leachate (the most cytotoxic); 46223 Berry's Creek 1, Sediment; 46224 Kearny Marsh, Sediment; 46225 Kingsland, Sediment] exhibited cytotoxic behavior. Unfortunately, we do not have chemical analyses for these samples and it is thus difficult to interpret the results obtained for these samples.

Five samples [75661 HR3, River; 75662 HR5, River (most genotoxic); 75658 HR 1, River; 75657 Berry's Creek, Tidal Creek; 75955 Berry's Creek 1, Tidal Creek] exhibited genotoxic behavior, particularly the former two samples. The most surprising result was that according to the chemical analyses, the most genotoxic sample (75661 HR3, River) did not contain the target pesticides or target tested volatile and semi-volatile compounds. Moreover, three samples that were non-geno/cytotoxic (75659 Kearny Marsh, Freshwater Marsh; Penhorn Creek, Tidal Creek; 75673 Chamber, Grab) showed positive results for the presence of the target pesticides. According the to chemical analyses, the concentrations of the target pesticides found in all samples were of the same order of magnitude, with slight differences. In addition, the concentrations of the target pesticides were detected at a very low level of 2-17 ppb. These low levels of pesticides appear to be below the detection limits of the tested bioassays (our optical fibers and Vitotox). The absence of a correlation between the bioassays and chemical analyses brings us to the conclusion that the cause of the genotoxicity shown by our bioassay could probably not be attributed to any one of the pesticides found to be toxic by chemical analyses but rather to other, as yet unknown, substances that are presently not recognized by the chemical analyses.

Metabolic activation (S9 fraction of the rat liver extract) was used only for Vitotox, since we still need to optimize the procedure with S9 for our method. The results with metabolic activation were either similar to the results without activation or were even worse (lower induction factors). The experiments showed that there were no common precursor chemicals for geno/cytotoxicants in the tested samples.

10.2. Effect of salinity effect on the measurements

It is likely that the presence of salts in the samples will influence the physiological behavior of the bacteria, especially of that of the nonresistant bacterium (i.e., *E. coli*) used in above-mentioned tests. Therefore, we examined the effect of different concentrations of salt on the measurements at the same inducer concentration (mitomycin C, 320 ppb). The results indicate that salt concentrations higher than 4.5% NaCl (w/v) caused cell death (Fig. 18). A concentration of 2.2% NaCl (w/v) also produced an inhibition effect, but it was not lethal. Results for solutions of physiological saline or lower strengths were similar to those for the control (water + mitomycin C) curve, while the optimal salt concentration was that of normal physiological saline. These findings imply that the salinity of environmental samples must indeed be checked so as to confirm their suitability for testing with a bioassay such as Vitotox or our fiber probe biosensors.

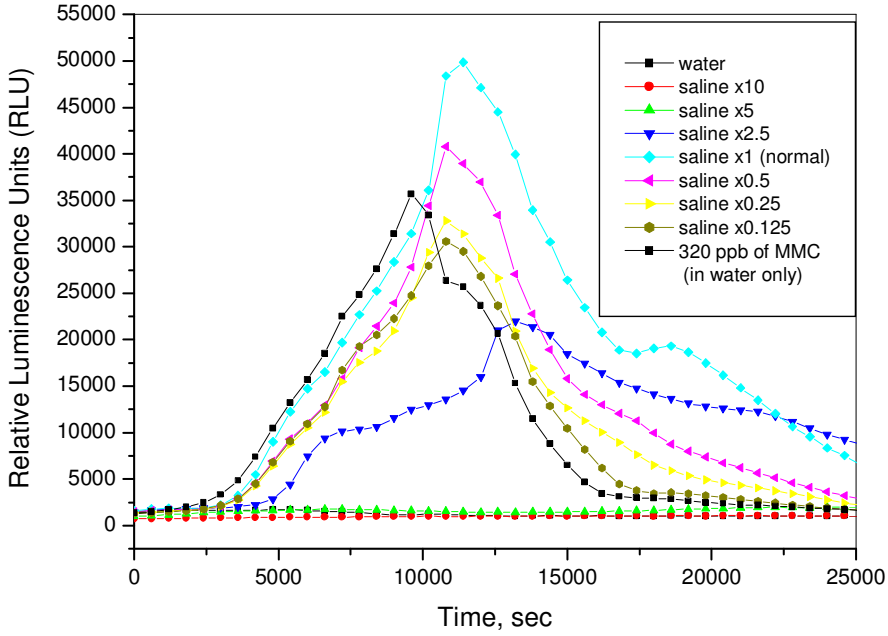

Fig. 18. Effect of salinity effect on the measurements. Mitomycin C was used as the inducer at a concentration of 320 ppb.

Note: If the bioassay shows a cytotoxic effect, we recommend that both the pH and the salinity of the sample be checked, for example, by estimating salinity in terms of TDS (total dissolved solids). This parameter could give an indication of the osmotic properties of the sample, which may influence the outcome of the bioassay

10.3. Storage studies

To determine whether it would be possible to store our optrodes, reporter cells encapsulated in alginate beads were stored under different conditions. The results are presented in Fig. 19 (a,b,c,d).

The results show that immobilized bioreporter *E. coli* DPD1718 retained its luminescence response for least 2 weeks when stored in a rich organic medium (LB). During the first four days, the luminescence level fell to a value of 50% of that on day 0, but thereafter it remained constant for another ten days.

Storage in physiological saline (0.9%, w/v NaCl), poor inorganic medium (AB) or Tris buffer (pH 7.4) caused a marked decline in luminescence response by the third day. Thereafter, no detectable signal was obtained.

Cell viability data indicate that cells maintained in LB medium retained their viability in the first 4-5 days, with a slight decline in the following 10 days. Storage in other media gave acceptable cell stability in the first 3 days. However, the number of CFUs (colony forming units) for these media was lower than that for LB.

These results confirm that the number of viable cells in the immobilizing matrix does not necessarily reflect the biochemical activity of the immobilized cells.

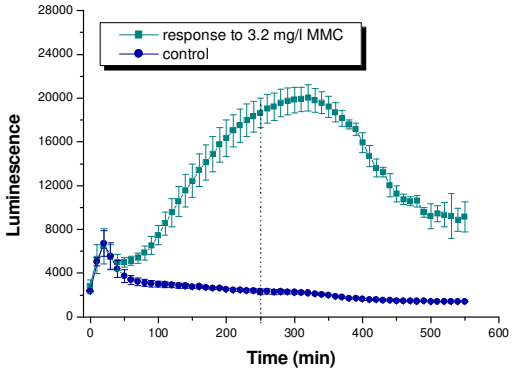

Fig. 19 (a). Typical response (after 10 days storage in LB)

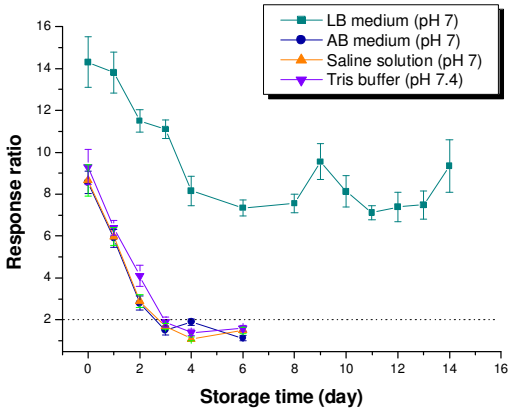

Fig. 19 (b) Response ratio during storage

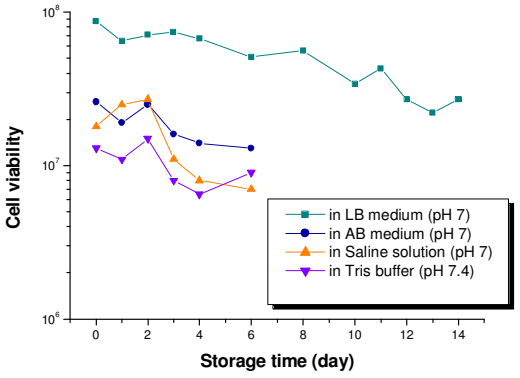

Fig. 19 (c). Cell viability during storage

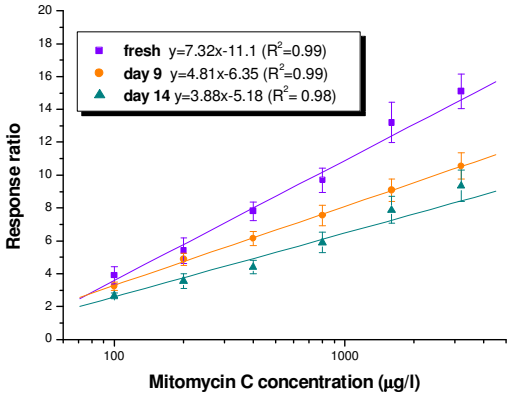

Fig. 19 (d). Calibration curves at different storage time points

11. CONCLUSIONS AND ACCOMPLISHMENTS

- During the research period, we constructed two instruments:

  1) a multiple fiber maker that facilitates simultaneous preparation of a number of fibers (22 fibers/in a few min); and

  2) a portable photodetector for field measurements.

  Most samples were tested by two bioassays, i.e., our optical fiber method and the commercial Vitotox kit. We found very good correlations between the results obtained with the two methods, with the Vitotox test showing slightly better sensitivity.

- Highly genotoxic samples (induction factors higher than 4-5) were not found, but five samples gave positive results for the genotoxicity tests. The most genotoxic samples were found from the river sources (75661 HR3, River; 75662 HR5, River). Four samples were found to be cytotoxic, while the most cytotoxic sample was the 74948 1E-Manhole, Leachate.

- The bioassay results did not correlate well with the chemical analyses, probably because not all possible pesticides were tested by chemical analyses or because other substances that were not recognized by the chemical analyses may have been present.

- Metabolic activation with S9 did not facilitate any improvement in the measurements. This result indicates that the samples probably did not contain precursors that would become genotoxicants after activation.

- The salinity and pH of the sample could affect the bacteria during the test. We recommend that pH and TDS measurements for samples showing cytotoxic behavior be taken into account in order to delineate the source of the cytotoxic effect.

- The initial storage experiments showed the ability of our sensors to keep their functionality when stored in a rich medium (LB) at 4^{o}C for at least 10 first days, but at the cost of a loss of activity of 50%. Additional storage experiments should be conducted with the aim of improving the shelf life of the sensors in terms of time and sensitivity.

12. ACKNOWLEDGMENTS

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