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ORIGINAL PAPER

A multi-channel bioluminescent bacterial biosensor for the on-line detection of metals and toxicity. Part II: technical development and proof of concept of the biosensor

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Abstract This research study deals with the on-line detection of heavy metals and toxicity within the context of environmental pollution monitoring. It describes the construction and the proof of concept of a multi-channel bioluminescent bacterial biosensor in immobilized phase: Lumisens3. This new versatile device, designed for the non-stop analysis of water pollution, enables the insertion of any bioluminescent strains (inducible or constitutive), immobilized in a multi-well removable card. The technical design of Lumisens3 has benefited from both a classical and a robust approach and includes four main parts: (1) a dedicated removable card contains 64 wells, 3 mm in depth, arranged in eight grooves within which bacteria are immobilized, (2) this card is incubated on a Pelletier block with a CCD cooled camera on top for bioluminescence monitoring, (3) a fluidic network feeds the card with the sample to be analyzed and finally (4) a dedicated computer interface, BIOLUX 1.0, controls all the elements of the biosensor, allowing it to operate autonomously. The proof of concept of this biosensor was performed using a set of four bioluminescent bacteria (*Escherichia coli* DH1 pBzntlux, pBarslux, pBcoplux, and *E. coli* XL1 pBfiluxCDABE) in the on-line detection of $CdCl_2$ 0.5 μM and As_2O_3 5 μM from an influent. When considering metals individually, the "fingerprints" from the biosensor were as expected. However, when metals were mixed together, cross reaction and synergistic effects were detected. This biosensor allowed us to demonstrate the simultaneous on-line cross detection of one or several heavy metals as well as the measurement of the overall toxicity of the sample.

Keywords Multi-channel biosensor · Bacteria · Heavy metals · Bioluminescence · Toxicity

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Introduction

In 1977, Karube et al. [1] introduced a new concept of biosensor which used microorganisms as the bioelement. Since this pioneering work, 593 publications have been devoted to microbial biosensors, 64% of focusing on environmental applications, mainly biological oxygen demand and specific pollutant monitoring (ISI Web of

Sciences 2009 and personal database). Biosensors ought to contribute to the Water Quality Framework since such devices are normally designed for the on-line monitoring of pollution as required by European legislation.

The use of bacteria in the specific detection of a pollutant or of the overall toxicity of water has been widely studied and applied, but very often in relation to bioassays and rarely in relation to biosensors [2–11].

On-line biosensors that use bioluminescent bacteria for measuring have also been developed with a view to detect pollutants and it has been possible to observe a technological approach in the design of type 1 or type 2 biosensors (Fig. 1). The first article to appear was published by Heitzer et al. [12] and was followed by several others where bacteria were in liquid phase (Type 1 biosensor [13, 14]) or immobilized Type 2 biosensor [15–17]).

An approach combining the responses of several different bacteria in order to extract a "pollution fingerprint", similar to DNA/DNA hybridization techniques, undoubtedly dates back to 2001 [18] when stress-sensitive bacteria were used, but also to the later work of Biran et al. [19] and then Elad et al. [10] who put forward a statistical method in the interpretation of data. However, the use of on-line measurement within an integrated system has not yet been advanced.

To our knowledge, very few papers have been dedicated to the detection of metals by biosensors using recombinant bioluminescent bacteria. Leth et al. [20] immobilized bacteria from the *Alcaligenes eutrophus* CH34 bacterium (now *Cupriavidus metallidurans*) to detect copper. The "multi-strain" approach was attempted in bioassay by Ivask et al. [9], and also by Hakkila et al. [21] in a biosensor format.

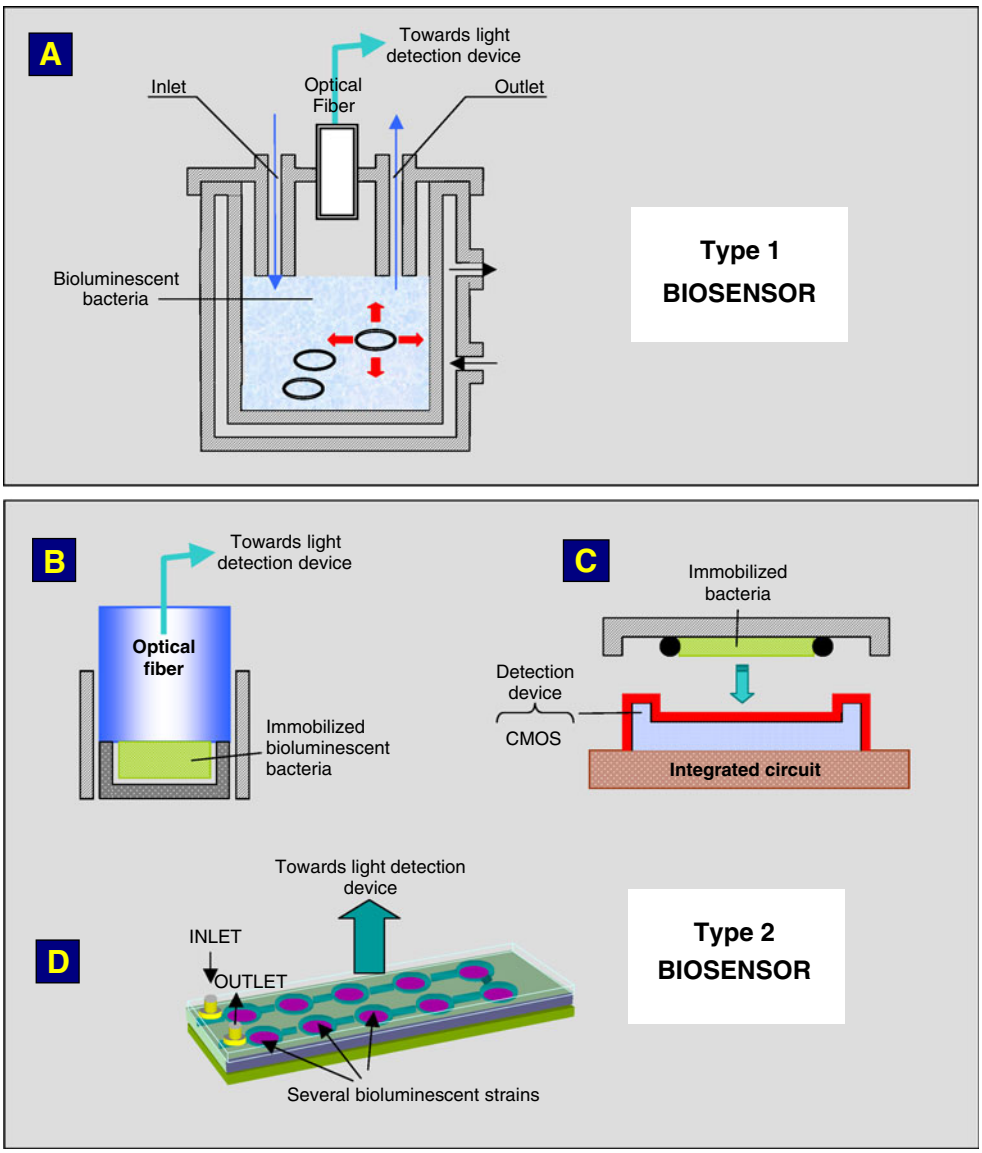


Fig. 1 Figures showing the two kinds of biosensors (types 1 and 2). *CMOS* complementary metal oxide semiconductor

This article reports on the technological development and the proof of concept of a type 2 biosensor using a card which is not in contact with the optical transducer. This biosensor is very simple in design to ensure that it is sturdy enough for work in the field.

Experimental

Biosensor technical design and instrumentation

The new Lumisens3 biosensor is an autonomous device that includes all the required components for the on-line analysis of toxicity and metallic pollution in water from the environment.

The biosensor consists of four technical parts: the measuring chamber, the removable multi-well card, the fluidics, and the computer interface which controls the device and processes the results (Fig. 2).

The black measuring chamber is equipped with a cooled CCD (charge-coupled device) camera (SPTO RT SE 6, Optilas) placed above the multi-well card. This arrangement allows a comprehensive detection on the whole surface of the card along with a significant flexibility in terms of the position of the measuring points. The CCD camera (fitted out with an Optilas lens at a 30 cm focal point) is cooled to −28 °C using Peltier elements. These elements reduce the electronic background noise from the camera. The images are collected and processed via an acquisition interface every 10 min with a 2-min integration time.

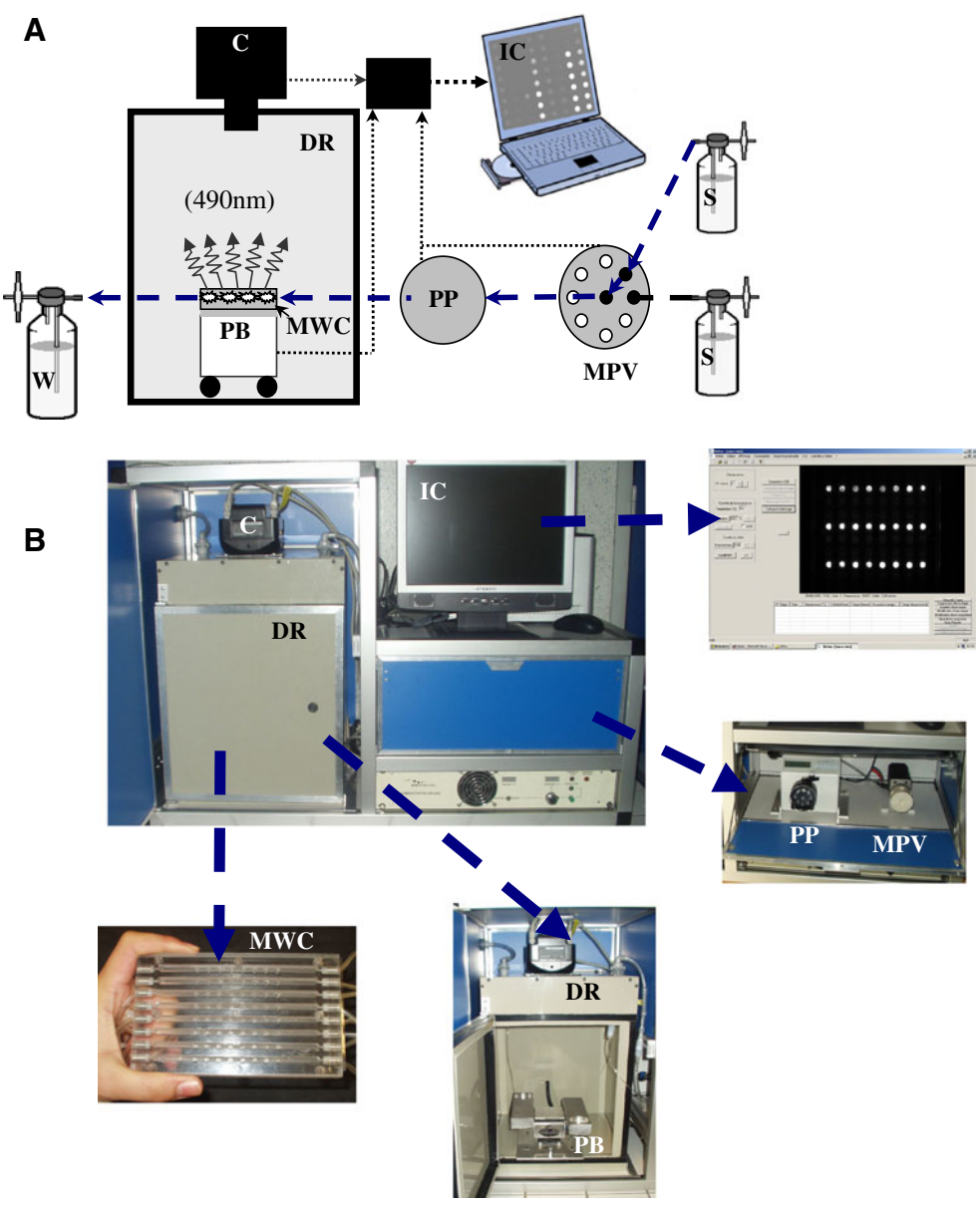


Fig. 2 Schematic representation of Lumisens3 biosensor (**a**) and photos of the biosensor (**b**). *C* Cooled CCD camera, *DR* dark room, *S* samples to be analyzed, *W* waste, *PP* peristaltic pump, *MPV* multi-position valve, *IC* informatics control, *PB* Peltier block, *MWC* removable multi-well chip

In order to construct the multi-well, preliminary studies were undertaken as to the choice of material and the definition of the minimum size of the bacteria-containing wells and their spacing. We machined three specific cards with several wells (2, 3, 4, 5, 6, 7, 8, 9 mm diameters). Each well-containing bacteria was 3 mm in depth, the lower limit of our drilling machine. The material was either on transparent polycarbonate lying on a black floor, on black Ertacetal or on white Teflon. Teflon was withdrawn because of the light diffusion around the wells; this phenomenon was not observed with Ertacetal and the transparent polycarbonate that offered the best contrast image. The conclusions of this study demonstrated that the wells should have a diameter of 4 mm minimum and be spaced 3 mm apart to optimize the detection of bioluminescence using a CCD camera [22] (Fig. 3). The material selected to build the card was transparent polycarbonate, thereby avoiding any heavy metal contamination from omnipresent plastic dyes [23]. The useful surface area of the card is limited because of the Peltier element which is responsible for regulating the temperature. In order to escape any edge effects when regulating the temperature, the initial useful

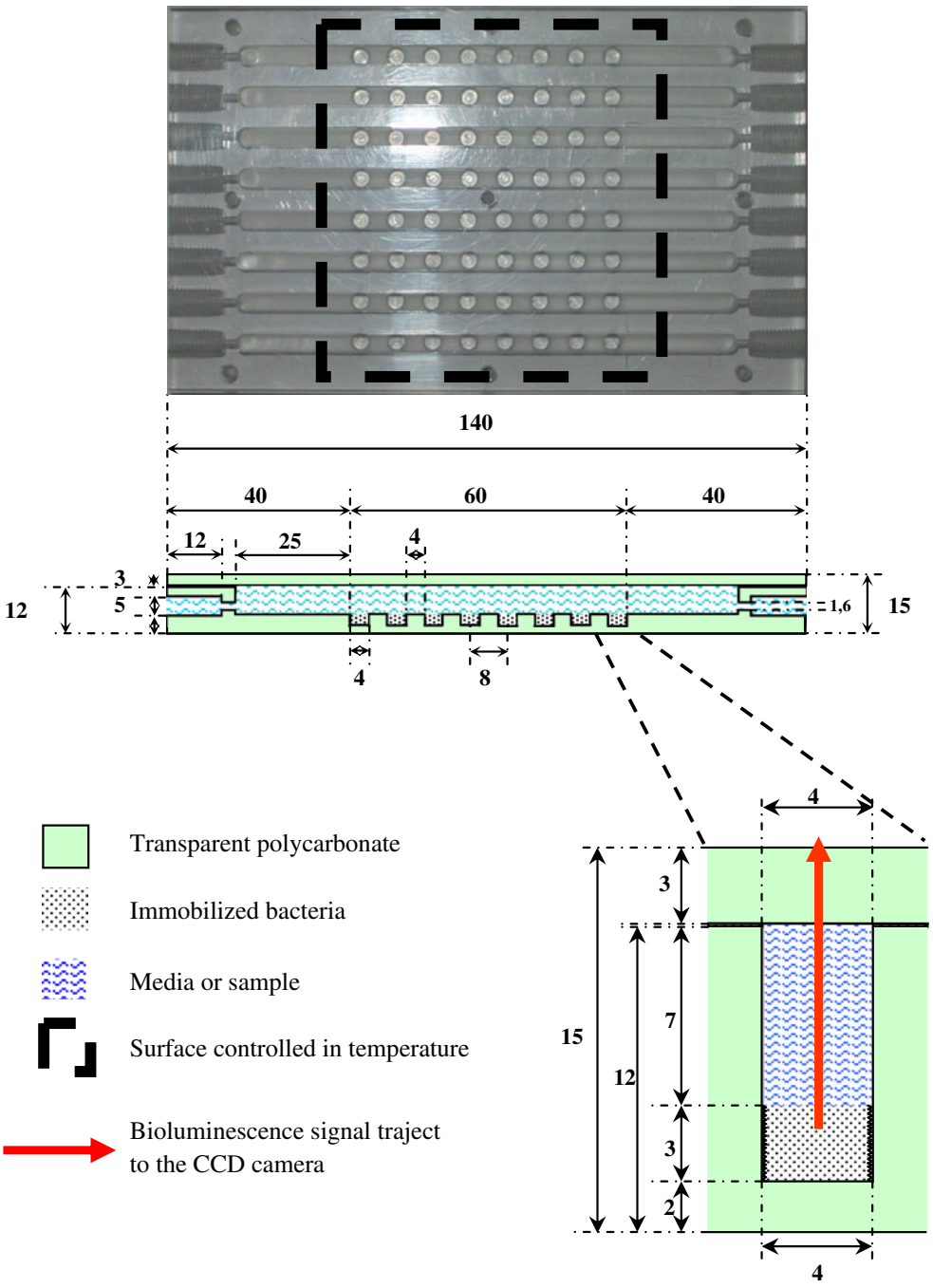


Fig. 3 Photo and schematic representation of the multi-well card designed for Lumisens3 biosensor (values are in millimeters)

surface area (75×75 mm) was decreased to 65×65 mm. Taking the size of the wells and the spacing into account, a maximum of 64 wells are included on the card. The 64 wells are allotted to eight circulation grooves which are supplied by a streamer-shaped fluidics circuit. The bioluminescence signal is measured above the card. The watertightness between the lid and the card is ensured by a silicone grease seal. Screws keep the structure airtight.

The circulation of the fluids within the biosensor is enabled by an eight-roller peristaltic pump (Meredos) which smoothly moves the liquid in a flow rate from 1.25 to 80 mL min^{-1}. The type of input medium is selected by a multi-position solenoid valve (VICI Valco, Interchim) with six inlet ports and one outlet port. These two elements can receive information manually or via an RS232 port on a computer interface. The other fluid circulation components consist of plastic tubes (Tygon RS3603) and various PTFE joints (Bioblock, France). Each part of the circuit can be removed and cleaned separately.

The computer interface, BIOLUX 1.0, was programmed in C^{++} (Fig. 4). This software lets the biosensor operate in two different modes. The user can either directly and independently control the different parts of the biosensor (pump, solenoid valve, Peltier block, and CCD camera) or program the analysis/regeneration sequences via the programming interface. The software constantly displays the flow rate and temperature values, as well as the selected solenoid valve for the duration of the experiment. The CCD camera acquisitions can be obtained periodically (for example, every 10 min) or at different times throughout the experiment. Each image contains the relevant data from the acquisition: date, flow rate, temperature, selected solenoid valve, exposure time, and the gain for the CCD. Results are processed from the card images obtained in bitmap format (8 bytes, 256 gray levels). A cache preset is applied to the image in order to visualize each well separately. Calculating the quantity of light emitted by each well is performed by counting the value of each pixel

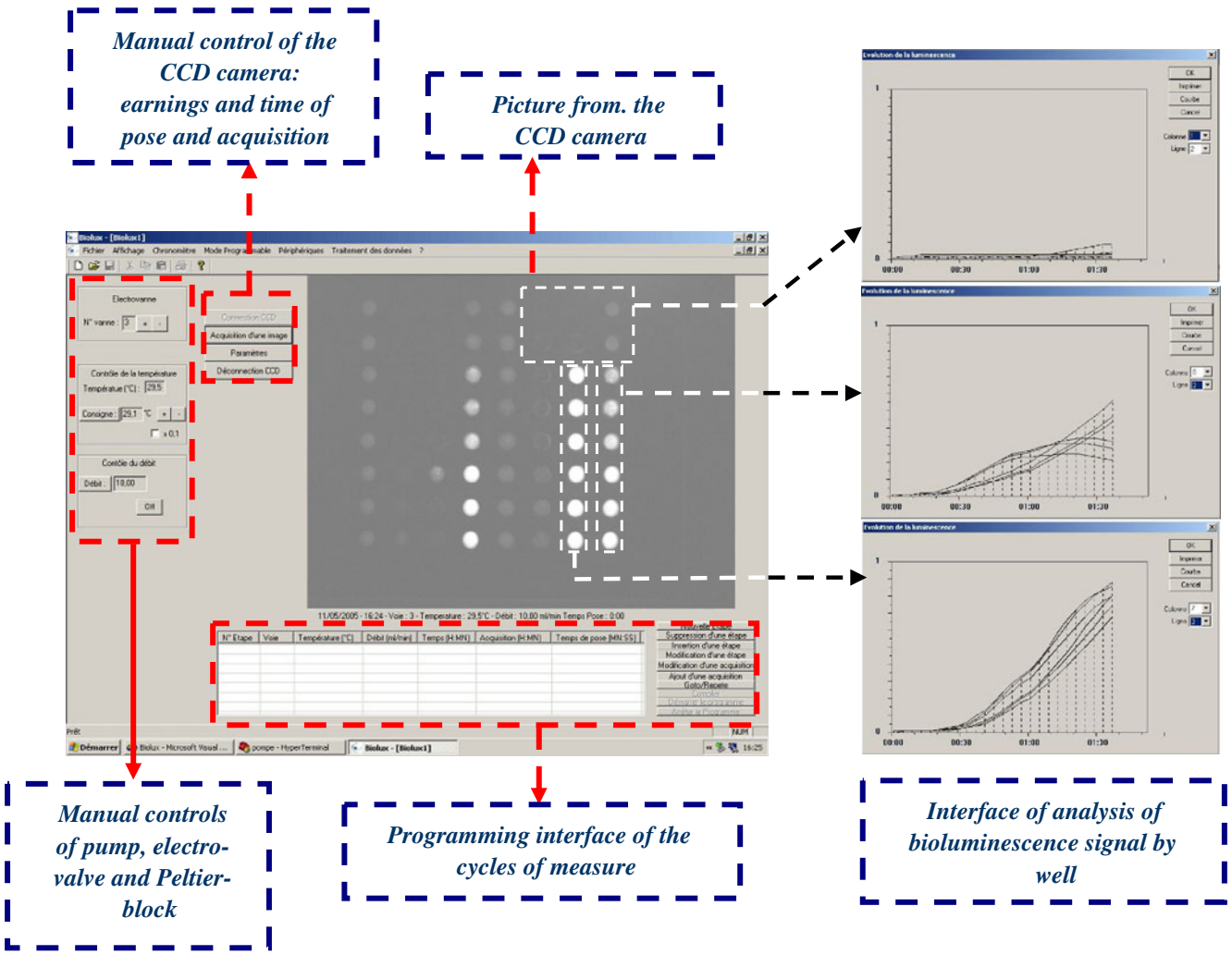


Fig. 4 Screenshots of the software Biolux 1.0 interface and description of its associated functions

contained in the relevant zone. Black is given a 0 value and white, a value of 256. The pixel average is then transformed into a value between 0 and 1. The set of bioluminescence intensities calculated from the card can consequently be displayed by the software. During an experiment, each image is dealt with in this way. How the luminous intensity per well evolves can then be visualized as a function of time at the end of the experiment and the data can either be displayed by the software or exported in Excel format by multiplying the bioluminescence values by 1,000.

Bacterial strains and media

The strains for the constitutive or inducible expression of the bacterial bioluminescence are listed in Table 1. Besides the construction of strains as described in the first part [24], the *E. coli* DH1 pBfi*luxCDABE-P* without promoters was constructed as a negative bioluminescence control.

An acetate medium with a C/N/P ratio of 100/10/1 was used for the growth of microbial cells, both for bioassay or biosensor applications. One liter of tap water was filtered through a 0.45 μm filter (Sartorius) and supplemented with 2.834 g acetate (Panreac), 1.1919 g NH_4Cl (Merck), 0.028 g K_2HPO_4 (Merck), 5 g NaCl, 0.5 g yeast extract (Merck) and 0.1 g tryptone (Biokar Diagnotics). The pH was adjusted to 7 and the medium was sterilized by autoclaving at 100 °C for 30 min. When applied to the biosensor, this media was diluted ten times to bring carbon and energy sources and in the meantime to limit cell division in the agarose matrix without affecting the detection limit.

Batch cultures were prepared at 30 °C and shaken at 250 rpm in 100-mL Erlenmeyer flasks. Bacterial growth was monitored using a spectrophotometer (Unicam) and absorbance measurement was performed at 620 nm.

Chemicals

Metals used in this study were: $CdCl_2$ (Panreac) and As_2O_3 (Sigma). The purity of the metals was greater than 95%. Metal stock solutions were prepared at a high concentration (1 M or 0.5 M) in acidified distilled water (pH≈5–6) and conserved at +4 °C for 6 months in brown bottles. Working dilutions of metals were made daily in distilled water acidified to pH≈5–6 and then added to the samples at the appropriate concentration.

Immobilization of the strains

Strains were immobilized in agarose matrix as described previously [24]. Shortly afterwards, the immobilization solution, prepared in fresh acetate medium, contained a bacterial suspension at OD_{620nm}=0.1 mixed with agarose (2% *W*/*V* final concentration). 40 μL of this solution was poured into each well on the multi-well card in compliance with the same immobilization plan (Fig. 5). After immobilization the multi-well card was immediately used in the biosensor.

Operation of the biosensor

The measurement cycles were programmed using the BIOLUX 1.0. software with a flow rate of 5 mLmin^{-1}, allowing a good agarose stability (data not shown). Induction medium (acetate medium diluted ten times with the appropriate heavy metal concentration) was fed into the

Table 1 Strains and plasmid used in this study

Strain (genotype)	Plasmid	Description	Function	References
E. coli XL1 blue (*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *lac*$^-$ F' [*proAB*$^+$ *lacI*^{q} *lacZ* ΔM15 Tn10 (Tet^r)])	pBtac2	Ap^R, p*tac* promoter, constitutive expression vector	Negative bioluminescence control	Roche
	pBfiluxCDABE	Ap^R, *ptac::luxCDABE*, constitutive expression of the *Vibrio fischeri* bioluminescence *lux* operon	Global toxicity control	[24]
E. coli DH1 (F$^-$, *recA*1 *endA*1 *gyrA*96 *thi*-1 hsdR17 *supE*44 *relA*1)	pBarslux	Ap^R, *parsR::arsR::luxCDABE*, bioluminescent sensor inducible by arsenic	Heavy metal detection	[24]
	pBzntlux	Ap^R, *pzntA::luxCDABE*, bioluminescent sensor inducible by cadmium and mercury	Heavy metal detection	[24]
	pBfiluxCDABE-P	Ap^R, *luxCDABE V. fischeri* bioluminescence *lux* operon *without promoter*	Negative bioluminescence control	This study
	pBcoplux	Ap^R, *pcopA::luxCDABE*, bioluminescent sensor inducible by copper	Heavy metal detection	[24]

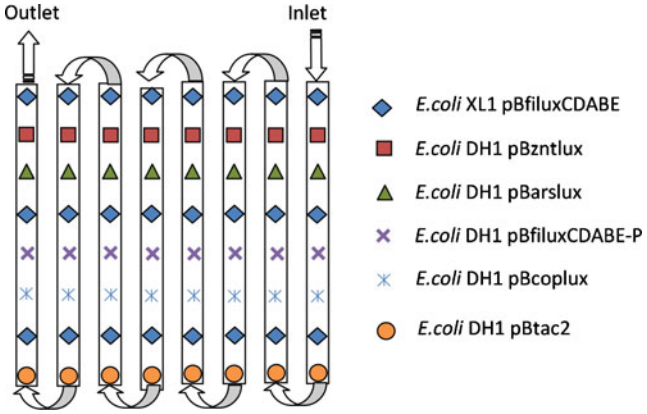


Fig. 5 Immobilization plan for strains in Lumisens3 multi-well card

biosensor for 100 min to induce bioluminescence. While the biosensor was operating, one picture of the card was taken every 10 min and then processed with the BIOLUX 1.0 software. Bioluminescence data were expressed as arbitrary units and then transferred in Excel format at the end of each experiment. Induction ratio is the ratio between the peak of bioluminescence recorded after induction with the metal versus the background bioluminescence just before the induction. This background was stable all over the experiment.

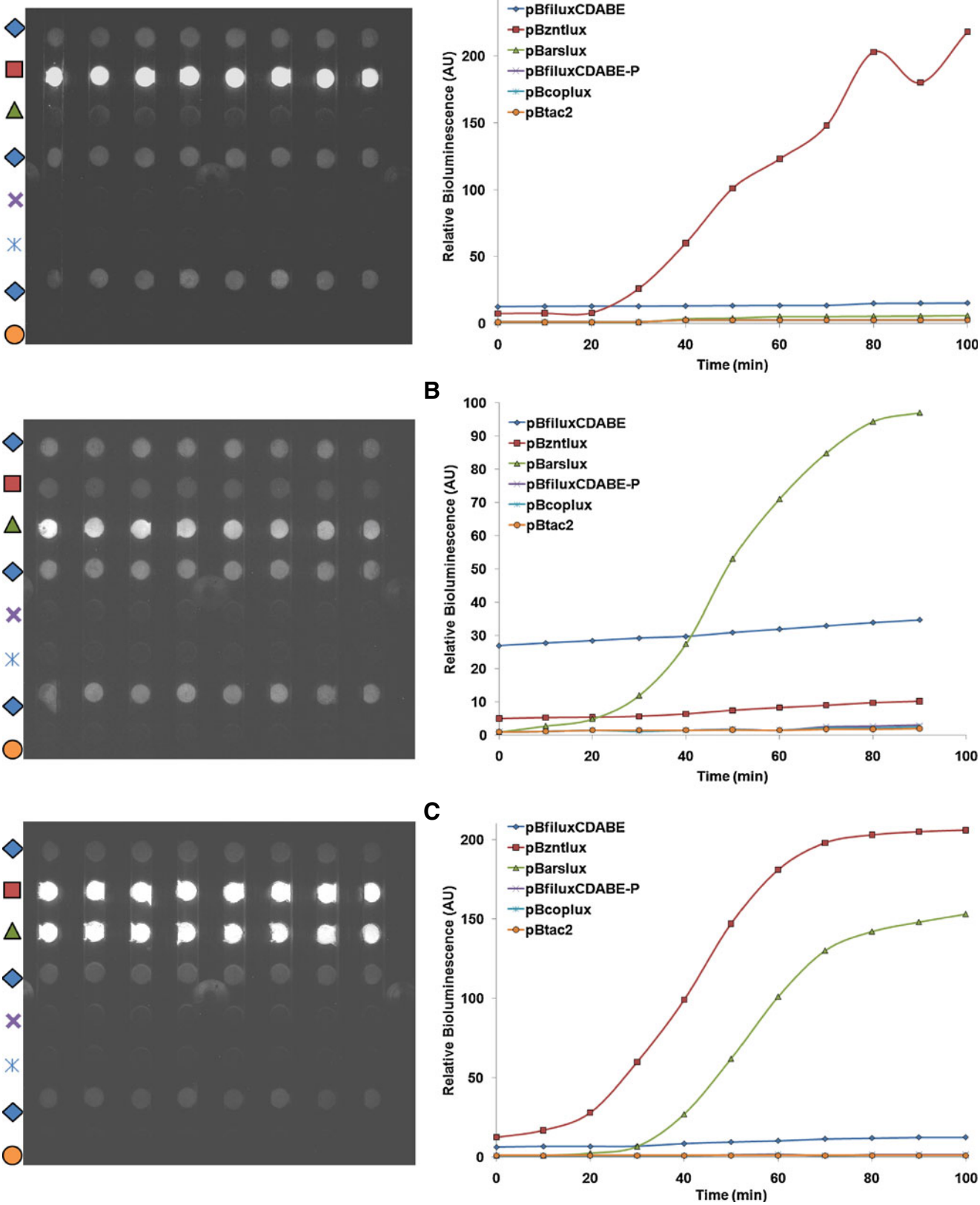
Results

The aim of this study was to demonstrate the value of a multi-channel biosensor in the multiple measurement of both the overall toxicity and the various metals that are present in a sample of fresh water. The inducible and constitutive strains that were used in the biosensor are: *E. coli* DH1 pBzntlux, pBarslux, pBcoplux, and *E. coli* XL1 pBfiluxCDABE [24]. Two other bacteria, *E. coli* DH1 pBtac2 and *E. coli* pBfiluxCDABE-P, were added to the multi-well card as controls for the proof of concept. These two constructions correspond to the negative bioluminescence controls using either the original cloning plasmid or the *luxCDABE* operon cloned in the same plasmid, but without a promoter (pBfiluxCDABE-P).

The results presented in Fig. 6 have come from three independent experiments. Figure 6a indicates the response of the immobilized strains in the multi-well card during the passage of an acetate medium (diluted ten times) containing 0.5 μM of $CdCl_2$. In these conditions, the cadmium-specific inducible strain, *E. coli* DH1 pBzntlux, displays a bioluminescence signal which increases during the time of induction until it reaches a level of 220 AU (IR=33). A substantial bioluminescence signal, almost nine times the base level before induction, is also measured for *E. coli* DH1 pBarslux after 100 min. The *E. coli* DH1 pBcoplux shows no significant bioluminescence signal, either during the course of the induction or at the end. The control strain for toxicity, *E. coli* XL1 pBluxfiCDABE, has a stable bioluminescence signal and therefore the cadmium sample is not toxic under the conditions used. The control strains show no bioluminescence signal. This first experiment validates the cross detection of cadmium by *E. coli* DH1 pBzntlux and pBarslux, but not by *E. coli* DH1 pBcoplux. In the experimental conditions used, the latter does not produce a signal which can be measured by the biosensor in the presence of cadmium. The tests that were previously conducted with this strain in microplate format or in liquid phase however demonstrated that it was sensitive to cadmium at the levels of concentration used during the experiment. This absence of any signal may be the effect of immobilization in the agarose, which in the case of this strain, may cause, even in microplate format, a considerable reduction in the bioluminescence signal as has already been shown [24]. *E. coli* DH1 pBcoplux can therefore produce a signal, but it is not possible for this signal to be measured by our CCD camera, as the camera is not sensitive enough and the concentration of cadmium present in the water is not high enough.

Figure 6b shows the results from the analysis of an acetate medium, diluted ten times and containing 5 μM of arsenic III. For this experiment, the previous strains were immobilized in the multi-well card following the same procedure as before. As expected, *E. coli* DH1 pBarslux emitted a bioluminescence signal which rose during the experiment. The induction ratio for this strain reached 62 at the end of the experiment. The other inducible strains, *E. coli* DH1 pBcoplux and *E. coli* DH1 pBzntlux, did not display significant induction signals (less than 2) because the strains did not react to this metal [24]. The other toxicity control strains and the negative control strains for bioluminescence did not show any significant variations in their bioluminescence signals. The amount of metal used was therefore not toxic to the bacteria. Arsenic III was successfully detected by the biosensor under the particular experimental conditions used.

In the final proof-of-concept experiment, a mixture of cadmium (0.5 μM $CdCl_2$) and arsenic III (5 μM As_2O_3) were introduced on line into the biosensor on a card containing strains that had been freshly immobilized with the established plan. The results from this experiment as presented in Fig. 6c at first show a strong increasing bioluminescence signal from the *E. coli* DH1 pBzntlux and *E. coli* pBarslux strains. After 100 min of recording, the induction ratios reached 80 for *E. coli* DH1 pBzntlux and 1362 for *E. coli* DH1 pBarslux respectively. The other strains did not display a significant variation in their bioluminescence signals. The mixture of metals was therefore not toxic to the bacteria and was clearly detected


A
B
C
pBfiluxCDABE
pBzntlux
pBarslux
pBfiluxCDABE-P
pBcoplux
pBtac2
Relative Bioluminescence (AU)
Time (min)
0
20
40
60
80
100
50
150
200
250
10
30
70
90

◀ **Fig. 6** Fingerprints of $CdCl_2$ 0.5 μM (**a**), As_2O_3 5 μM (**b**) and of a mixture of these two heavy metals (0.5 μM $CdCl_2$ and 5 μM As_2O_3) (**c**) obtained with Lumisens3 biosensor within 1/10× acetate medium and at a flow rate of 5 $mL\,min^{-1}$ at a temperature of 30 °C. Bioluminescence measurements were recorded every 10 min (each point is the average of the bioluminescence of eight wells. The standard error was under 5% and was not added) *On the left* pictures taken after 50 min of experimentation, *on the right* bioluminescence signal measured every 10 min for each strain

by the specific strains. The response from *E. coli* DH1 pBarslux was considerably stronger than before (induction factor of 9 for the arsenic on its own at the same concentration). This strain was the only one that responded to both the metals tested. The stronger signal may be due to a synergistic action from the two metals on the strain, which is only revealed if the strain responses are combined.

Discussion

The results for the detection of cadmium and arsenic prove that the concept of on-line detection using multiple inducible or constitutive bioluminescent bacteria is possible and this is despite the fact that the CCD camera was probably not sensitive enough.

In determining the metals, the "multi-strain" approach was attempted in bioassay format by Ivask et al. [9] and in a biosensor format by Hakkila et al. [21].

To our knowledge, the biosensor, as developed in this article, is the first on-line measuring device to couple a number of bioluminescent bacteria in the control of metallic pollution and its toxicity. If we take into account the non-specificity of our genetic constructions (one bacteria can detect several metals), ultimately the multi-strain approach based on combined responses is the only one possible to achieve a more precise identification of the metal. Finally, the multi-strain response also allows us to take into consideration the synergistic or antagonistic effects.

The core of the biosensor is represented by the card on which the bacteria are immobilized. In our opinion, microfluidics, an area which has been largely developed within different disciplines of biology [25, 26] is not appropriate for use in environmental work. Indeed, there are two limitations. Firstly we had to adapt to the requirements of the sample volumes from the environment and to clogging problems caused by bacterial aggregates that varied between 100 and 1,000 μm in size. Moreover, while using the biosensor, an increase in the number of bacteria took place and a 300 μm thick film is formed on the surface of the gel [27], thereby limiting the use of a card with micrometric dimensions. The second limitation is in relation to the well size, as the wells have to be designed in order to easily immobilize the bacterial membranes which were highly viscous and which created pipetting problems. Nevertheless, the compromises that related to the practical aspects of the card design have led to a 3 mm thick agarose, largely restricting the diffusion of oxygen necessary for both bacterial metabolism and the bioluminescence reaction [28].

The technical design of Lumisens3 has benefited from both a classical and a robust approach. The biosensor is quite versatile because all types of bioluminescent micro-organisms can be used. Nevertheless, data interpretation will need specific software that will allow cross-reactions from all the bacteria to be interpreted, more specifically when a complex mixture of metals coming from an environmental sample is tested. This software is under development in our laboratory.

The biosensor is currently limited by the following:

- The shelf life of the encapsulated bacteria (not the purpose of this study) seems to be no more than several days (data not shown), while vacuum drying allowed a 12-week storage time [29] to, for example, 8 months after lyophilization [30].
- The drift of bacterial metabolism while using the bacteria inside the card with the possible contamination from other bacteria from the environmental sample.
- The biofilm formation inside the card on the surface of the agarose, pointed out by Affi [27] influences the contamination of the feeding tubing.
- A demonstration of the possibility to use the biosensor during several days or weeks.

When these limitations will be overcome, biosensors using microorganisms would certainly be mature for environmental purposes.

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