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Methods

OPTIMAL CONDITIONS FOR STABILITY OF PHOTOEMISSION AND FREEZE DRYING OF TWO LUMINESCENT BACTERIA FOR USE IN A BIOSENSOR

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Abstract—Bioluminescent bacteria have been used for many years for biotoxicological analysis. One of the main concerns with this microorganism is the low experimental repeatability when subjected to external factors. The aim of the present study was to obtain accurate, sensitive, and repeatable measurements with stable signals (during the detection and over days) for application in a water-analysis device for the detection of pollutants. Growth conditions were tested and optimized. An optimal freeze-drying procedure for the constitutive bioluminescent bacteria *Vibrio fischeri* and *Photobacterium phosphoreum* was developed. The luminescence stability after rehydration was also investigated. Freeze drying was found to be a critical process in survival and signal stability of luminescent bacteria; for this reason, different suspension fluids and various bacterial pellet/suspension fluid ratios (g/ml) were evaluated. The toxicity of heavy metals and organic compounds in water was determined to investigate the applicability of a test based on bacteria obtained in this way, comparing the data with legal limits. A scale-up process was developed with industrial technology: freeze-dried bacteria that emitted a stable luminous signal after rehydration were obtained. Moreover, the median effective concentration (EC50) was calculated with these bacteria. Environ. Toxicol. Chem. 2011;30:801–805. © 2011 SETAC

Keywords—Bioluminescence Biosensor Freeze drying Toxicity *Vibrio*

INTRODUCTION

Biological sensors, markers, and detectors are increasingly being used to detect and monitor the environmental impact of hazardous materials. Bacteria, because of their rapid response and low cost, are excellent tools for toxicity screening and assessment.

Bacterial tests can be used as a preliminary, rapid screening method in a test battery and are generally less time-consuming and less expensive than chemical analyses. In addition, whole-cell bacterial biosensors measure only the bioavailable fraction of toxic compounds and allow the assessment of the effect of a toxicant on a living organism [1,2], which has advantages over traditional methods. Bioluminescent bacterial assays based on *Vibrio fischeri* are more sensitive than other bacterial assays (nitrification inhibition, respirometry, adenosine triphosphate (ATP) luminescence, and enzyme inhibition) against various chemicals [3].

In vivo luminescence is a sensitive indicator of xenobiotic toxicity because it is directly coupled to respiration by the electron transport chain and thereby reflects the cell's metabolic status: luminescence will decrease in the presence of noxious substances. The mechanisms underlying the interaction between luminescence and toxicity are not yet clear, although several mechanisms have been suggested [4].

Because different organisms respond differently to toxicants, bioassays are needed that employ diverse organisms. Organisms from various groups have been used in bioassays: bacteria, nematodes, cladocerans, fishes, amphiphods, algae, and plants. Bioassays based on higher organisms have longer reproductive times, have longer sensing times, and are more expensive than using bacterial cells.

Biotests with natural luminescent bacteria are now widely used, and most are based on *Vibrio fischeri*, *Vibrio harvey*, and *Photobacterium leiognathi*. Commercial kits, mainly for aquatic toxicity testing, have been produced based on *V. fischeri* [5]. In short- and long-term tests, they give a measure of acute and chronic toxicity, respectively. They are usually sufficiently sensitive to detect most of the compounds toxic to humans and mammals. Even in cases in which they are not particularly sensitive, they are early indicators of toxicants.

A bacterial bioluminescent biosensor must fulfill several requirements: the biosensing cells must be maintained during storage and transport without loss of activity, the bioluminescence must be measured in a manner that minimizes interference from ambient light, and simple operation methods are needed to apply the biosensor to real sample analysis without pretreatment [6,7]. Freeze drying is the most suitable method for commercial delivery of the cells because of the ease of storage and transport. Moreover, freeze-dried cells can be immediately used after rehydration. The aim of the present study was to determine the optimal growth conditions and freeze-drying procedure for *V. fischeri* (Lux2) and *P. phosphoreum* (Lux3); these were selected for their light emission stability and viability between 12 different strains, to obtain accurate measurements and stable signals when applied to a water-analysis device.

MATERIALS AND METHODS

Organisms and molecular identification

Vibrio fischeri, Lux2, was DSMZ 7151 (German Collection of Microorganisms and Cell Cultures). *Photobacterium phosphoreum*, Lux3, was isolated from Mediterranean seawater in our laboratory. Molecular identification was made using oligonucleotide primers for amplification of the 16S rRNA gene and subsequent sequencing; primers were 357F (5′-CCT ACG GGA

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GGC AGC AG-3′) and 907R (5′-CCG TCA ATT CCT TTG AGT TT-3′) [8]. The polymerase chain reaction (PCR) was performed in a 25-μl volume with 0.2 mM dNTPs, 0.5 U Taq DNA polymerase (Sibenzyme), 1× Taq polymerase buffer (Sibenzyme), and 0.4 μM of each primer with 2 μl of DNA template. The amplification program began with 95°C for 5 min, and PCR steps consisted of 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, followed by 2 min at 72°C and storing at 4°C until analyzed.

Amplified PCR products were purified with a PCR Purification Kit (Qiagen) and sequenced by MWG-Biotech. Sequence analysis was performed using the NCBI (National Center for Biotechnology Information) BLAST database and RDP database (Ribosomal Database Project) to confirm strain identity.

Growth of cultures

We used AB medium (NaCl 30 g/L, glycerol 3 ml/L, Difco casitone 5 g/L, Difco yeast extract 3 g/L, KH_2PO_4 1.179 g/L, $Na_2HPO_4{\cdot}2H_2O$ 5.395 g/L) in a continuously shaken Erlenmeyer flask (140 rpm). For plate counts, 18 g/L agar was added. The best growth conditions are between 18°C and 26°C, as reported elsewhere [9]. Bacteria were grown at 22°C to obtain maximal luminescence, which occurs at the end of the exponential growth phase, i.e., after 17 h.

Luminescence measurement

Measurements were done with a luminometer (multilabel counter, model Victor Light 1420; PerkinElmer), with computerized data collection (Microsoft Excel software). Samples were analyzed in 96-well black polystyrene Multilabel microplates (ThermoLabsystem). Stability assays were conducted with an LKB-Wallac 1250 luminometer equipped with a photomultiplier tube detector to measure light emission and a paper recorder for signal visualization. We evaluated signal stability, recording the emission profile for 30 min; the variables considered were sample volume, bacterial concentration,and temperature of the solutions.

Freeze drying

The freeze-drying process was developed using a freeze dryer (Hetossic model CD52) according to the following procedure: the bacterial culture was centrifuged and resuspended in two specific suspension fluids, SF3 (lactose 120 g/L, soluble starch 20 g/L, NaCl 10 g/L, pH 7) and SUC (sucrose 100 g/L, NaCl 30 g/L, pH 7), with or without the addition of buffer. The buffers used were the same used for toxicity assays: Tris 0.01 M, Hepes 0.01 M, or phosphate/diphosphate (PBS) 0.01 M. In summary, we obtained Tris-SF3, Tris-SUC, Hepes-SF3, Hepes-SUC, PBS-SF3, and PBS-SUC cryoprotective solutions. Then, 1 ml of the suspension was placed in small vials and kept at −80°C for at least 3 h. The first freeze-drying step requires that the sample be brought to −60°C for approximately 24 h under vacuum; the second step is a dehydration step that takes 24 h. Artificial sea water was prepared as suggested by DSMZ, medium 246: NaCl 28.13 g/L, KCl 0.77 g/L, $CaCl_2{\cdot}2H_2O$ 1.6 g/L, $MgCl_2{\cdot}6H_2O$ 4.8 g/L, $NaHCO_3$ 0.11 g/L, $MgSO_4{\cdot}7H_2O$ O 3.5 g/L.

Chemicals and pollutants

All chemicals used were of analytical grade, and the concentrations of the solutions are expressed in milligrams per liter. We tested the following compounds in different concentrations, obtained by diluting a stock solution (1,000 mg/L) in Tris-buffered saline (Tris/NaCl [Tris(hydroxymethyl)aminomethane 0.01 M + NaCl 0.05 M], pH 7): 2,4-dichlorophenoxyacetic acid; 2,4,5-trichlorophenoxyacetic acid; 2,4-dinitrophenol; fluoroacetate; phenol; $PbCl_2$, $SeCl_4$, Hg_2Cl_2, $K_2Cr_2O_7$, As_2O_5, As_2O_3, $CuCl_2$, $3CdSO_4{\cdot}8H_2O$, and $ZnSO_4{\cdot}7H_2O$. In the case of 2,4,5-trichlorophenoxyacetic acid, mild heating and continuous agitation were necessary to obtain complete solubilization.

Toxicity assay

The bacteria strains we used are of marine origin and therefore require an environment with adequate salt content. Therefore, rehydration using saline or sea water is not advisable, because suspension fluids are rich in NaCl content. Rehydration with saline solution or artificial sea water would cause an excess of NaCl and osmotic stress to bacterial cells. Further dilutions were made using only water or Tris plus NaCl, to maintain adequate salt content. For this reason, freeze-dried bacteria were first rehydrated in 1 ml sterilized distilled water. Before starting each assay, the light emission by the resuspended bacteria was measured (20 μl of bacteria and 180 μl Tris-buffered saline in a microplate well) to determine whether the light level was optimal for the analysis (between 10,000 and 20,000 relative light units [RLU]). Then, if necessary, the bacteria were diluted in 0.05 M NaCl aqueous solution before sample testing. Light emission was measured in microplate wells containing 20 μl bacteria and 180 μl pollutants.

Among the various buffers tested, the one that resulted in the highest bacterial sensitivity to the pollutants was Tris, pH 7, supplemented with NaCl 0.05 M to reduce osmotic stress. Toxic chemical compounds were prepared by dissolving the corresponding salt in Tris buffer. The light emission of each well was read by the luminometer every 5 min for approximately 60 min. The luminometer provides the results of each reading in Excel format, and these results were used to calculate the median effective concentration (EC50). We tested these buffers: Tris/NaCl (Tris[hydroxymethyl]aminomethane 0.01 M + NaCl 0.05 M), pH 7; Hepes/NaCl (4-[2-hydroxyethyl]piperazine-1-ethanesulfonic acid 0.01 M + NaCl 0.05 M), pH 7.00; and PBS/NaCl ($H_2PO_4^-$/HPO_4^{--}) 0.01 M + NaCl 0.05 M, pH 7.

EC50 calculation

The EC50 was calculated by interpolating the light emission values versus toxicant concentration using the linear regression equation ($y = mx + b$), as in the following formula

$$\mathrm{EC50} = (y_{\max}/2 - b)/m, \quad (1)$$

in which $y_{\max}$ is the light emission value of the blank (0 mg/L) and m is the slope of the line. We calculated the EC50 of the signal intensity recorded at 15 and 60 min.

Industrial tests

To test the potential of the biosensing element as a commercial device, a biotechnology company, Actygea (Gerenzano, Varese, Italy), setup the conditions to produce freeze-dried preparations of *V. fischeri* Lux2 on an industrial scale. The culture conditions were the same as the experimental assay except that the volume was increased to 10 L by using a 20-L bioreactor with the following operating conditions: temperature 22°C, 400 rpm, and 12 L/min air. When light emission reached the maximal level, the biomass was collected by centrifugation at 4,000 × g, and the wet pellet of cells (3.5 g/L) was suspended in the SF3 suspension fluid at the ratio

1 g:20 ml. One milliliter of the bacterial suspension was dispensed into vials and freeze dried for 36 h. Three vial formats were used: 20-ml vials (10 ml working volume), 9-ml vials (5 ml working volume), 7-ml vials (4 ml working volume). After freeze drying, the survival of *V. fischeri* Lux2 was verified by suspending one lyophilized vial in 1 ml sterile saline solution (NaCl 9 g/L in water) and plating serial dilutions of the suspension on AB agar medium.

RESULTS

Organisms and molecular identification

Amplification of 16S rRNA gene and subsequent sequencing provided evidence that the strain Lux3 belongs to the *Photobacterium* genus and particularly to the species *Photobacterium phosphoreum.*

Freeze-drying conditions and stability assays

The light emission of rehydrated bacteria had to meet two main requirements. First, to define the magnitude of the reduction of light emission, the signal must be constant during the assay conducted in the absence of toxicants. Second, it must be possible to register a signal for at least 7 d by keeping the rehydrated suspension at 4°C. The first requirement was reached and was improved during the research by adding Hepes or Tris buffers in the suspension fluids used for freeze drying; the use of these buffers in this step led to improvement by shortening of the time necessary to reach signal stability after rehydration: time was reduced from 90 min to 1 h. Morever, the signal fluctuations in 1 h of analysis were reduced from 10 to 4%. The third buffer used, PBS, revealed a signal near zero in all the samples once rehydrated. Cell viability decreased up to 10^4 cfu/ml (plate count) when comparing cell counts before and after freeze drying. Therefore, we investigated the optimal pellet/SF (g/ml) ratio, when the biomass was resuspended in the suspending fluid after centrifugation. We tested several pellet:SF ratios and measured light emission over days; despite the considerable reduction in cell viability, Lux2 maintained a satisfactory viability and luminescence for as long as 17 d (Fig. 1); Lux3 maintained luminescence up to day 8 after rehydration (Fig. 2).

The two strains behaved differently: Lux2 maintained luminescence at pellet:SF ratios of 1:20 and 1:40, and no luminescence at ratios of 1:2 and 1:4; there was some, albeit not satisfactory, luminescence at 1:200. Lux3 maintained luminescence at pellet:SF ratios of 1:2 and 1:4 ratio; it did not maintain light emission at ratios of 1:40 and 1:200.

Miyamoto-Shinohara et al. [10] reported that cells of *Pelagiobacter* sp. have higher survival rates when washed with seawater before applying vacuum and suggested that washing removed polysaccharides that trap water molecules on the surface. Therefore, we washed Lux3 cells with sterile seawater before the freeze-drying step at −80°C and compared their survival rate at a pellet:SF ratio of 1:4 (one of the optimal ratios for the maintenance of viability and light emission for this strain) using the two suspension fluids versus the survival rate of Lux3 cells not washed in seawater. As shown in Figure 3, maintenance of intensity of the luminous emission up to 8 d was better in washed cells. In washed cells, the signal remained stable for 30 min from measurement onset and the intensity of the light emission decreased less than in nonwashed cells over days. The slight fluctuations of the signal in washed cells did not affect the accuracy of the detection of pollutant concentrations. Our results also showed that the values of optical densities of the two bacterial suspensions (after 30 min) were superimposable, demonstrating that variations in light emission could not be due to bacterial sedimentation.

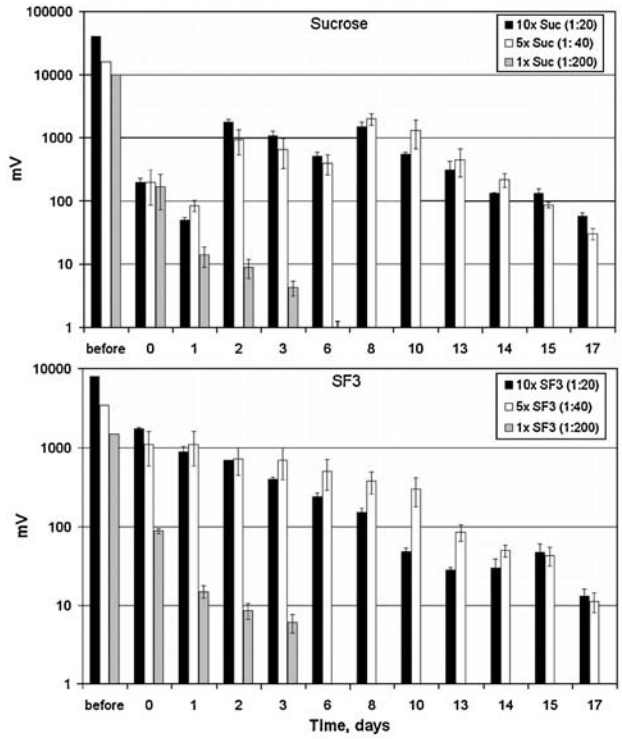

Fig. 1. Lux2 17 d after rehydration. Light emission of rehydrated vials of Lux2 monitored for 18 d, assuming day 0 as the day of reconstitution. Black bars: Light emission (expressed in mV) of the bacterial culture with a pellet:SF ratio of 1:20. White bars: Light emission (expressed in mV) of the bacterial culture with a pellet:SF ratio of 1:40. Gray bars: Light emission (expressed in mV) of the bacterial culture with a pellet:SF ratio of 1:200. Top (SUC) and bottom (SF3) represent the different suspending fluids we used. Error bars represent standard deviation; samples were analyzed in two replicates.

Industrial tests

The survival rate of *Vibrio fischeri* Lux2 produced in the Actygea laboratories was approximately 90% in three lyophilized lots and satisfactory (50%) in one lot. As shown in Table 1, the microbial titer before freeze drying (after the −80°C step) was 1.6×10^9 cfu/ml. All the vials, before and after freeze drying, were luminescent, and the light was visible to the naked eye. Dehydration of samples was visibly better in the case of vials of lots 1, 2, and 3, probably because their larger bottom area allows the liquid to spread better and a thinner layer of suspension is more easily dried compared with smaller vials that contain the same suspension volume. In fact, the survival of cells was low (6%) and viability was reduced to 10^2 cfu/ml in the vials with a smaller working volume. The survival rate of cells freeze dried with the industrial protocol was higher than that of cells obtained in our laboratory tests (10^4 cfu/ml).

Thirty days after these assays, we rehydrated 20 vials (four randomly selected from each lot), and measured the light emission for 14 d and cell viability by plate count. Light (more than 10 mV) was emitted up to d 10. Cell viability was reduced by 3 log units (from 10^9 to 10^6) in most vials; however, the reduction in cell concentration did not affect the stability of the signal.

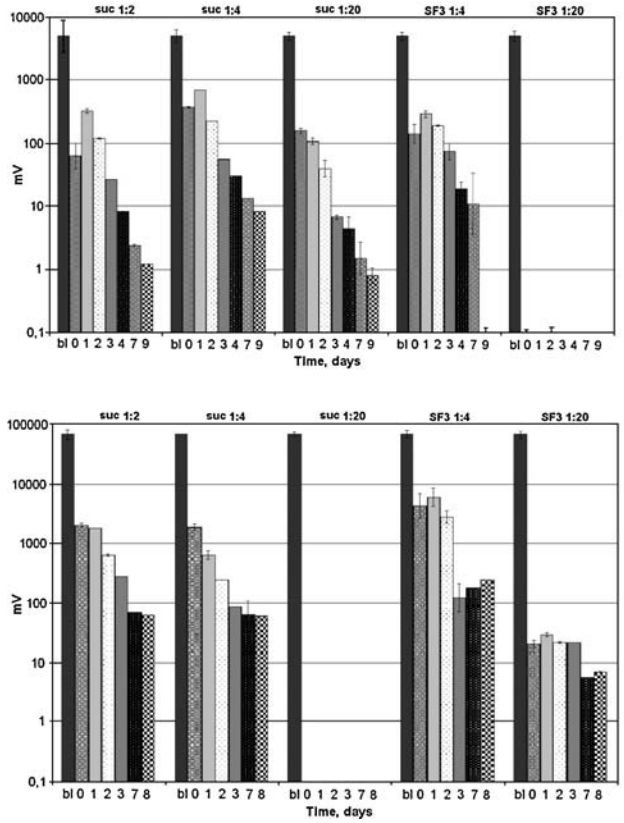

Fig. 2. Lux3 9- and 8-d analysis after rehydration. Top: Light emission of rehydrated vials of Lux3 monitored for 9 d. Bottom: Replicate of the assay up to 8 d. Each group of bars represents samples with different bacterial concentrations and suspension fluids. Black bars: Light emission (expressed in mV) of the bacterial culture before freeze drying. Gray bars: Emission measured through the days after rehydration, assuming day 0 as the day of reconstitution. Error bars represent standard deviation; samples were analyzed in two replicates. bl = Before lyophilization.

Biotoxicity assays

We compared our data with the EC50 of Microtox® (Strategic Diagnostics), which is based on measurement made after 15 min. As reported in Table 2, our EC50 has shown to be lower than Microtox for 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, phenol, Se(IV), Cr(VI), Cu(II), and Cd(II), both at 15 and at 60 min, for Lux2 and Lux3. Our EC50 were higher for 2,4-dinitrophenol, fluoroacetate, and Pb(II). For Hg(II), As(V) and Zn(II) the results were variable, depending on the bacterial strain and the exposition time (15 or 60 min). As shown in Table 2, for some toxicants under specific conditions, we were not able to calculate EC50. We could not calculate EC50 for As(III) at all.

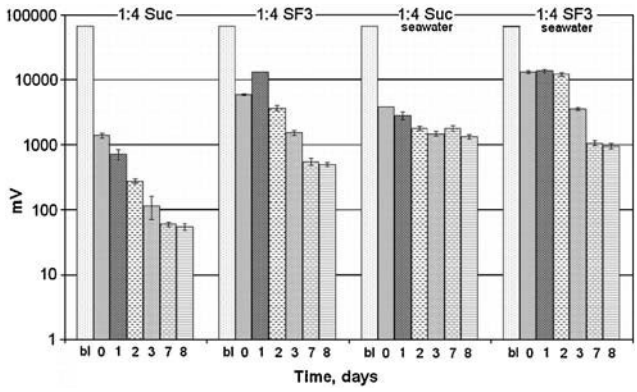

Fig. 3. Lux3 1:4 not washed and washed with seawater before lyophilization. Light emission from Lux3 with and without a wash with sterile seawater measured 8 d after reconstitution. Each group of bars represents samples with different bacterial concentrations and suspension fluids. Light emission (expressed in mV) of the bacterial culture before freeze drying. Gray bars: Emission measured during the days after rehydration, assuming day 0 as the day of reconstitution. Error bars represent standard deviation; samples were analyzed in two replicates. bl = Before lyophilization.

Table 1. Freeze-dried lots, bacterial survival, and percentage yield

Lot	Vial total volume (ml)	Vial working volume (ml)	Microbiological count after freeze drying	Survival (%)
1	20	10	2.1×10^9	90
2	20	10	1.7×10^9	90
3	20	10	8.0×10^8	50
4	9	5	1.6×10^9	85
5	7	4	9.0×10^7	6

In summary, two problems emerged from the biotoxicity assays: variability of the results of EC50 assays and in some cases difficulty in identifying a decrease in light emission, probably because of the intrinsic resistance of the bacteria to toxicants and heavy metals. There were no significant differences between the two measurements (after 15 and 60 min), even if in some cases it was possible to see a slight decrease in EC50 value across time, in Lux2 analysis. Therefore, the sensitivity of the bioluminescent bacteria to the toxic substances tested does not change over time.

DISCUSSION

The growing demand for biosensor devices able to detect pollutants and environmental contaminants has prompted research on sensing elements. In this context, we monitored the effect of freeze-drying conditions on the maintenance of viability and on the intensity of light emissions from two sensing elements (*V. fischeri* [Lux2] and *P. phosphoreum* [Lux3]) for several days after their rehydration. We found that an appropriate suspension fluid used at correct concentrations maintains cell viability at a level to produce a suitable luminous signal for at least 8 d. The results obtained from investigating the influence of different buffers on lyophilization led to improvement in two parameters: the time for reaching the signal stability after rehydration and the signal fluctuations in 1-h analysis. The fading effect shown by PBS on PBS-SF3 and PBS-SUC cryoprotective solutions is still under investigation.

The yield of the freeze-drying process has been reported to be lower when using *Vibrio* species than the yield obtained with other bacterial groups [11]. The cell survival rates and luminescence maintenance were enhanced in the industrial preparations over our laboratory-scale tests. The higher survival rate could result in a hypothetical higher stability and a longer lasting light emission. The wider surface on the bottom of the vials probably allowed a more uniform spread of the suspension and permitted more efficient drying of the liquid. The decrease in cell viability that occurred one month after freeze drying did not affect the signal.

To explain the high EC50 of Cr(VI) we referred to Fulladosa et al. [12], who attributed *V. fischeri* resistance to hexavalent chromium in part to the capacity of the bacterium to reduce Cr(VI) to Cr(III). In a study of the effects of As(III) and As(V) on *V. fischeri*, chemical speciation was found to be correlated with toxicity [13]; this could explain why it was difficult to obtain a clear EC50. In the same study, a correlation was

Table 2. EC50 of the heavy metals and toxic chemical compounds reported for Lux2 and Lux3 strains obtained with our procedure and those reported for Microtox®[a]

Chemicals	*n*	EC50 (mg/L) Lux2 at 15 min ± SE (*CV*)	EC50 (mg/L) Lux2 at 60 min ± SE (*CV*)	EC50 (mg/L) Lux3 at 15 min ± SE (*CV*)	EC50 (mg/L) Lux3 at 60 min ± SE (*CV*)	Microtox® EC50 (mg/L) at 15 min
2,4-Dichlorophenoxyacetic acid	11	39.1 ± 3.4 (29.7%)	37.7 ± 11.4 (29.2%)	56.3 ± 3.3 (34%)	59.8 ± 18 (32.1%)	100.7 [14]
2,4,5-Trichlorophenoxyacetic acid	7	43.8 ± 16.6 (13.2%)	37.8 ± 14.3 (13.5%)	35.8 ± 13.5 (17.4%)	NP	51.7 [14]
2,4-Dinitrophenol	11	33.2 ± 10.01 (25.3%)	22.9 ± 3.3 (25.8%)	25.1 ± 7.6 (24.3%)	NP	7.5 [15]
Fluoroacetate	11	63.3 ± 19.1 (37.4%)	52.1 ± 3.3 (37.2%)	NP	61.6 ± 18.6 (39.5%)	9.3 [16]
Phenol	8	NP	NP	4.7 ± 1.7 (40.4%)	NP	28 [17]
$PbCl_2$(II)	6	NP	44.5 ± 18.2 (15.5%)	31.4 ± 12.8 (20.8%)	NP	0.43 [16]
$SeCl_4$	9	27.2 ± 9.1 (53.3%)	12.3 ± 4.1 (51.2%)	15.7 ± 5.2 (57.9%)	NP	NK
Hg(II)	4	—	0.10 ± 0.05 (47.6%)	44.2 ± 22.1 (37.3%)	0.5 ± 0.25 (36.9%)	0.17 [18]
Cr(VI)	7	61.3 ± 23.2 (14,7%)	50.1 ± 18.9 (15%)	88.17 ± 33.33 (16.5%)	61.46 ± 23.11 (15.5%)	3,930 [13]
As(III)		NP	NP	NP	NP	8.7 [14]
As(V)	4	23.2 ± 11.6 (68.6%)	6.8 ± 3.4 (66.2%)	NP	NP	22.1 [14]
Cu(II)	4	NP	0.58 ± 0.29 (43.1%)	NP	0.46 ± 0.23 (38.6%)	3.8 [16]
Cd(II)	5	NP	10.14 ± 2.23 (52.3%)	NP	10.08 ± 4.5 (48.2%)	59.3 [14]
Zn(II)	4	22.3 ± 11.2 (39%)	0.50 ± 0.25 (38.7%)	5.26 ± 2.63 (42.4%)	0.71 ± .36 (38.9%)	3.6 [19]

[a] Maximum pollutant concentration: 1,000 mg/L. *CV* = coefficient variation; NP = not possible to calculate the EC50; NK = not known; *n* = No. of replicates. EC50 = median effective concentration.

observed between toxicity and pH; i.e., changes in pH of the pollutant solutions resulted in improvement or reduction of the EC50. In our case, we did not evaluate changes in pH because we had to standardize a single working procedure. Nevertheless, we were able to define the EC50 value for most of the tested pollutants. In some cases, the sensitivity to various kinds of pollutants of both strains tested was lower than that reported in other studies (see Table 2); this was more evident for standard solutions of metals, which suggests inappropriate pH in our working conditions.

In conclusion, we have defined the conditions for a freeze-dried sensing element based on bioluminescent bacteria, which is commercially interesting, competitively sensitive compared with other systems, and easy to transport. This result represents a starting point for the use of these bacteria in remote-controlled, automated biosensors for the control of toxicity in environmental monitoring applications. The main advantages of our system concern the possibility of using the same bacterial suspension for approximately two weeks; when using a 96-well plate in a luminometer, it is possible to analyze more chemicals at the same time, and analysis can be performed at room temperature. We plan to perform further studies to improve this device.

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