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SHORT RESEARCH AND DISCUSSION ARTICLE

Design of a toxicity biosensor based on *Aliivibrio fischeri* entrapped in a disposable card

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Abstract The degradation of the marine environment is a subject of concern for the European authorities primarily because of its contamination by hydrocarbons. The traditional methods (ISO 11348 standard) of general toxicity assessment are unsuitable in a context of in situ monitoring, such as seaports or bathing zones. Consequently, to address this issue, bacterial biosensors appear to be pertinent tools. This article presents the design of an innovative bioluminescent biosensor dedicated to in situ toxicity monitoring. This biosensor is based on the entrapment of the wild marine bioluminescent bacterial strain *Aliivibrio fischeri* ATCC® 49387™ in an agarose matrix within a disposable card. A prestudy was needed to select the most biological parameters. In particular, the regenerating medium's composition and the hydrogel concentration needed for the bacterial entrapment (mechanical resistance) were optimized. Based on these data, the ability of the bacterial reporter to assess the sample toxicity was demonstrated using naphthalene as a chemical model. The biosensor's results show a lower sensitivity to naphthalene (EC_{50}=95 mg/L) compared with the results obtained using the reference method (EC_{50}=43 mg/L). With this architecture, the biosensor is an interesting compromise among low maintenance, ease of use, appropriate sensitivity, relatively low cost and the ability to control online toxicity.

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Introduction

Environmental quality has become an increasingly important concern for our modern societies. The direct consequence of this collective awareness has led to a more exigent regulatory framework, notably in the domain of water quality (Decision 2455/2001/EC). In most cases, the parameters of water quality can be assessed using physico-chemical methods. However, toxicity measurements can only be treated using a biological approach.

The estimation of this parameter requires biological markers (Kokkali and van Delft 2014), such as enzymes, cells (prokaryote or eukaryote) or complex organisms. One of the most widespread biological markers is the seawater bioluminescent strain *Aliivibrio fischeri* (formally *Vibrio fischeri* or *Photobacterium phosphoreum*). When it is in contact with a toxic sample, bioluminescence is inhibited. Therefore, it is possible to establish a relationship between the light inhibition rate and the relative toxicity.

According to this simple concept, a standard (ISO 11348 2007) and several commercial bioassays have been developed, such as the Microtox® M500 (Modern Water, UK), LUMIStox® (Hach Lange, Germany), TOXmini® (microLAN, The Netherlands) or ToxAlert 10® (Merck, Germany) (Bulich and Isenberg 1981; Hao et al 1996; Jennings et al 2001) systems. Nevertheless, these bioassays are essentially constrained to laboratory applications and appear to be inappropriate in the context of in situ monitoring, such as in the release quality control in wastewater treatment plants.

To fill this gap, online systems were developed to continuously monitor the effluent toxicity (Kokkali and van Delft

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2014) using systems such as Microtox® CTM (Modern Water, UK) and iTOXcontrol® (microLAN, The Netherlands). The cultivation of the bacteria has been integrated into the system (bioreactor), which allows for the automation of the measurement sequences. The main drawback is the initial knowledge that is required to use these methods and perform the required maintenance.

In this context, we designed an innovative biosensor based on a disposable card with entrapped bioluminescent bacteria. The implemented configuration ensures a simple utilization by novices and significantly limits the maintenance operations (replacement of the disposable card and the tubes). A proof of concept was performed using a naphthalene chemical model.

Experimental

Growth medium and bacterial strain

For the growth medium, 1 L of distilled water was supplemented with 21.1 g of NaCl (Merck, Germany), 0.58 g of KCl (Merck, Germany), 1.2 g of $CaCl_2{\cdot}2H_2O$ (Merck, Germany), 3.6 g of $MgCl_2{\cdot}6H_2O$ (Sigma-Aldrich, USA), 0.08 g $NaHCO_3$ (Merck, Germany), 2.62 g of $MgSO_4{\cdot}7H_2O$ (Sigma-Aldrich, USA), 5 g of tryptone (Biokar Diagnostics, France) and 3 g of yeast extract (Biokar Diagnostics, France). Then, 15 g/L of agar (Biokar Diagnostics, France) was added to the solid medium. The pH was adjusted to 7 using a solution of HCl (Sigma-Aldrich, USA) or NaOH (Merck, Germany) before autoclaving at 120 °C for 20 min.

The microbial cultures of *A. fischeri* ATCC® 49387™ were seeded from pure bacterial colonies. The cultures were agitated for 15 h at 25 °C and under a constant agitation of 250 rpm.

Regenerating media

Two types of media, RM10 and RM100, were tested in this study to determine a compromise between the stability of the bioluminescent signal and the cellular growth in the biosensor (indirectly, the bacterial release potential). The composition of these media was similar to that of the growth medium, except for the addition of tryptone (Biokar Diagnostics, France, 0.5 and 0.05 g/L in RM10 and RM100, respectively) and yeast extract (Biokar Diagnostics, France, 0.3 and 0.03 g/L, respectively).

The pH was adjusted to 7 using a solution of HCl (Sigma-Aldrich, USA) or NaOH (Merck, Germany) before autoclaving at 120 °C for 20 min.

Naphthalene solution

A stock concentrated solution of naphthalene (Sigma-Aldrich, USA) was prepared in the organic solvent dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) at 100 g/L. For the toxicity test, this solution was diluted to 2 g/L in fresh regenerating medium. This step was performed in an ultrasonic bath (Thermo Fisher Scientific, USA, FB15052, frequency 37 kHz) for 2 h. The diluted solutions were store at room temperature and were protected from light for a maximal period of 15 h.

Description of the biosensor

The biosensor was built around a disposable card that was specifically designed for this study and used as an immobilization holder for the bioluminescent cells. From this card, the first design step of the toxicity biosensor was to anticipate the use constraints and propose a suitable human-computer interface (Supplementary data, Fig. SD-1). To do so, three-dimensional modelling and technical plans were created using computer-aided design software (SolidWorks 2010, Dassault Systèmes, France).

This biosensor was composed of three compartments (Fig. 1c). The upper part hosts all of the required connections to the power supply, the temperature controllers (iTron, JUMO, France) and the ports (serial, USB and IEEE) that are necessary for computer control. The intermediate floor is the biosensor's heart. Temperature control is an essential parameter of the bioluminescence biochemical reaction. Consequently, to maintain a stable temperature of 25 °C, two heating modules (Heating foils, RS Components France, France) were integrated in this second level. The preheating module (Fig. 1b), which was installed upstream of the disposable card, was responsible for the thermal regulation of samples. The second heating module, which was positioned under the immobilized bacteria, created a constant temperature in the card (Fig. 1a). A disposable card support was designed to allow quick replacements by the user (Fig. 1a). The card was positioned in front of the light sensor (SPOT Insight™ charge-coupled device (CCD) Camera, 1N18, SPOT Imaging Solutions, USA). This position was adjusted with respect to the focal length of CCD camera to maximize the bioluminescence capture. To minimize measurement interferences due to environmental light sources, the bioluminescence monitoring was carried out in a light-tight room. The lower compartment was the fluidic part of the biosensor. The sample selection was performed with a computer-controlled multi-position solenoid valve (SD valve, Valco Instruments Co. Inc., USA). The sampling and the sample transfer to the disposable card containing the bioluminescent cells were performed using a peristaltic pump (Peristaltic Pump TL, Medorex, Germany). The transfers were performed in Tygon® tubes (R-3603, internal diameter 1.6 mm, external diameter 3.2 mm, wall thickness 0.8 mm, Saint Gobain, France).

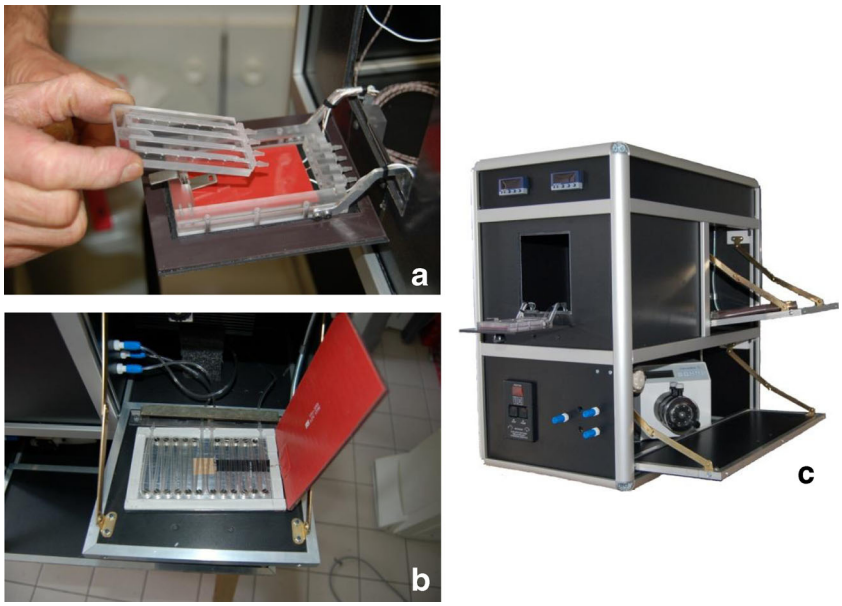

Fig. 1 General view (**c**) of the biosensor and targeted views of the intermediate compartment. **a** Disposable card and its holder. **b** Preheating module

Immobilization procedure

As a first step, a 2 and 4 % (*w*/*v*) agarose solution (low gelling temperature type VII-A, A0701, Sigma-Aldrich, USA) was prepared in fresh regenerating medium and heated using a microwave until dissolved. Subsequently, the agarose solutions were placed in a dry bath at 35 °C for 10–15 min (FALC instruments, Italy). This temperature limits the heat shock of the bacterial cells and prevents the polymerization of the agarose.

At the same time, the bacterial cells were cultivated in the growth medium to an optical density of $OD_{620\ nm}$=1.9 (U-1800, HITACHI, Japan). Subsequently, a 500 μL aliquot of the bacterial suspension was then mixed with 500 μL of the agarose solution at 35 °C and was transferred to the wells on the disposable card (10.6 μL per well) to obtain a final optical density of $OD_{620\ nm}$=0.85 with an agarose concentration of 1 or 2 %. The agarose's polymerization was performed in a temperate room for few minutes before being used in the biosensor.

Implementation of the biosensor

The disposable card containing the entrapped bacteria was positioned in the biosensor inside the light-tight room (Fig. 1a). During the first 15 h, the cells were fed the regenerating medium (RM10 or RM100) at a flow rate of 2.5 mL/min using the peristaltic pump and were maintained at a constant temperature of 25 °C to obtain a cell density suitable for bioluminescence production (phenomenon related to the quorum sensing). This medium allows a continuous supply of nutrients and oxygen and possesses a composition similar to the growth media, except for the tryptone and yeast extract, which were reduced (Cf. "Regenerating media" section).

After this stabilization period, the immobilized cells were then fed a contaminated sample for 1 h to assess its toxicity. This intoxication phase was followed by a regeneration step aimed at restoring the cells with the regenerating medium for 2 h (Cf. "Regenerating media" section). The feeding method was selected using a SD valve controlled by the Vcom V-1.1.01 software (Valco Instruments Co. Inc., USA).

Every 30 min, the SPOT advanced V-4.6 software (SPOT Imaging Solutions, USA), which controls the CCD camera, captured the bioluminescence (integration time of 60 s) as a digital image. These pictures were then converted into numerical signals using the Kodak image analysis software (Scientific Imaging System, Eastman Kodak Company, USA, Version 3.6).

Maintenance

All Tygon® tubes of the biosensor were replaced before the subsequent use. Just before use, a tube decontamination step was performed with an ethanol solution (70 % *v*/*v*, Sigma-Aldrich, USA), followed by a washing step using sterilized distilled water to ensure the sterility and innocuousness of the tubes. The disposable cards were sterilized in an individual bag by autoclave (120 °C, 20 min).

Standard test

Reference tests, which were based on freeze-dried *A. fischeri* ATCC® 49387™ (ATCC, USA), were carried out using the same naphthalene-contaminated samples to be compared with the biosensor's results. These tests were performed according to a standard procedure (ISO 11348-2 2007) using the LUMIStox® device (Hach Lange, USA).

Data analysis

The bioluminescence data were treated and presented graphically using Microsoft Excel 2007 (Microsoft, USA). The statistical analyses required in this work were performed using the XLSTAT statistical analysis software (Addinsoft, France).

Results and discussion

Optimization of biosensor's parameters

A prior study (supplementary data, Fig. SD-2) proposed two candidate media, RM10 and RM100, which allow pertinent bacterial growth characteristics in the framework of the biosensor's development: a high and stable level of bioluminescence (a sufficient nutrient feeding) and low bacterial growth (limitation of the bacterial release, controlled evolution of physiologic state of the cells).

These media and two agarose concentrations (1 and 2 %) were simultaneously tested in the biosensor to assess their impact on the bioluminescence production and the bacterial release (risks of gene transfer or disruption of the ecological balance). The results are presented in Fig. 2. The stabilization of the bioluminescence signal depends on the regenerating medium used. For medium RM10, which contains more nutrients, the stabilization of the bioluminescence level in the biosensor was achieved after an incubation time of 11 h instead of 13 h with the RM100 medium. In fact, the expression of *A. fischeri*'s bioluminescence genes is controlled by a signalling molecule, *N*-(3-oxohexanoyl)-l-homoserine lactone (AHL), that is involved in quorum sensing (Milton 2006; Yan et al 2007). This molecule is naturally released by the strain in the surrounding environment. When the concentration of AHL is sufficient, the bioluminescence genes are no longer repressed, and the cells can express bioluminescence. In other words, when the cell density is insufficient, the bacteria lose their capacity to produce bioluminescence. Consequently, a lag phase is necessary to ensure a viable bioluminescence production in the biosensor.

These results show that the composition of the regenerating medium significantly influences the bioluminescence production of *A. fischeri* (Kruskal-Wallis test, significant difference, p value=0.007). Indeed, RM10 induces a higher signal (120,000 relative light unit (RLU)) than RM100 (35,000 RLU). From these results, the bioluminescence levels are not influenced by the polymer concentration (Kruskal-Wallis test, non-significant difference, p value=0.226).

Concerning the bacterial release, the association of the highest tested concentration of agarose (2 %) with RM100 allows for the minimization of this phenomenon (supplementary data, Fig. SD-3). Despite a greater average release with RM10 than with RM100, the phenomenon is significantly mitigated with the highest polymer concentration.

Consequently, going forward, RM10 was used as regenerating medium in the biosensor, and the bacterial cells were immobilized in a 2 % agarose hydrogel.

Toxicity assessment

The primary aim of this study was to demonstrate the use of a bacterial bioluminescent biosensor in the context of toxicity monitoring. The biosensor described in this paper was designed to perform measurement sequences without external intervention and in in situ conditions. To assess its performance in the framework of the water toxicity monitoring, the results obtained with the biosensor were compared with the results provided by the reference method, which was the ISO 11348-2 standard (Fig. 3).

The calculated EC_{50} values of naphthalene were of 43 and 95 mg/L according to the reference method and the biosensor, respectively. The biosensor is thus less sensitive to naphthalene exposure than the reference method is. This difference may be related to an important variation between the standard protocol and the procedure implemented in the biosensor.

Several parameters may be implicated. First, the ISO 11348-2 reference method implemented in this study is based

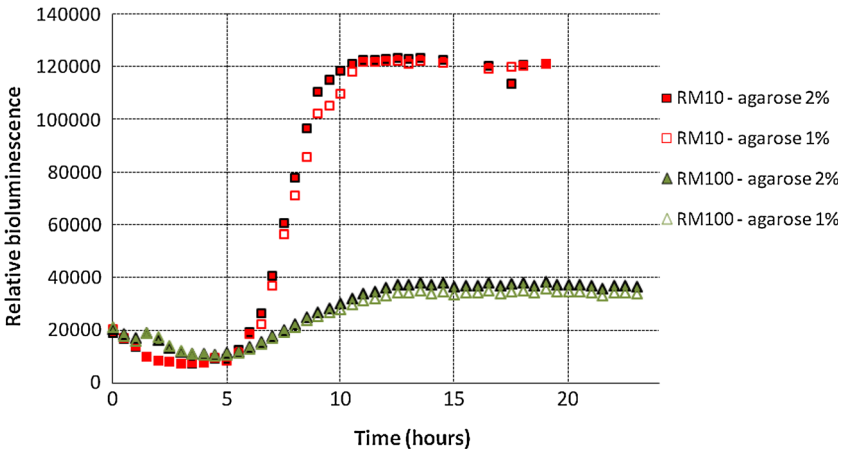

Fig. 2 Effect of the regenerating media and the polymer concentration on bioluminescence production and bacterial release (n=4). The standard deviation is below 5 % for each condition. Experimental conditions: the cells used were freshly prepared and immobilized in a solid agarose matrix. The temperature was set at 25 °C

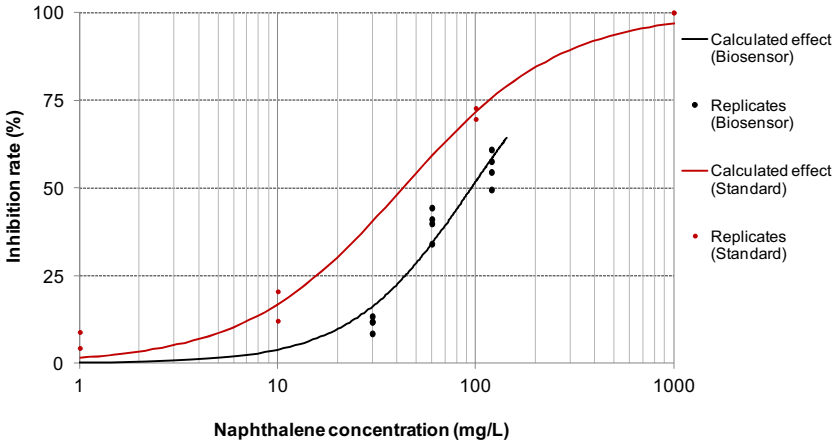

Fig. 3 Dose-response relationship calculated from the data obtained using the standard methods (n=2) or with the biosensor (n=4). The experimental conditions: standard method: the cells used are free (direct contact with the sample) and lyophilized. The setpoint temperature was 15 °C, and the exposure duration was 30 min (Kruskal-Wallis test, nonsignificant difference, p value=0.208); biosensor: the cells used were freshly prepared and immobilized in a solid agarose matrix (diffusion limitation through the matrix). The temperature was set at 25 °C, and the exposure time was 60 min (Kruskal-Wallis test, nonsignificant difference, p value=0.887)

on lyophilized cells, whereas in the biosensor, the toxicity measurement is performed with fresh bacteria. In the context of another study (Jouanneau 2011; Jouanneau et al 2011), a similar phenomenon was observed. After a freeze-drying cycle, the bacterial cells showed an increased sensitivity to the tested metals. Second, the contact mode between the bacteria and the sample differs between the measurement methods. In the reference method, bacteria are free (in the media), which ensures direct contact with the sample. Consequently, the effect of the toxic compounds on the bioluminescent cells present in the analyzed sample is maximized. With the biosensor, the bacteria are entrapped in a solid agarose matrix (polymer network). Affi et al (2009) demonstrated that this configuration tends to limit the diffusion speed through the matrix and decreases the bioavailability of the sample's pollutants for the immobilized cells. Finally, the temperature of the assays was different. The reference method requires an assay temperature of 15 °C, but with the biosensor, the temperature is fixed at 25 °C (the optimal temperature of growth for *A. fischeri*). This value was selected in the biosensor to limit the stabilization period.

Despite a significant sensitivity loss, the primary interest of the toxicity measurement using the biosensor lies in its capacity to perform several successive automatized analytical cycles. After a toxic exposure, the bacteria are regenerated with RM10 medium (to restore them). Therefore, after a regenerating cycle, the bioluminescent bacteria are operational for a new toxicity measurement.

Conclusion

The toxicity is a parameter that has generated increasing interest in the domain of water quality monitoring. Nevertheless, this parameter can only be assessed by using a biological approach. The bioassay based on the bioluminescence inhibition of *A. fischeri* is one of the most widespread toxicity tests. However, its online version requires know-how.

The main aim of this study was to propose an easy-to-use biosensor that is able to monitor effluent toxicity. To achieve that goal, several technological choices were implemented. The heart of the biosensor is based on bioluminescent cells, *A. fischeri*, that are entrapped in a solid agarose matrix. The bacteria are immobilized into a disposable card that was designed to ensure contact between the cells and the samples or the regenerating medium. After use, the bacterial card can be easily replaced, which significantly simplifies maintenance.

The first step of this study was to define the most suitable parameters (composition of the regenerating medium and agarose concentration) that ensure a stable and high bioluminescence signal (toxicity measurement) and to limit the growth of bacteria and, consequently, their release to the environment (risk of gene transfer or modification of environmental biotope). In this context, regenerating medium RM10 and an agarose concentration of 2 % were selected. These conditions allow, after a regenerating step, the re-use of the bioluminescent cells for a new analysis, which is unlike the reference method ISO 11348.

The second step compared this new biosensor with a reference method that is based on a similar approach (ISO 11348-2 2007). The results obtained with the biosensor are less sensitive than the reference method. Indeed, the EC_{50} obtained with immobilized cells is approximately equal to twice the reference value. The differences of experimental conditions are likely, as explained in "Toxicity assessment" section, the origin of this phenomenon (contact mode, temperature, exposure time).

Nevertheless, the biosensor was tested on only one chemical, naphthalene, which is not sufficient to demonstrate or

know its real interest in a context of water monitoring. Consequently, the goal of future works will be to implement complementary experiments (others chemicals, real samples) to complete the testing phase.

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