

Bacterial Bioluminescent Biosensor Characterisation for On-line Monitoring of Heavy Metals Pollutions in Waste Water Treatment Plant Effluents

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1. Introduction

The detection of pollutants in the environment is becoming a health and economic issues. The sector of water realises more than 700.000 analyses per year with an average price of approximately 15 euros/analysis. In 2001, the European Community published in the official journal a list of priority substances to be detected within the water framework (Decision N° 2455/2001/EC). Heavy metals like cadmium, lead and mercury belong to this list. The current measurement techniques are not applicable to the on line analysis of these pollutants because they are too expensive and complex to be applied in the field. Our laboratory develops since ten years alternative measurement methods of chemical pollutants in water and pathogenic bacteria in the food industry either as bioassay or biosensor.

2. Generalities about bioluminescence, heavy metals and biosensors

2.1 Bacterial bioluminescence

The mechanism of the bacterial bioluminescence is studied since 1944 (McElroy & Ballentine, 1944). Today eleven bioluminescent bacterial species classified in four genus are listed. Three genus are coming from marine origin: *Vibrio*, *Photobacterium* and *Alteromonas*. The fourth, more recently discovered is of terrestrial origin: *Photorhabdus* (ex *Xenorhabdus*) (Meighen & Szittner, 1992).

From the biological and ecological point of view, the interest of the bioluminescence is related to the symbiotic character of the bacteria. It was suggested that the metabolic function of the bacterial luciferase was connected to its capacity to the electrons transfer to oxygen rather than to its light output. Thus, it would be an alternative pathway for the electrons transport under low oxygen pressure like in fish organs (Hastings et al., 1985; Meighen, 1993) and the hosts would draw advantage from the light emitted by using it for communication or defence (Nealson & Hastings, 1979; Meighen, 1994; Visick et al., 2000).

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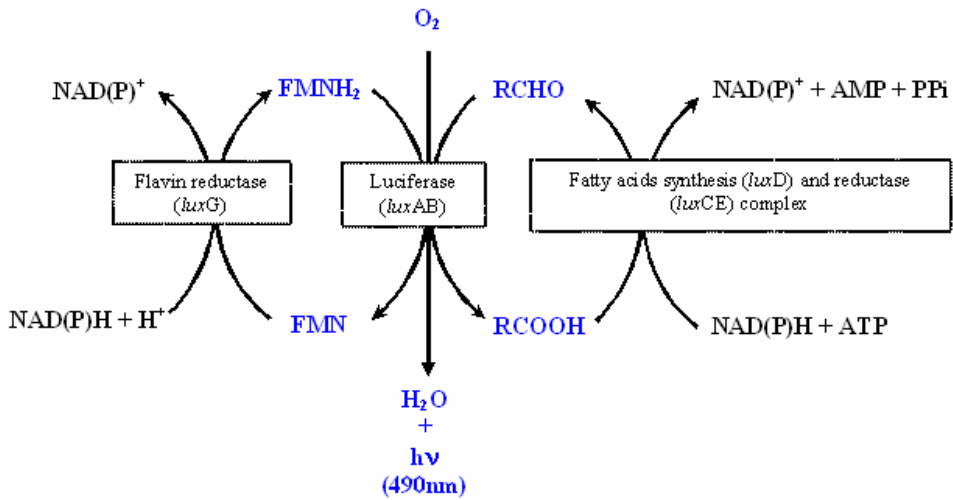


Fig. 1. Bioluminescence reaction (adapted from Sung & Lee, 2004 and Tu & Mager, 1995)

The reaction of bioluminescence is catalyzed by the luciferase enzyme coded by the *luxAB* genes (Figure 1) (Blum, 1997). Three key substrates modulate the light reaction: the dissolved oxygen provided by the surrounding environment, the reduced flavine mononucleotide coming from the bacterial metabolism and a long chain aldehyde such as decanal coded by the *luxCDE* genes (Meighen, 1991). When spectral emission from the whole bacteria was recorded from 400 to 750 nm with a highly sensitive spectrometer initially devoted to Raman scattering, two peaks were clearly identified, one at 491-500 nm (± 5 nm) and a second peak at 585-595 (± 5 nm) with the Raman CCD (Thouand et al., 2003).

2.2 Heavy metals and bacterial resistance

a. Heavy metals

Among metals it is necessary to separate metals and metalloids essential to the biological life (for example copper, zinc, iron, selenium...), from non essential metals such as for example cadmium, mercury. However all metals according to their concentration have a toxicity with respect to living organisms.

Heavy metals are naturally present both in the environment and in water at trace levels (5ng. L^{-1} Cd in water, INERIS, France), but the anthropogenic activity increases metals concentration found in the natural environments (Audry et al., 2004). Because of their great solubility in water heavy metals are easily absorbed by the living organisms and are found accumulated in human at the end of the food chain (Kurniawan et al., 2006). In fact the industrial wastes are mainly responsible for the current pollution of the rivers by heavy metals that are normally collected and treated by waste water treatment plants (WWTP). However, Karvelas et al, 2003, pointed out that even a fraction is adsorbed in the activated sludges, between 37 to 77% (depending to the metal) are rejected into the river. The WWTP can thus be a considerable source of pollution in spite of the existing standards of rejections and the treatments carried out.

b. Bacterial heavy metals resistance mechanisms

Basically, heavy metals action in the bacterial cytoplasm leads to important oxydative stresses (Nies, 1999). The minimal inhibition concentrations of heavy metals/metal for *Escherichia coli* are reported in table 1.

MIC (mM)	Heavy metals
0.01	Hg ²⁺
0.02	Ag ²⁺ , Au ³⁺
0.2	CrO ₄ ²⁻ , Pd ²⁺
0.5	Pt ⁴⁺ , Cd ²⁺
1.0	Co ²⁺ , Ni ²⁺ , Cu ²⁺ , Zn ²⁺
2.0	Tl ⁺ , UO ₂ ²⁻ , La ³⁺ , Y ³⁺ , Sc ³⁺ , Ru ³⁺ , Al ³⁺
5.0	Pb ²⁺ , Ir ³⁺ , Os ³⁺ , Sb ³⁺ , Sn ²⁺ , In ³⁺ , Rh ²⁺ , Ga ³⁺ , Cr ³⁺ , V ³⁺ , Ti ³⁺ , Be ²⁺
10.0	Cr ²⁺
20.0	Mn ²⁺

Table 1. Minimal inhibition concentrations (MIC) of heavy metals to *Escherichia coli* (Nies, 1999)

The entry of heavy metals into the bacterial cell is done by the regulation systems of the divalent cations or oxyanions. Indeed, heavy metals are not distinguishable for these transmembrane proteins, from the other divalent cations or oxyanions (for example: SO₄²⁻, HPO₄²⁻, Fe²⁺, Mg²⁺, Mn²⁺, etc...). Bacteria have developed two kinds of resistance mechanisms to cure this passive entry. The first of them is fast, non specific and is based on the gradient of concentration or on proto-motive force. The second mechanism is more specific, inducible and is activated in the presence of a metal and generally requires ATP hydrolysis.

This second inducible mechanism of resistance is subdivided in three types.

- The first of them is a more or less specific system of cation/oxyanion efflux by ATPases pumps (ATPase CPx-type).
- The second system is based on the complexation capacity of some divalent heavy metals with the -SH group of the glutathion or the metallothionines for cyanobacteria (Cavet et al., 2002).

Lastly, the third mechanism, more specific for oxyanions, allows a reduction of them in a less toxic form. These reduced forms are then evacuated from the cytoplasm with a specific ATPases pumps (Nies, 1995; Nies, 1999; Nies, 2003).

The bacterial mechanisms of regulation and resistance to heavy metals are summarised in figure 2. The efflux systems using ATPases pumps are preferred in the bacteria because their energy cost per cell is lower than the complexation of metals by the glutathion or the metallothioneins. Indeed one ATP molecule is enough to evacuate a divalent metal with an ATPase pump belonging to the family of the ATPases CPx-type, whereas 16 ATP are necessary to complex only one divalent metal (Nies, 1999).

It is important to note that the mechanisms of inducible resistances, known to be specific, are thus not activated by only one metal even if their name seems to make it believe (Nies, 2003). In the same way, only one metal can activate several systems of resistance (Moore et al., 2005; Wang & Crowley, 2005).

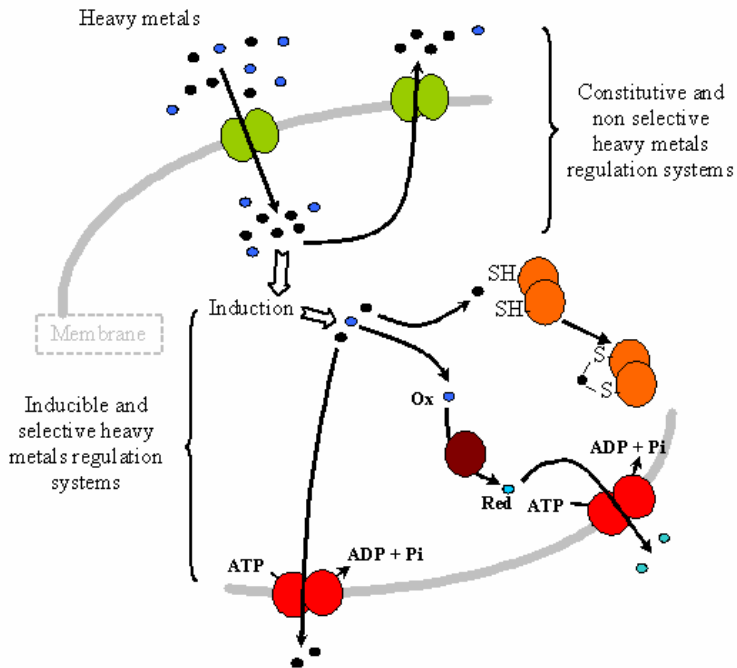


Fig. 2. Main heavy metals regulation and resistance mechanisms in bacteria.

c. Molecular basis of the ZntA/ZntR system of *Escherichia coli*

The action of the MerR regulators consists first of all to the repression of the target promoter in the absence of the metal. This inactivation is due to a distortion of the DNA double helix on the RNA polymerase fixation site. The regulator, having fixed the metal pollutant, changes its conformation allowing the RNA polymerase to fix to the promoter. The regulation of the *pzntA* promoter, by the ZntR protein is an example of the mechanisms of regulation of the MerR type (Brocklehurst et al., 1999; Outten et al., 1999; Binet & Poole, 2000) (Figure 3).

The regulators of the MerR type are activators/repressors. This family of proteins has at its N-terminal part a site for DNA fixation and the ligands are fixed to the C-terminal.

2.3 Detection of heavy metals with bioluminescent bacteria: choice of a heavy metal-bacterial resistance mechanism.

The inducible bioluminescent bacteria are used to detect specifically a pollutant or a family of pollutants or even the toxic effects. The action of the pollutants on the cells induces in this case the synthesis of the genes of bioluminescence and thus the emission of light. Thus, the choice of an inducible resistance system to build a sensitive strain is foremost justified by its sensitivity to metals *in vivo*. The sensitivity depends on the origin of the genetic system and the host strain.

To obtain an interesting sensitivity, the resistance systems coming from less resistant bacteria such as *Escherichia coli* should be preferred. Indeed, the strain *Ralstonia metallidurans* CH34 (Mergeay et al., 2003) which harbors a great number of resistance systems from the

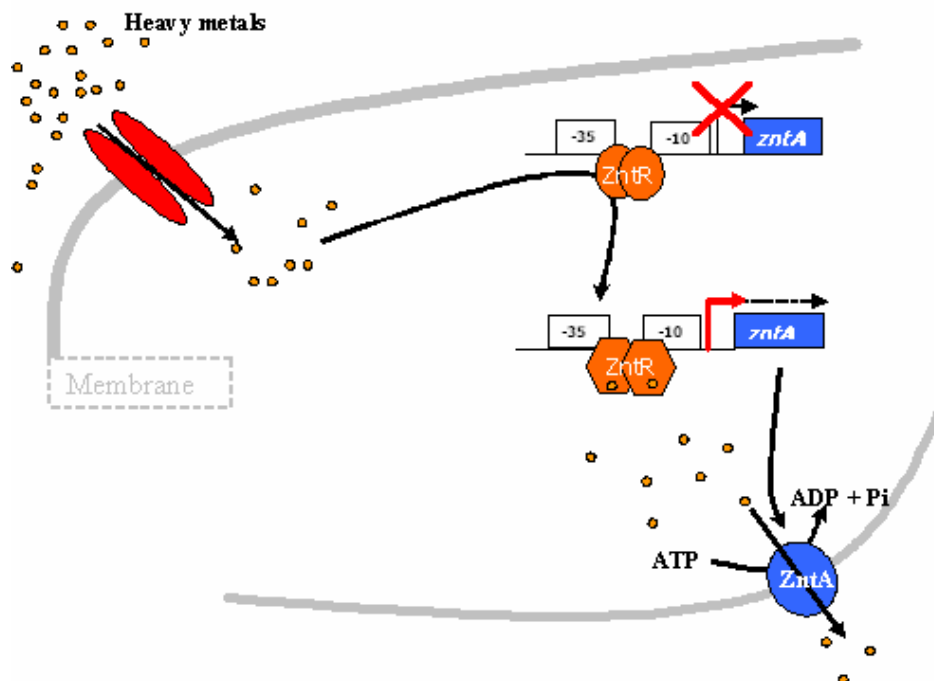


Fig. 3. Regulation of the *pzntA* promoter by the ZntR protein

pMOL28 and pMOL30 plasmids was the object of an insertion of the transposon Tn4431 containing the *luxAB* genes (Corbisier et al., 1993; Tauriainen et al., 1998). The detection limit of the strain obtained was 1mM of Pb^{2+} against 30nM of Pb^{2+} for mechanisms resulting from the less resistant bacteria such as *Escherichia coli* and *Staphylococcus aureus*.

Table 2 reports the detection limits of three bioluminescent strains for heavy metals detection using various mechanisms of resistance from *Escherichia coli*: ZntA/ZntR, CopA/CueR and the *ars* operon. The three plasmidic constructions based on these resistance systems make it possible to detect a range of eleven heavy metals with detection limits sometimes lower than the standards for water. However, it should be noted that the ranges of detection of the CopA/CueR system and the *ars* operon are not studied completely in comparison with the ZntA/ZntR system. Indeed, the *ars* operon is also activated by bismuth (Bi) and antimony (Sb) and the CopA/CueR system is also activated by gold (Au) (Wu & Rosen, 1993).

Various developments are proposed or were proposed on the market such as: the BIOMET® kit developed from transcriptional fusions between the *luxCDABE* operon and various resistance systems to heavy metals of the *Ralstonia metallidurans* strain (Vito, Belgium, <http://www.vito.be/>). One other kit using the inducible bioluminescent strains for arsenic or mercury (Biologically Heavy metal Assay kit) is distributed by Aboatox company following the works of Tauriainen et al. (1997, 1998).

The team of S.Daunert (Lexinton, Irvine, the USA) developed an original microfluidic and analysis system integrated in a compact disc allowing arsenic detection with the arsenic-sensitive *E.coli* pSD10 (*parsR::arsR::gfpuv*) (Rothert et al., 2005). Ivask et al. (2009) proposed

a set of 13 inducible strains (Gram - and Gram +) covering a broad range of sensitivity. Lastly, Hakkila et al. (2004) described a bioassay with 8 inducible bioluminescent strains to heavy metals encapsulated to disposable fiber optics.

Metals tested	<i>E. coli</i> heavy metals resistance mechanisms			European standards for water*
	ZntA/ZntR	CopA/CueR	Ars operon	
	DL <i>E. coli</i> MG1655 pzntlux (Riether et al., 2001)	DL <i>E. coli</i> MG1655 pcoplux (Riether, Dollard et al., 2001)	DL <i>E. coli</i> pJAMA-arsR (Stocker et al., 2003)	
Cd	0.010µM	/	/	0.045µM
Hg	0.3µM	/	/	0.005µM
Pb	0.03µM	/	/	0.005µM
Sb	10µM	/	/	/
As(V)	/	/	0.4µM	0.135µM
A(III)	/	/	0.2µM	0.135µM
Zn	1µM	/	/	nc
Ni	10µM	/	/	0.34µM
Co	10µM	/	/	nc
Cr(VI)	30µM	/	/	1µM
Cr(III)	30µM	/	/	1µM
Ag	/	0.3µM	/	/
Cu	/	3µM	/	30µM

Table 2. Detection Limits (DL) of the genetic constructions based on heavy metals resistance mechanisms of *E. coli*. *: Values obtained from INERIS (Institut National de l'Environnement Industriel et des Risques); /: unavailable data; nc: metal not concerned by a standard.

2.4 Bioluminescent bacterial biosensors

A biosensor is an integrated and autonomous device able to provide a quantitative or semi-quantitative analytical information by using a biological element of recognition (the bioelement) retained to transducer (Figure 4) (Thévenot, 2001). The biosensors are distinguished from bioassay that are disposable kits. The microbial biosensors can be classified according to whether the bioelement is in liquid phase or immobilised phase. The liquid phase biosensors remains on the principle of reactor cultures in batch or continuous modes.

In the reactors culture, sets of probes ensure the traditional control of the temperature, oxygenation and the pH. In many systems, measurements of bioluminescence and sometimes optical density are done in a second reactor for the pollutant measurement. Thus, if the sample injected is toxic, the bacteria in culture are not affected and the analyses can continue (Gu et al., 1999; Gu et al., 2002). Only one apparatus allows the simultaneous measurement of the bioluminescence and optical density in the same reactor (Thouand et al., 2003; Horry et al., 2004; Picart, 2004).

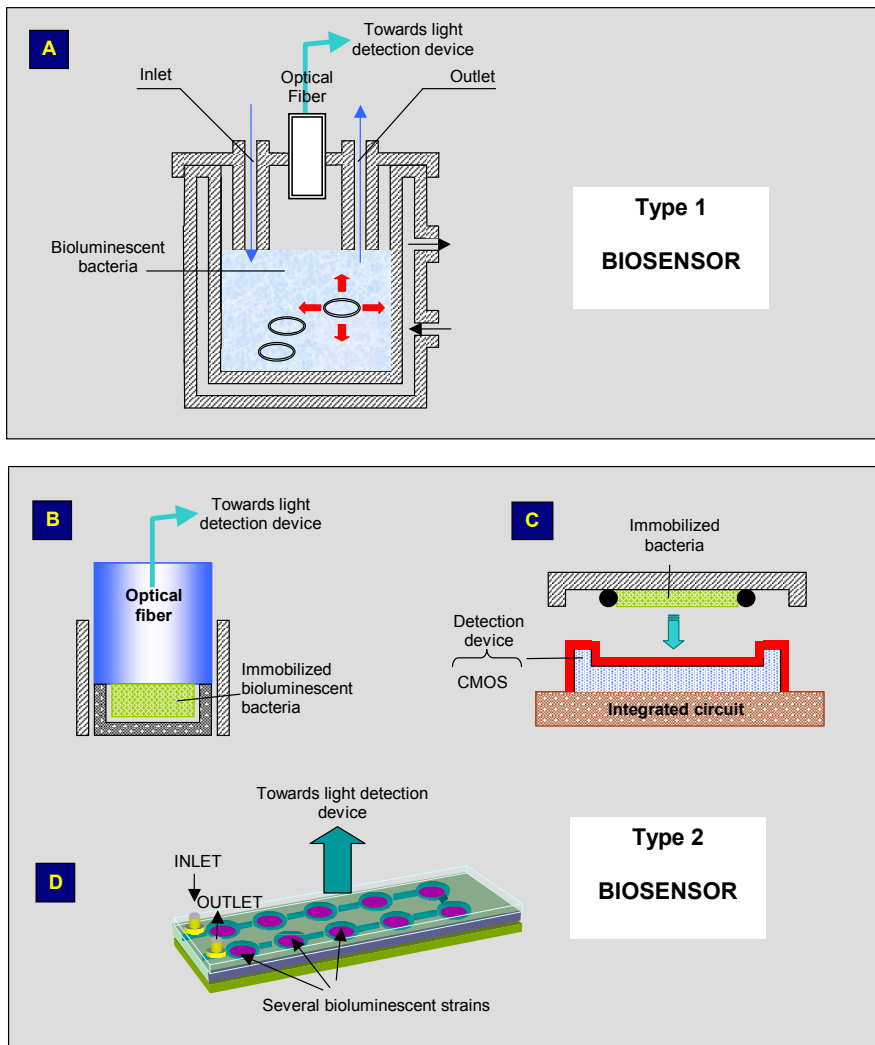


Fig. 4. Figures showing the two kinds of biosensors (type 1 and 2). CMOS = complementary metal oxide semiconductor

These biosensors often can use only one bioelement at the same time that limits the number of analyses, excepted a multi-reactors platform developed by (Lee & Gu, 2005). Another drawback of these systems is the dilution of the analysed sample after injection into the culture volume inside the reactor.

Contrary to the biosensors in liquid phase, the biosensors in immobilised phase allow a direct contact between the micro-organisms, the transducer but also with the sample. The photomultipliers, associated with a beam of fiberoptics are mainly used as physical transducer (Polyak et al., 2001; Horry et al., 2006) but also the CCD camera (Charrier, Thesis, 2006) or the CMOS (Complementary Metal Oxide Semi-conductor (Simpson et al., 1998).

Few bioluminescent bacterial biosensors multichannel match with the strict definition provided by the IUPAC were developed excepted for example Charrier (Thesis, 2006) and the systems approaching the biosensor but of single use, therefore being able to be regarded as bioassay (Kamidate et al., 2001; Philp et al., 2003; Tani et al., 2004; Lee & Gu, 2005; Maehana et al., 2006; Rabner et al., 2006).

3. Experimental procedures

3.1 Molecular biology

V.fischeri luxCDABE operon was amplified from plasmid pRL1114 (*luxCDABE V.fischeri*, Kn^R) (Fernandez-Pinas & Wolk, 1994) with Dynazime polymerase (Finzyme) and the following selected primers: 5'ACG TGA ATT CAT GAA TAA ATG TAT TCC AAT GA3', 5'ACG TCT GCA GTT AAT CCT TTA TAT TCT TTT GTA TG3'. The PCR fragment was purified (QIAquick), digested by NruI and EcoRI restriction enzyme (NEB) and introduced (T4 DNA ligase, NEB) in pBtac2 (Roche) vector under the control of the *ptac* promoter to obtain the plasmid pBfiluxCDABE. This plasmid transformed in *E.coli* DH1 allowed a constitutive expression of bioluminescence.

The *pzntA* promoter was amplified on a boiled preparation of *E.coli* DH1 by PCR using Deep Vent polymerase (NEB) and the following primers : 5'CAC TTC CTG ATC GTC CGC TCG CTG CT 3', 5'AGC ATG AAT TCG GCA TCC TCC GGT TAA GTT T3'. Plasmid pBfiluxCDABE was digested by NruI and EcoRI, purified and cloned into the linearized vector to obtain plasmid pBzntlux (Ap^R, *pzntA* :: *luxCDABE*).

3.2 Bacterial strains and media

E.coli DH1 strain (C.I.P. 104745, [F-, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44 relA1*]) was used for all the experimentations of this study.

LB medium supplemented with ampicillin (100µg.mL⁻¹, Sigma) or kanamycin (20µg.mL⁻¹, Sigma) was used for molecular biology. Solid LB was supplemented with 15g.L⁻¹ of bacteriologic agar (Biokar Diagnostics).

An acetate medium with a C/N/P ratio of 100/10/1 was used for the growth of *E.coli* DH1 pBzntlux for biosensor applications. 1L of tap water was filtered through a 0.45 µm filter (Sartorius) and supplemented with 2.835g acetate (panreac), 1.1919 g NH₄Cl (Merck), 0.028 g K₂HPO₄ (Merck), 5 g NaCl, 0.5 g yeast extract (Merck) and 0.1 g tryptone (Biokar Diagnostics). The pH was adjusted to 7 and the medium was sterilized by autoclaving at 100°C for 30 min. For biosensors application, the feeding media was acetate medium diluted ten times (1/10X) supplemented with ampicillin (final concentration 50µg.mL⁻¹), in order to limit cell division in agarose matrix.

Batch cultures were prepared at 30°C and shaken at 250rpm in 100ml Erlen flasks. Bacterial growth was monitored using a spectrophotometer (Unicam) and optical density measurement was reached at 620nm (OD_{620nm}).

3.3 Cell immobilization procedure

Agarose hydrogel matrix was used for cells immobilization in the biosensor chip according to previous published results (Pernetti, 2003; Horry et al., 2006).

In a first step, *E.coli* DH1 pBzntlux was cultivated 14 hours in acetate medium at 30°C. The next morning, OD_{620nm} of the culture was measured and cells were diluted with fresh

medium to $OD_{620nm}=0.2$. At the same time a 4% agarose solution was prepared by dissolving at 90°C, 0.4g agarose LGT (Agarose Type VII-A Low Gelling Temperature of 26°C; Sigma, reference A0701) in 10mL of acetate medium on a warming magnetic stirrer. 5mL of this solution was preserved at 37°C in a water bath with a constant agitation. Then 5mL of bacterial solution diluted to $OD_{620nm}=0.2$ then added to the 5mL 4% agarose solution preserved at 37°C. After homogenisation, the final solution obtained was at an $OD_{620nm}=0.1$ for a final agarose concentration of 2%. In a final step, this solution was pulled in the Lumisens2 biosensor chip (Horry et al., 2006). Agarose immobilized cells were used immediately in Lumisens2 biosensor for samples analysis without any conservation time.

3.4 Chemicals

Metals used were: $CdCl_2$ (Panreac), $HgCl_2$ (Prolabo France), $PbCH_3CH_2O$ (Panreac), $SnCl_2$ (Merck), $ZnCl_2$ (Merck), $NiSO_4$ (Labosi), $CoCl_2$ (Labosi), $K_2Cr_2O_7$ (Labosi), $CrCl_3$ (Acros), $AgNO_3$ (Prolabo France), $CuSO_4$ (Labosi), $FeSO_4$ (Labosi), $MnCl_2$ (Panreac) and As_2O_3 (Sigma). The purity of the metals was greater than 95%. Metal stock solutions were prepared at a high concentration (1M or 0.5M) in acidified distilled water ($pH\approx 5-6$) and conserved at +4°C in brown bottles. Working dilutions of metals were made daily in distilled water acidified to $pH\approx 5-6$.

3.5 Bioassay

Measurements of bioluminescence were made at controlled temperatures (30°C or 37°C) using a microtiter plate luminometer (Microlumate L96V, EGG Berthold). Heavy metal detection with *E.coli* DH1 pBzntlux, after an overnight culture at 30°C, bacteria were diluted to an $OD_{620nm}=0.05$ in fresh media. One hundred microliters of the dilution were added to a 96-well microtiter plate. Bacterial bioluminescence was induced after the addition of 25µL of a 5X metal solution.

3.6 Environmental samples treatment and analysis

Environmental water samples were all coming from the city waste water treatment plant of La Roche sur Yon (Moulin Grimaud, capacity of 83000 population equivalent). The samplings were carried out at the exit of the station, before the effluent rejection in the Yon river.

In laboratory, samples were first filtered on 0.45µm membrane (Pall Corporation) and preserved at 4°C during one or two days. Filtration was necessary to withdraw the largest particles which would clog the fluidic biosensors system.

Before their injection in the biosensor, samples were all supplemented with acetate media at 1/10X final concentration and, for cadmium detection control, with 0.5µM of $CdCl_2$.

DOC (Dissolved Organic Carbon) was measured in environmental samples with a TOCmeter : (COT-5000A Shimadzu). Total sulphate was measured with Hach Lange kit Sulfavér and total phosphate with the Briggs method.

3.7 Description and operation of Lumisens2 biosensor

Lumisens 2 (Horry et al, 2006) features three distinct parts: the fluidic layout, the disposable card and the optoelectronic equipment (Figure 5). The latter is composed of the power supply, the temperature regulation and the bioluminescence measurement device. The temperature, measured with a J thermocouple (TC Online), is regulated via a threshold

value given by the controller (Chauvin-Arnoult), connected to a heater (Radiospares), inserted close to the disposable card. The bioluminescence is monitored with a photon-counting photomultiplier (Hamamatsu) connected to the disposable card with a fiber optic (Oriel). The light measurements are automatically recorded with a dedicated LabView® interface throughout all of the experiments.

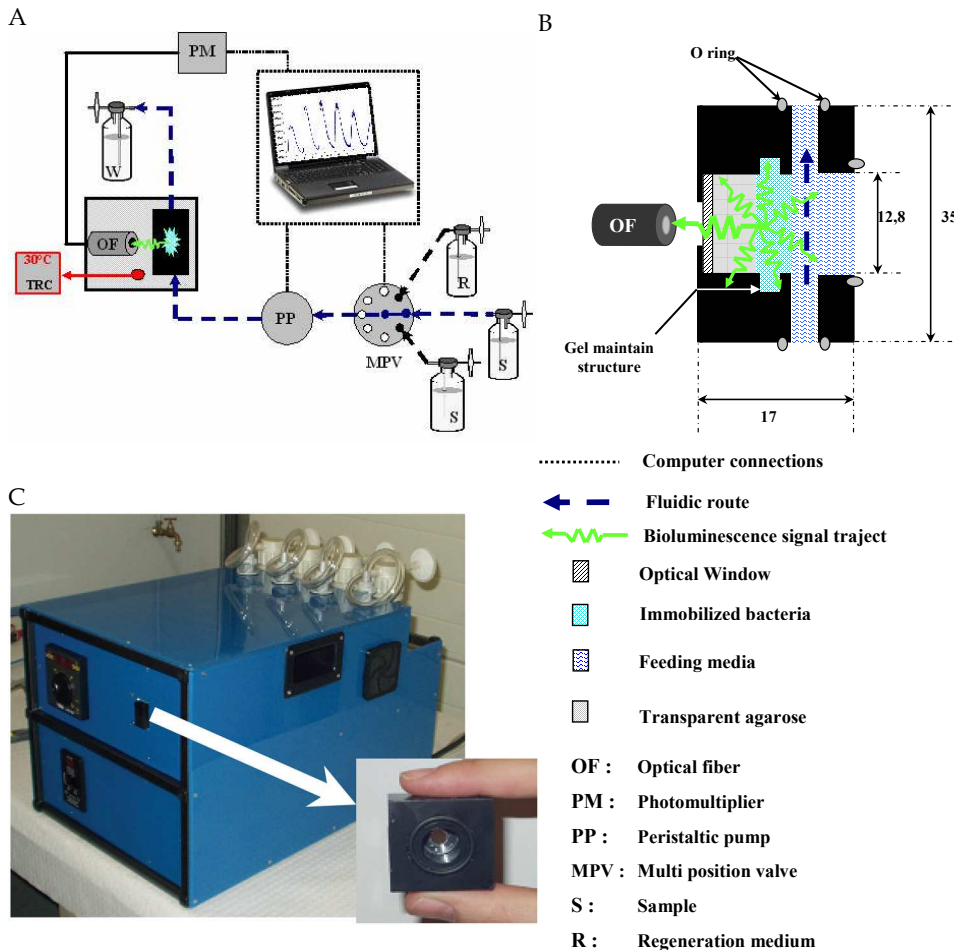


Fig. 5. Diagrams of Lumisens2 biosensor (A), its measurement chip (B) and Photo (C) modified according to Horry et al. (2006). (dimensions are in millimetres)

The bacteria are immobilized in a 35 × 35 × 17 mm disposable parallelepipedic card designed in Ertacetal® plastic. The card is bored through the center to let the fluids come in contact with the bacteria. A 13 mm diameter enclosure has been placed at the depth in which the bacteria are immobilized and includes a measuring chamber of 1.32 cm³. An optical window (Edmund Optics) is hermetically stuck to the bottom of the measuring chamber on which the membrane of the bacteria is coated and maintained because of a 1 mm groove.

Inserted into its fixed pad, the disposable card closes the hydraulic layout, and the immobilized bacteria are in contact with the fluid diffusing into the immobilization agarose matrix.

Three independent samples are linked to a remote controlled 10 channel multiposition valve (Valco AG) that allows their specific selection. Each channel can be periodically selected throughout the experiments with a Windows® Hyperterminal® protocol, allowing the design of sequences of liquid exposures. The liquids are drawn out of the disposable card with a peristaltic pump (Meredos, Germany) located between two anti-return valves to avoid the reflux of the liquids into the card.

Measurement cycles were programmed with a soft specially dedicated to Lumisens2. Experimentations were all realised with a flow rate of 5mL.min⁻¹. Induction medium was first injected in the biosensor during 100 minutes to induce bioluminescence. Then the biosensor switched automatically to the regeneration medium during 140 minutes to let the bioluminescence signal back to ground. Bioluminescence was measured each six seconds during all the experimentation and results were directly shown on the computer screen.

Constant oxygenation of the feeding medium was realised by a continuous bubbling of sterile filtered air (0.22µM filters, Sartorius) among the measurement cell.

4. Results

The aim of this study was to demonstrate the applicability of a bioluminescent bacteria for the on line cadmium detection in waste water treatment plant. The strain *E.coli* DH1 pBzntlux was immobilized in an agarose matrix and then used in the Lumisens 2 biosensor once described by Horry et al. (2006) for TBT detection. In our present study, every parameters describing the biosensor were carefully calculated before to applied the biosensor to environmental samples.

4.1 Specificity and detection limits of *E.coli* DH1 pBzntlux

The range of detected metals and their associated detection limits are listed in Table 3. Of the fourteen heavy metals used, eight were detected by *E.coli* DH1 pBzntlux. Detection limits for cadmium and mercury were under or equivalent to the recommended E.U. standards for water. Other heavy metals like lead, zinc, tin, cobalt, nickel and chromium (VI) were detected at levels above their E.U. limits. Chromium(III), silver, copper, iron, manganese and arsenic were not detected by *E.coli* DH1 pBzntlux. *E.coli* DH1 pBzntlux detects a large number of heavy metals and as a consequence it cannot be used as a specific sensor, rather it should still be considered as a general warning system for heavy metal water pollution.

4.2 Flow rate effect on the bioluminescence measurement

A first study was undertaken in order to evaluate the effect of various flow rates on the bacterial bioluminescence rate (Figure 6). The light production rates were comparable whatever the flow tested according to a statistical analysis (Test of Levene, $\alpha = 0,01$).

The fluids circulation rates inside the Lumisens2 biosensor thus do not influence significantly the detection efficiency by the immobilized bacteria in the range of the flows tested. However one can note a lower variability for 5mL.min⁻¹, that was selected in all our following experiments.

Metal tested	Specificity	Detection Limit	EU Pollution Standards*
CdCl ₂	+	0.005μM	0.045μM
HgCl ₂	+	0.005μM	0.005μM
PbCH ₃ CH ₂ O	+	5μM	0.005μM
SnCl ₂	+	5μM	?
ZnCl ₂	+	5μM	NC
NiSO ₄	+	50μM	0.34μM
CoCl ₂	+	50μM	NC
K ₂ Cr ₂ O ₇	+	50μM	1μM
CrCl ₃	-	-	1μM
AgNo ₃	-	-	?
CuSO ₄	-	-	30μM
FeSO ₄	-	-	NC
MnCl ₂	-	-	?
As ₂ O ₃	-	-	0.135μM

Table 3. Specificity and detection limits of *E.coli* DH1 pBzntlux in bioassay.

+ = Heavy metal detected, - = Heavy metal not detected, NC = Not concerned by legislation

* Obtained from INERIS (Institut National de l'Environnement Industriel et des Risques)

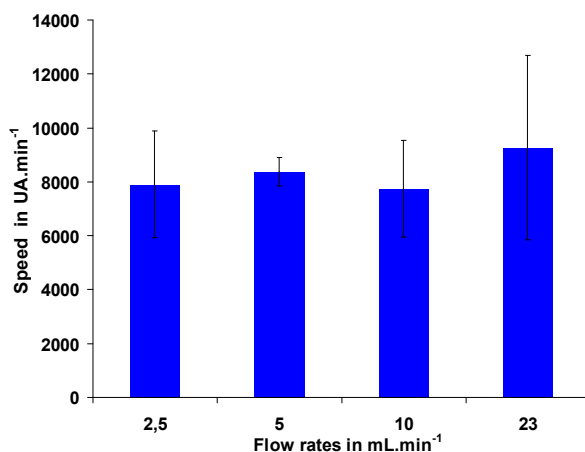


Fig. 6. Variation speed of the luminous intensity of *E.coli* DH1 pBzntlux according to the flow rate. (N=3 for each tested flow rate).

Experimental conditions: *E.coli* DH1 pBzntlux was immobilized in agarose 2% (OD_{620nm}≈0,1). Tested flow rates were 2.5mL.min⁻¹, 5 mL.min⁻¹, 10 mL.min⁻¹ and 23 mL.min⁻¹ at 30°C with acetate medium 1/10X supplemented with ampicilline (50μg.mL⁻¹) and 0.5μM CdCl₂

4.3 Reproducibility of the measurement

The phases of induction and regeneration have a low signal variability (lower than 10%). The maximum of variation (17.1%) of the signal is observed at the peak of bioluminescence (Figure 7).

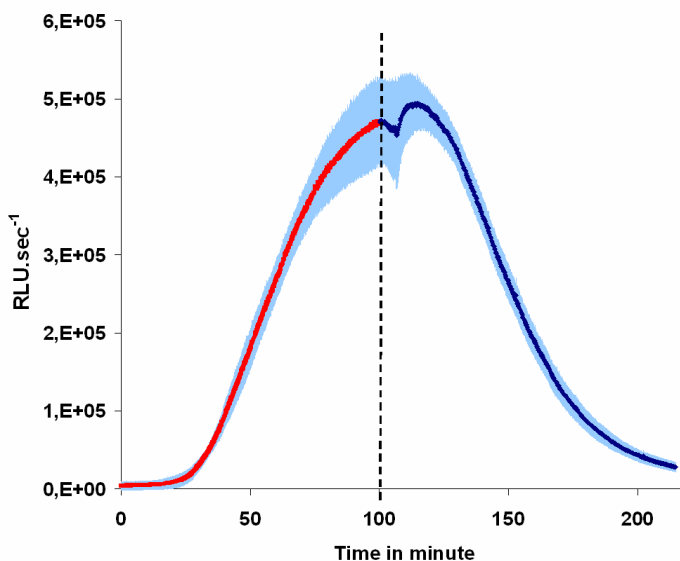


Fig. 7. Reproducibility of cadmium measurement by Lumisens 2 biosensor using *E.coli* DH1 pBzntlux. Results are the average of 5 independent experiments.

Experimental conditions : *E.coli* DH1 pBzntlux was immobilized in agarose 2% ($OD_{620nm} \approx 0,1$). Used flow rate was $5\text{mL}\cdot\text{min}^{-1}$. Induction medium (—) containing acetate medium 1/10X supplemented with $50\mu\text{g}\cdot\text{mL}^{-1}$ ampicilline and $0.5\mu\text{M}$ CdCl_2 was first injected during 100 minutes. Then regeneration medium (—) containing acetate medium 1/10X supplemented with $50\mu\text{g}\cdot\text{mL}^{-1}$ ampicilline was injected. Skye blue bands represent standard deviation (—).

One can note a significant reproducible light decrease signal with the switch from induction to regeneration medium linked to a variation of oxygen in the bottle. The total time of the measurement cycle is 220 minutes, including 100 minutes of induction with the medium of test and then 120 minutes of regeneration. It should be noted that the time of arrival to the peak of bioluminescence is lengthened compared to the bioassay (45 minutes in liquid phase against 60 minutes in immobilized phase)

4.4 Repeatability of the measurement

In an application point of view, it is necessary to know the repeatability of the measurement for one single agarose membrane. The cycle of measurement 100 minutes of induction then 120 minutes of regeneration was thus repeated on the same membrane until extinction of the bioluminescence signal (Figure 8).

The intensity of the detection peaks decreased in an important way during time. However, 5 analyzes producing a significant peak of bioluminescence were monitored in less than 20 H. This signal decrease affecting the repeatability could be attributed to:

- parameters related to the environment of the bacteria:
 - Toxicity of cadmium by accumulation in the membrane
 - Availability of oxygen in the measuring chamber

- parameters related to the bacteria:
 - Growth of the bacteria in the membrane
 - Lack of aldehyde for the bioluminescence reaction
 - Adaptation of the microorganisms to cadmium

These parameters were successively studied during various experiments in order to quantify their role in the signal.

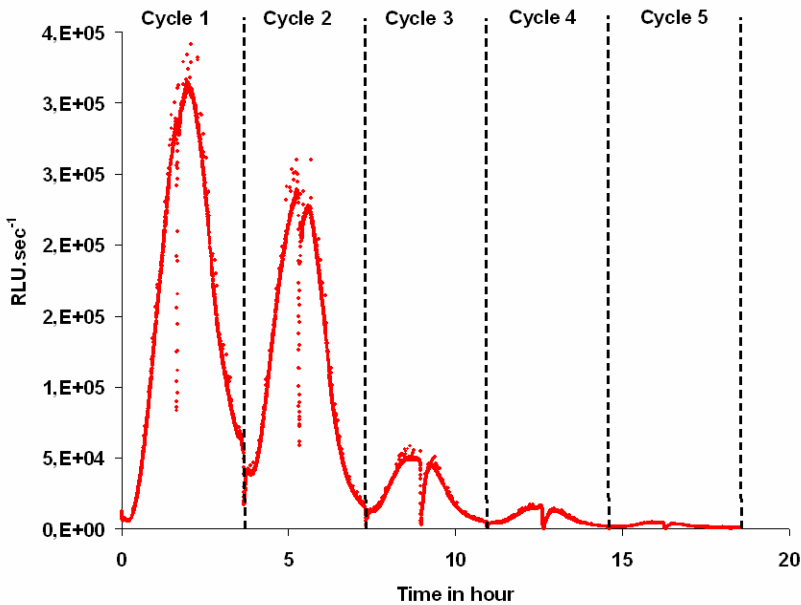


Fig. 8. Cadmium measurement repeatability. Experimental conditions were as described in Fig 7.

4.5 Influence of several parameters on the repeatability

a. Cadmium concentration monitoring inside the membrane

The accumulation of cadmium inside the membrane could cause an increasing toxicity for the bacteria. The cadmium concentration was measured by ICP-MS (Inductively Coupled Plasma - Mass Spectrometry) at different time in the membrane (without bacterium) having undergoes several cycle- metal addition/washing (Figure 9).

The agarose membrane contains an initial quantity of residual cadmium (51.6 nM), ten times higher than the detection limit of *E.coli* DH1 pBzntlux (5nM). This can partly explain the flush of bioluminescence at the beginning of the metal addition.

As expected, the assay medium contains a cadmium concentration close to 500 nM (470 nM) that was never reached inside the membrane (305.1 nM and 356.7nM instead of 470 nM). Moreover, after regenerations, the metal concentration does not go down to the initial Cd concentration (51.6nM) but remains at concentrations from 83 nM to149 nM (Figure 9).

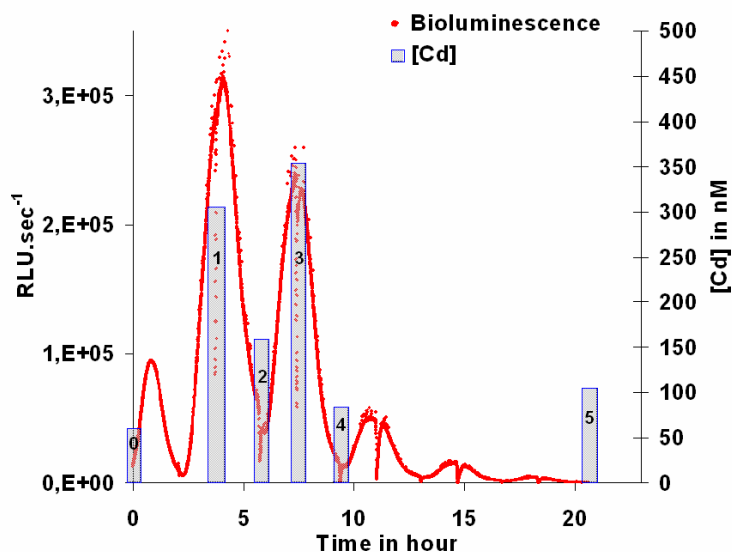


Fig. 9. Concentrations in cadmium in membranes without bacteria during several cycles of measure. (The bioluminescence curve of bioluminescence, with the initial peak is presented only for indication).

Experimental conditions: Cadmium titrations were realized on membranes without bacteria by ICPMS in the departmental laboratory of analysis of Vendée (LDA85). For each measure, the membrane to be analyzed underwent the deliberate number of cycles and was then taken off for analysis.

Hence, cadmium was not accumulated during all the cycles, but a fraction remained blocked in the agarose, while this material is not considered as an adsorbant according to Perneti et al. (2004).

b. Oxygen effect

Oxygen is necessary both for the catabolism of the bacteria and the reaction of bioluminescence. The oxygen brought to the bacteria comes only from the assay and the regeneration media and dropped during the biosensor measurement (Figure 10).

During the first induction, the oxygen concentration at the biosensor output decreased significantly (from 5.8 to 5.2mg.L⁻¹) whereas it remained stable during the following regeneration (5.4mg. L⁻¹). The oxygen fall was confirmed with the following cycle and can be at the origin of the decrease of bioluminescence.

To address this steady lack of oxygen inside the card, a permanent bubbling device was designed to bring air inside the card. Figure 11 compares the results obtained with and without air bubbling.

These results show a very significant improvement of the living time of the card with a constant air feeding allowing eleven cycles of measurement instead of five without air bubbling. However the bioluminescence signal disappeared after more than forty operating hours. Oxygen even in excess in the measuring cell did not seem to be the only limiting factor of the living time of the membrane.

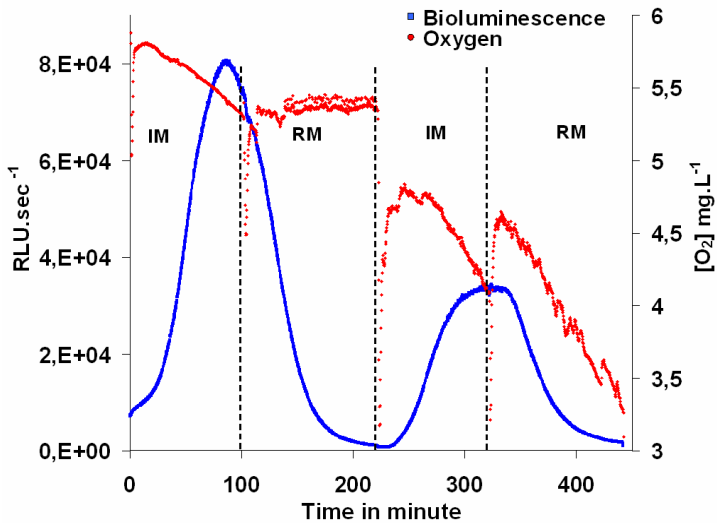


Fig. 10. Evolution of oxygen concentration at the exit of the Lumisens2 biosensor measurement cell.

Experimental conditions were as described in Fig 7. Oxygen measurements were realized by a O_2 probe (SCHOTT 9009/2) connected with an IT interface Reacell 1.11 (Jeulin).

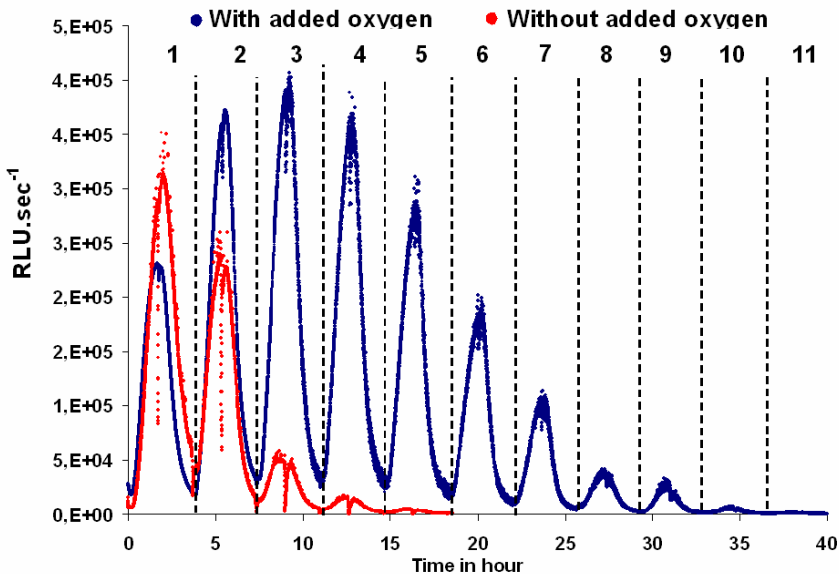


Fig. 11. Repeatability of cadmium detection by Lumisens 2 biosensor and *E.coli* DH1 pBzntlux with and without oxygenation of the feeding medium.

Experimental conditions were as described in Fig 7.

c. Bacterial growth inside the agarose matrix

The carbon provided by the circulating medium (acetate medium 1/10X: DCO=148mg.L⁻¹) is low, but could allow the cellular division. In order to check this hypothesis, the absorbance (OD 620nm) was measured in situ during a whole experiment after each medium substitution (Figure 12).

As supposed the bacteria divided in the membrane and the optical density doubled in 24 hours (from 0.068 to 0.127) with a low growth rate ($\mu=0.081\text{h}^{-1}$). As a consequence the opaqueness of the resulting agarose membrane led to a quenching of photon and caused a fall of the quantity of light collected by the fiber optic beam. Other consequences are possible, like a more important adsorption of cadmium onto the bacteria.

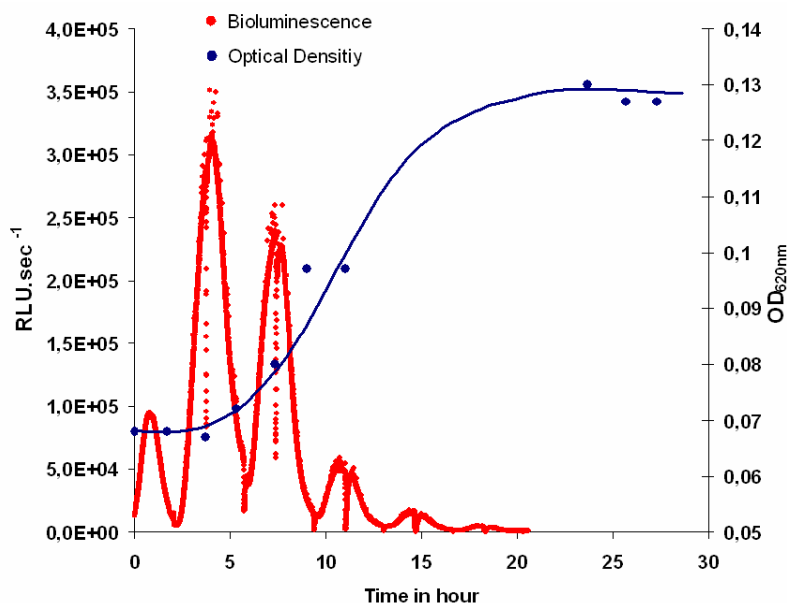


Fig. 12. Evolution of OD_{620nm} in the agarose membrane of bacteria during a repeatability experiment without added oxygen (The bioluminescence curve is presented only for indication).

Experimental conditions were as described in Fig7. OD620nm was monitored at each switch between induction and regeneration medium.

d. Other parameters influencing the repeatability

Three other parameters were tested due to their possible effects on bioluminescence :

- A lack of oxygen already addressed above but increased to avoid any limitation.
- A lack of tetradecanal (resulting from the *luxC,D* et *E* genes activities) involved in the bioluminescence reaction. The tetradecanal is not produced at saturated concentration in the *Vibrio* genus and could be limited in *E.coli* DH1 pBzntlux too (Meighen, 1999).

- An adaptation to the Cadmium concentration. Another fundamental parameter, which can affect the answer of the bacteria during long and repeated exposures to the same cadmium concentration, is their adaptation to the toxic. Indeed, the ZntA/ZntR system that we used for the detection of heavy metals belongs to systems induced naturally to evacuate the cadmium from the cytoplasm of the cell.

These different hypothesis were tested and the results are presented in the Figure 13.

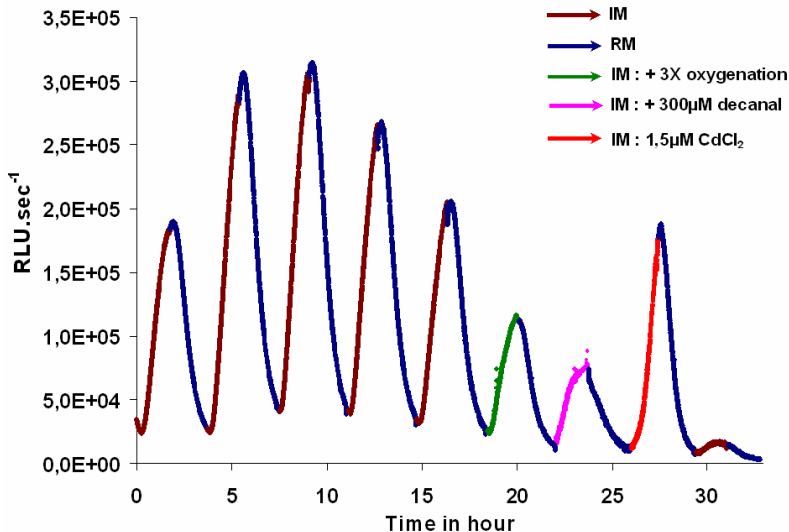


Fig. 13. Parameters influencing the repeatability of the cadmium detection by Lumisens2 biosensor using *E.coli* DH1 pBzntlux. IM: Induction Medium; RM: Regeneration Medium. Experimental conditions were as described in Fig 7 with constant oxygenation of the feeding media.

The induction with more oxygen or the addition of decanal did not improve the signal. On the other hand, an induction carried out with more cadmium (1.5 μM instead of 500 nM) made it possible to obtain the same signal as the first cycles of analysis. This result would consolidate the assumption according to which the bacteria adapt to the metal concentration tested (500nM CdCl_2).

4.6 Detection limit

The period of 15 to 20 hours, where the signal of the bacteria is stable, was used to determine the limit of detection of Lumisens2 for cadmium. For that, 5 cadmium concentrations were successively used on the same membrane and the experiment was repeated three times to determine the limit of detection when metal was added either in distilled water or in the waste water treatment plant of la Roche sur Yon (France) effluent (Figure 14). The detection limit of the biosensor for cadmium was 50 nM with or without environmental matrix. This value was close to the European standard for water (45 nM) but was ten times more important than the limit of detection observed for cadmium in bioassays in liquid or immobilised phase (5nM).

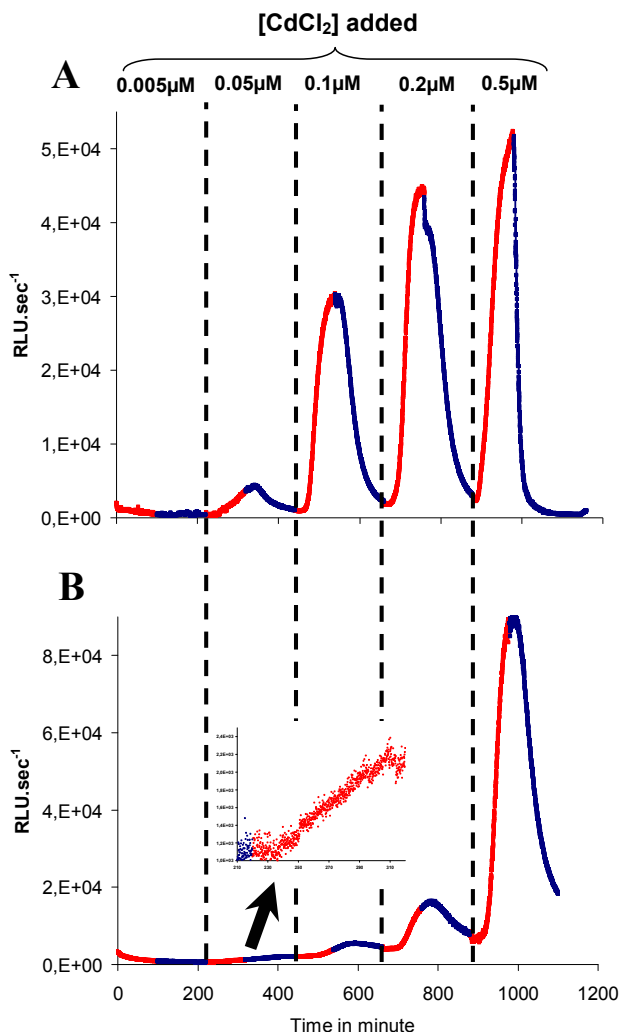


Fig. 14. Detection limit of Lumisens2 biosensor for cadmium detection either in distilled water (A) or in waste water from waste water treatment plant (B) using strain *E.coli* DH1 pBzntlux.

4.7 Application to the detection of cadmium in a waste water treatment plant output.

In a last stage, we wished to associate the biosensor for the detection of cadmium in an effluent from the urban waste water treatment plant (WWTP) of la Roche sur Yon (France). Since technically it was not possible to bring the biosensor on site, the samples of the WWTP were collected twice a day (morning and evening) during 4 days and tested in the laboratory. For each day, a card containing the *E.coli* DH1 pBzntlux bacteria was introduced into the Lumisens 2 biosensor and was used all the day without exceeding 5 cycles of

measurements to avoid the bioluminescence decrease. During these cycles, the two samples were introduced directly to check the presence of Cd in the effluent and after addition of 500 nM of CdCl₂ in order to confirm the response of the bacteria.

The figure 15 shows that the bacteria of the biosensor did not detect metals because of a cadmium-free effluent or a concentration lower than the detection limit (50nM). On the other hand, the bioluminescence was monitored when metal was added in the effluent (for example Day 1) that confirmed the measurement's capacity of the bacteria.

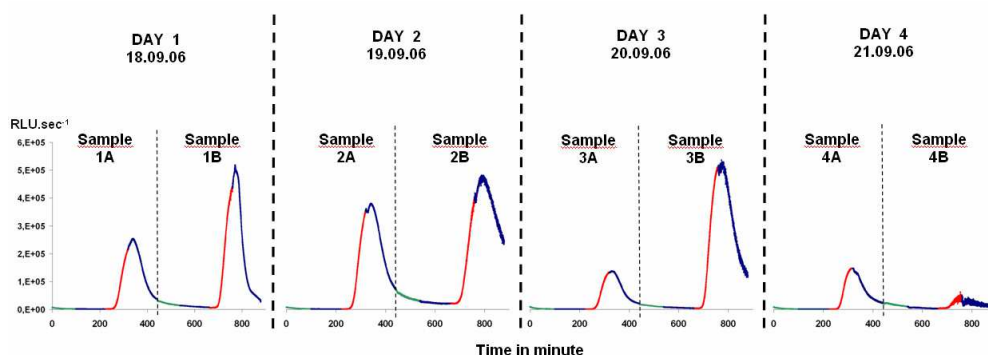


Fig. 15. Detection of heavy metals in waste water treatment plant samples from city of La Roche sur Yon with Lumisens 2 biosensor and *E.coli* DH1 pBzntlux.

It also appears that the produced bioluminescence ranged between 100,000 and 500,000 RLU.s⁻¹ according to the samples which received the same metal concentration. The 4B sample was positive while bioluminescence was weak but remained significant compared to the other samples.

Experimental conditions: Each day two samples of water were taken at the exit of the waste water treatment plant of La Roche sur Yon. For each day, samples were analysed with one chip of Lumisens 2 biosensor. Analysis cycles first start with the injection of the raw sample, in order to determine the presence of heavy metals, during 100 minutes (green curve). In a second step, regeneration medium was introduced to wash the membrane during 120 minutes. In a third step, the same sample, supplemented with 0.5 μM of CdCl₂, was introduced in the biosensor during 100 minutes and in a final step the membrane was washed during 120 minutes. Flow rate was 5 mL.min⁻¹ with constant oxygenation and the temperature was 30°C.

	1A	1B	2A	2B	3A	3B	4A	4B
DOC (mg.L ⁻¹)	9.115	6.839	7.828	8.990	8.468	8.589	7.566	14.020
Sulfate (mg.L ⁻¹)	34	43	74	38	34	60	60	25
Phosphate (mg.L ⁻¹)	0.425	0.625	0.421	0.365	0.563	0.587	0.374	0.447

Table 4. Chemical analysis results of all samples taken from waste water treatment plant of La Roche sur Yon. DOC analyses were realized with TOCmeter (COT-5000A Shimadzu), sulfate analyses with Hach Lange kit and phosphate analysis with the Briggs method.

The average bioluminescence was 312,000 RLU.sec⁻¹ with a variability of 64%, exceeding the variability between membranes (see figure 7). We sought to identify external factors able to influence the signal of bioluminescence such as the dissolved carbon, the sulphates and phosphates, all three chelators of metals (Table 4). No correlation was identified between the fall of the optical signal and a strong content of one of the three measured elements. No explanation could be given and it is finally possible to conclude that measurement is currently of all-or-nothing basis. In other word, if a metal is present at a concentration ranging from the limit of detection and the limit of toxicity, the biosensor is able to measure it without to quantify the concentration by the means of the luminous intensity.

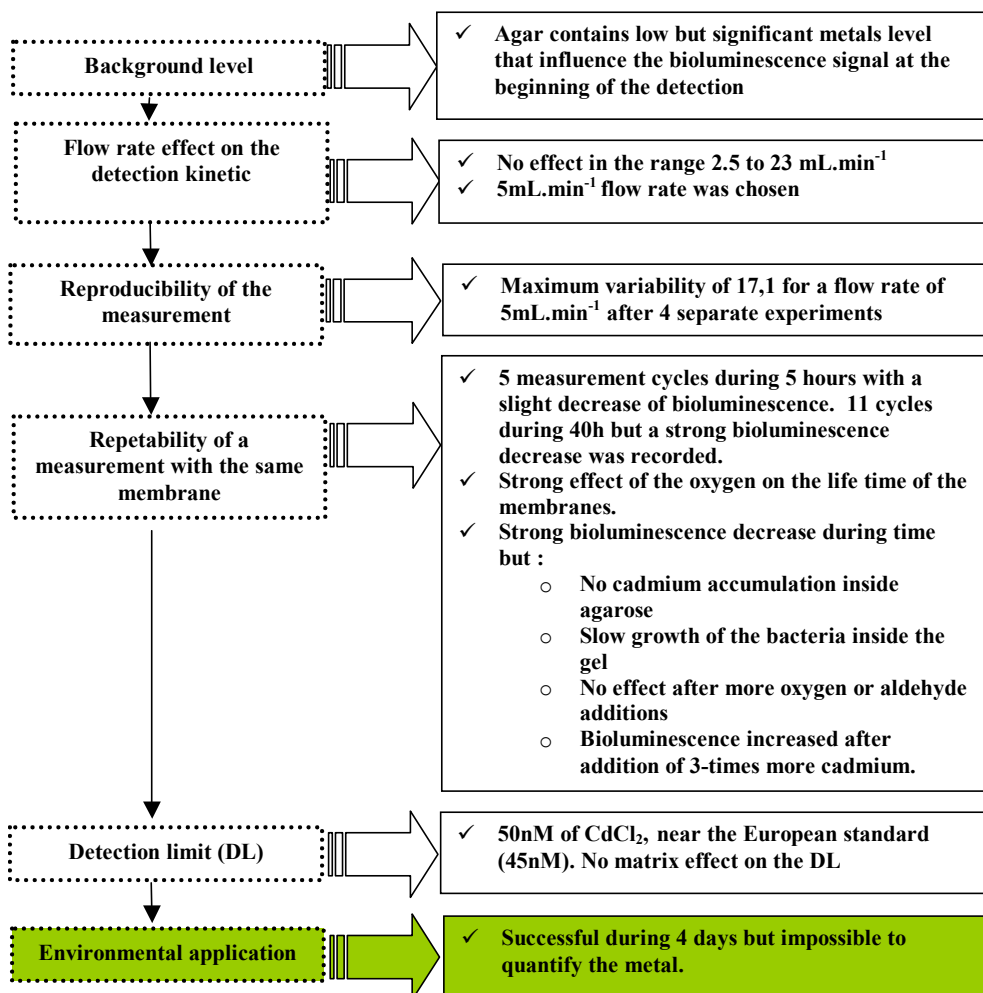


Fig. 16. Summary of the main results obtained in this study with the *E.coli* DH1 pBzntlux immobilized in the Lumisens 2 biosensor.

5. Discussion

The study carried out with Lumisens2 made it possible to better understand and characterise the on line detection of a metal with an immobilised inducible bioluminescent strain: *E.coli* DH1 pBzntlux. The main results are summarised in figure 16.

The reproducibility of the measurement of cadmium at the selected flow rate is acceptable. The lifetime of the same membrane in a continuous mode is forty hours and during this period, eleven analyses are possible. Lee et al., (2004) bring back a life time of 60 hours with their biosensor with only six possible analyses. Heitzer et al., (1994) describe a lifetime of 14 days but they shown only 3 possible analyses in twenty hours. Our results in comparison with the above data, are thus satisfactory. However, as we noted, a progressive fall of the signal of bioluminescence was observed after a prolonged use of the same membrane, that could be attributed to two parameters (combined or not) discussed below.

5.1 Bacterial growth effect

The growth of the bacteria inside the membrane caused a fall of the bioluminescence while acting on three factors: the “quenching” of the light by the bacteria, the increase in the consumption of nutrient and oxygen (Figure 17).

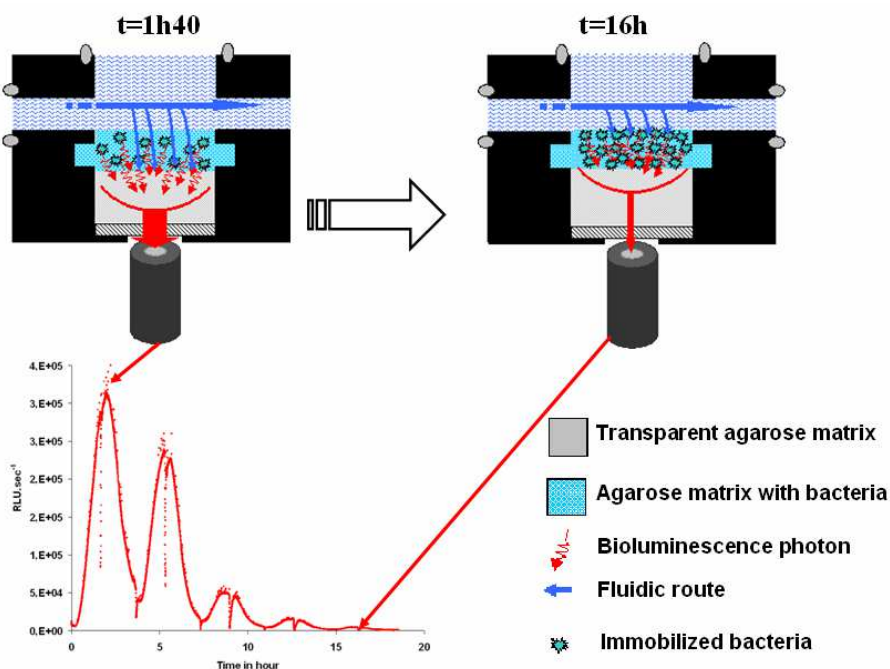


Fig. 17. Possible effects on cadmium detection of the bacterial growth in Lumisens2 Biosensor chip.

At the beginning of the experimentation, the immobilised bacteria are in low number in the membrane. The nutrient, oxygen and metal would be sufficient so that each bacterium emits a strong and detectable bioluminescence. However, after 16 hours of use, the growth of the

bacteria in the membrane of agarose limits the diffusion of the nutrients and metal. Affi et al. (2009) demonstrated that oxygen is the limiting factor since oxygen concentration moving from 100% saturation at the surface to 10% at the bottom off the gel.

The calculation of instantaneous S_0/X_0 ratio (ratio between the mass of carbon, S_0 versus the mass of bacteria, X_0) in the measuring cell is 309 at the beginning of an experiment and 265 at the end of the analysis. Those ratios allow the growth of the bacteria and a solution would be to limit the growth by decreasing the quantity of carbon available but certainly at the expense of the bioluminescence.

5.2 Effect of the adaptation to the metal

The adaptation of the bacteria to cadmium is a believable hypothesis because after addition of higher cadmium concentration (Figure 13), the levels of induction were restored. As shown in the figure 18, the presence of cadmium induces both the bioluminescence production and also the synthesis of the resistance mechanism to heavy metals *ZntA/ZntR* (Binet & Poole, 2000). The repeated exposure to metal would cause an important synthesis of the *ZntA* protein into *E.coli* DH1 pBzntlux cytoplasm. According to our assumption, the quantity of transmembrane ATPases pumps would become sufficient so that the cadmium once entered in the cytoplasm would be immediately evacuated outside the bacteria. This phenomenon would prevent the fixation of metal by the *ZntR* protein and the activation of the synthesis of the *luxCDABE* operon. The strain would thus be adapted to the presence of the same cadmium concentration. Moreover, the resistance system *ZntA/ZntR* is not alone and other systems of efflux or complexation of heavy metals can amplify this phenomenon of adaptation and thus take part in the progressive fall of the signal of bioluminescence (Nies, 1992; Ferianc et al., 1998; Nies, 1999; Spain, 2003; Wang & Crowley, 2005).

6. Conclusions

The Lumisens2 biosensor designed with the *E.coli* DH1 pBzntlux bacteria made it possible to better understand the on line detection of a metal. In spite of the signal bioluminescence decrease attributed to adaptation and growth effect, we showed that lifetime for the same membrane is 40 hours (eleven analyses), that the reproducibility of measurements was satisfactory with a reproducibility of 17% and finally that the limit of detection of cadmium was 50nM. A card can be used with a stable signal during one day and it is possible to monitor the pollution of an effluent of a WWTP during 4 days.

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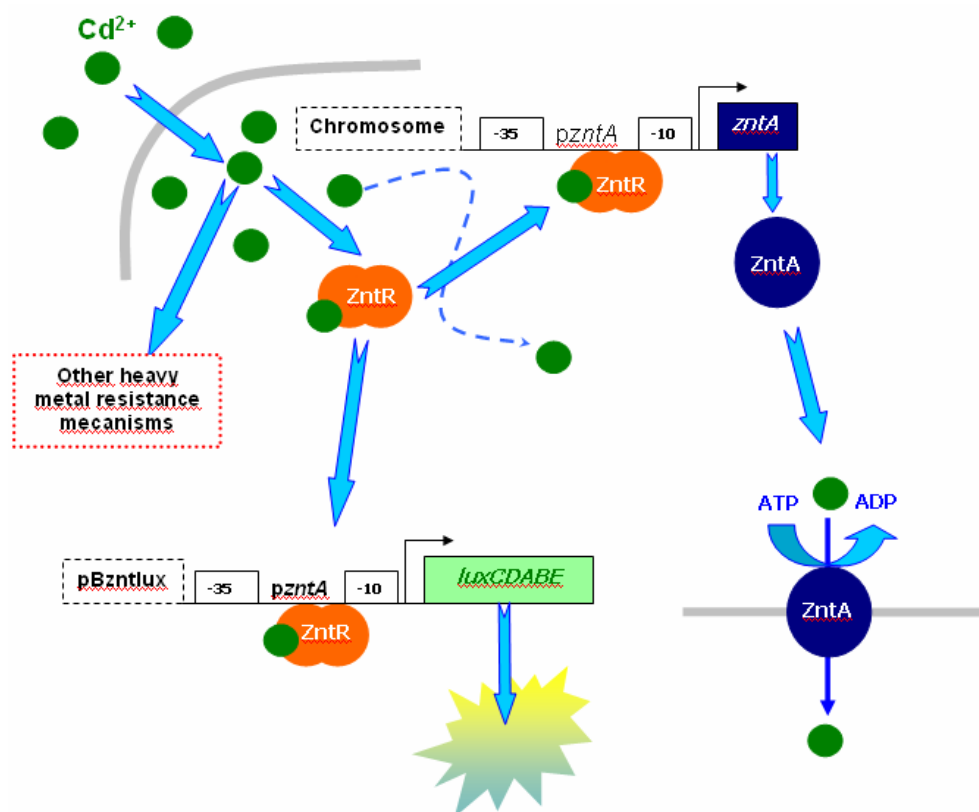


Fig. 18. Synthetic plan of cadmium activated mechanisms in *E.coli* DH1 pBzntlux.

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A biosensor is defined as a detecting device that combines a transducer with a biologically sensitive and selective component. When a specific target molecule interacts with the biological component, a signal is produced, at transducer level, proportional to the concentration of the substance. Therefore biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. Bringing together researchers from 11 different countries, this book covers a wide range of aspects and issues related to biosensor technology, such as biosensor applications in the fields of drug discovery, diagnostics and bacteria detection, optical biosensors, biotelemetry and algorithms applied to biosensing.

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