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Semi-autonomous inline water analyzer: design of a common light detector for bacterial, phage, and immunological biosensors

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Abstract The use of biosensors as sensitive and rapid alert systems is a promising perspective to monitor accidental or intentional environmental pollution, but their implementation in the field is limited by the lack of adapted inline water monitoring devices. We describe here the design and initial qualification of an analyzer prototype able to accommodate three types of biosensors based on entirely different methodologies (immunological, whole-cell, and bacteriophage biosensors), but whose responses rely on the emission of light. We developed a custom light detector and a reaction chamber compatible with the specificities of the three systems and resulting in statutory detection limits. The water analyzer prototype

resulting from the COMBITOX project can be situated at level 4 on the Technology Readiness Level (TRL) scale and this technical advance paves the way to the use of biosensors on-site.

Keywords Bioluminescence · Chemiluminescence · On-site water analyzer · Biosensors · Bacterial pathogens · Metals · Toxins

Introduction

Environmental protection requires efficient and rapid methods to detect accidental or intentional spill of toxic compounds in waters as well as pathogenic bacterial contamination. Analytical methods such as mass spectrometry commonly used to detect organic or metallic compounds are very sensitive but usually performed in specialized laboratories, not in real time on field. Similarly, current detection methods of living pathogenic bacteria in environmental samples require standard clinical cultures, incompatible with rapid, on-site measurements (Rompré et al. 2002). Biosensors integrated into inline water monitoring devices represent promising and complementary alternatives that could offer sensitive and rapid detection on-site used to quickly raise the alarm (Michelini et al. 2013). Significant advances have been reported in the design of whole cell-based biosensors used to detect heavy metals with genetically modified bacterial cells; these improvements concern higher sensitivity and selectivity (Daunert et al. 2000; Cayron et al. 2015 in this Special Issue), robustness and rapidity using cells in a dormant state until use (Camanzi et al. 2011; Prévéral et al. 2016 in this Special Issue) or adaptation to the analysis of samples in complex matrices using optimized bioluminescent reporters

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(Winson et al. 1998; Brutesco et al. 2016 in this Special Issue). However, progresses are still required to translate such biosensing technologies from laboratory bench tops to technological devices offering easy-to-use, cost-effective on-site detection. To date, various technical implementations have been proposed to embed laboratory biosensors into actual monitoring of water networks. For instance, the Lumisens 1 system by Horry et al. (2004) is based on a bioreactor maintain living bioluminescent *Escherichia coli* cells to detect organotin compounds; this cumbersome solution was later simplified into a disposable card with immobilized cells in the Luminens 2 system (Horry et al. 2007). Elad et al. described the immobilization of bacterial biosensors into polymeric biochips placed in flow-through chambers; the authors validated the simultaneous detection of various pollutants (paraquat, arsenite, nalidixic acid) under continuous water flow for several days (Elad et al. 2011). Miniaturization of integrated sensors is a promising route to develop robust, reliable and cheap monitoring devices; as an example, Moll et al. associated microfluidics and love-wave acoustic detection to design an immunological biosensor aiming at detecting online pathogenic bacteria (Moll et al. 2007), Patent FR2878622).

In this context, the main objective of the COMBITOX project funded by the French Agence Nationale de la Recherche (ANR) lies in the integration of three different types of luminescent biosensors into a semi-autonomous inline water analyzer prototype. The Editorial to this Special Issue provides a synthetic description of the aims and reaches of COMBITOX (Ansaldi et al. 2015). The collection of articles accepted for publication in the COMBITOX Special Issue of *Environmental Science and Pollution Research* summarizes the design of bacterial biosensors to detect toxic metals such as nickel, arsenite, or mercury (Cayron et al. 2015; Prévéral et al. 2016; Brutesco et al. 2016), a bacteriophage-based biosensor to detect enteric pathogenic bacteria (Franche et al. 2016) and an immunological biosensor to detect microcystin-LR (Demey et al. 2015; Bazin et al. 2016); the three of them rely on the emission of light in a close range of emission wavelengths, either through bioluminescence for the bacterial and bacteriophage biosensors with the bacterial luciferase (Baldwin et al. 1995) or chemiluminescence for the immunological biosensors with the horseradish peroxidase (Ryan et al. 1994). Because the light signal is emitted in a similar range of wavelengths (*c.a.* 490 and 430 nm for bioluminescence and chemiluminescence, respectively), we selected a single sensor to detect the light signals. The challenge here rather lies in the design and the optimization of a common reaction cell to accommodate the various biological reactions. Indeed, for both the bacteriophage and the bacterial biosensors, the water sample is usually incubated with the bioreagent (i.e., a bacteriophage solution or lyophilized bacteria) while bioluminescence emission kinetics is measured, whereas the immunological biosensor requires multiple

incubation/washing steps to remove unspecific antibody ligands after incubation with the water sample. This Technical Note describes the design of our prototype core unit associating a light detector and a reaction cell together with the operational procedures to bring the three types of biosensors into play. The water analyzer prototype resulting from the COMBITOX project can be situated at level 4 on the Technology Readiness Level (TRL) scale, i.e., “Component and/or breadboard validation in laboratory environment.”

Materials and methods

Principals and operational details about the bacteriophage, bacterial and immunological biosensors cited in this paper can be found elsewhere in this Special Issue (Demey et al. 2015; Prévéral et al. 2016; Bazin et al. 2016; Franche et al. 2016). We selected the substrate-independent, bacteriophage-based biosensor among the three different types we developed to optimize the core unit light detector/reaction chamber of the COMBITOX analyzer prototype because it is the one expected to require the highest sensitivity. Thus, the different light measurements performed here were done on *E. coli* TD2158 live cells after successful lysogenization of the genetically modified HK620 bacteriophage according to the protocol described elsewhere in this Special Issue (Franche et al. 2016).

Prototype design and construction

The French company AP2E (Aix-en-Provence, France) developed electronics, mechanics, automation and realized the COMBITOX prototype assembly. We selected the H1070 photomultiplier (PMT) from Hamamatsu for its robustness and high sensitivity in the 230–700 nm range (datasheet available at <http://www.hamamatsu.com>). For the record, bioluminescence and chemiluminescence reactions result in photons emission with a maximum emission peak at 490 and 430 nm, respectively. The current signal issued by the PMT is pre-amplified in situ and digitally converted by a 16 bits ADC connected to the black box where the biological reactions take place. The PMT is set at its maximum gain and powered by a reference voltage. For light emission measurements, the photosensor surface is placed above the reaction chamber (5 mm distance), provisionally described as a cuvette array containing the water sample to be analyzed.

Sample concentration by filtration

In order to maximize the light collection by the Hamamatsu PMT, the light-emitting objects (bioluminescent bacteria) contained in the incubation mix were concentrated in 2D onto a membrane surface prior to light measurements. To that avail, we used 0.45 µm Millipore® HA nitrocellulose membranes

(25 mm diameter), with a Millipore® XX2702550 Model 1225 Sampling Manifold Vacuum Filtration Cell Harvester. The water sample is concentrated on circular surface S equal to 4.91 cm^2 , which is 4.34 times bigger than the sensitive surface of the PMT ($S_{\text{PMT}} = 1.13 \text{ cm}^2$). Supposing a uniform deposition of the bacteria on the membrane surface, a volume V of 2.17 ml (i.e., $4.34 \times 0.5 \text{ ml}$) was filtered in order to obtain the same number of bioluminescent bacteria under the PMT sensitive surface than in the 0.5 ml volume inserted into the GloMax® 20/20 luminometer from Promega. We thus can readily compare the light emission of both apparatus. In order to correlate the bioluminescence intensity with the number of bioluminescent bacteria concentrated onto the membrane surface, we used the *E. coli* biosensor pRpoD-lux that constitutively emits light described elsewhere in this Special Issue (Prévéral et al. 2016; Brutesco et al. 2016).

Bioluminescence measurements

After 60-min incubation at $30 \text{ }^\circ\text{C}$ of a known number of *E. coli* TD2158 cells with the bacteriophages suspension, bioluminescence emission was measured either using a GloMax® 20/20 luminometer from Promega or the COMBITOX light detection unit. Cell counting was done with a spectrophotometer according to the following calibration: at an Optical Density measured at 600 nm of 1, 1 ml of a cellular suspension of *E. coli* contains 10^9 cells. In the luminometer, the incubation mix (0.5 ml) was placed in a 1.5-ml Eppendorf tube inserted in an integrating sphere ensuring maximum light collection. In the COMBITOX light detection unit, bioluminescence emission was measured by lowering the PMT at the surface of a $0.45 \text{ }\mu\text{m}$ Millipore HA nitrocellulose membranes after filtration of 2.17 ml of the incubation mix. The distance between the PMT and the membrane was set at 5 mm to prevent contamination of the sensitive surface of the detector.

Results

Optimized sample conditioning prior to luminescence measurements

The design of a universal light emission measurement unit is the most challenging part of the COMBITOX semi-autonomous inline water analyzer prototype. Although the reading output is the same for all biosensors—emission of light between 430 and 490 nm—the way by which the water sample interacts with the biosensor can be very different. For bacterial pathogens and metal detection (bacteriophage and bacterial biosensors, respectively), the water sample and the bioreagents (a bacteriophage suspension and lyophilized bacteria, respectively) are mixed and incubated in the same liquid

phase, whereas for toxin detection (immunological biosensor), they are in separate phases. For the latter, the specific antibodies used to detect the toxins are immobilized onto a chitosan foam. After incubation with the water sample, several washing/incubation steps of the chitosan foam with chemiluminescent reagents and washing buffers are necessary to initiate the chemiluminescence reaction.

The simplest way to operate the PMT is to bring its sensitive area as close as possible to the sample surface for light detection. For the immunological biosensor, this setup will be almost optimal for photon collection, especially if the chitosan foam containing the antibodies is thin. For the bacteriophage and bacterial biosensors, the sensor surface is placed above the liquid surface. Depending on the depth of the cuvette and absorbance properties of the water sample incubated with the bioreagents, only the light emitted in the top layers of the reaction volume will reach the light sensor. To prevent such effects and ensure maximal collection, we decided to concentrate all the light-emitting objects (bioluminescent bacteria) on a surface by filtrating the water sample on a $0.45 \text{ }\mu\text{m}$ nitrocellulose membrane after incubation with the bioreagents. Light emission is then measured at the membrane surface as described for the immunological biosensor. This protocol allows lower detection limits by increasing the reaction volumes (for the bacteriophage and bacterial biosensors) and/or addition of higher doses of bioreagents (for the bacterial biosensors) while discarding problems linked to the volume treated, light scattering or re-absorption by the reaction medium.

The three types of luminescent biosensors of the COMBITOX project derive from academic research and were optimized with laboratory-type luminometers, such as the GloMax® 20/20 from PROMEGA. The design and the improvements of the custom-made light sensor for the analyzer

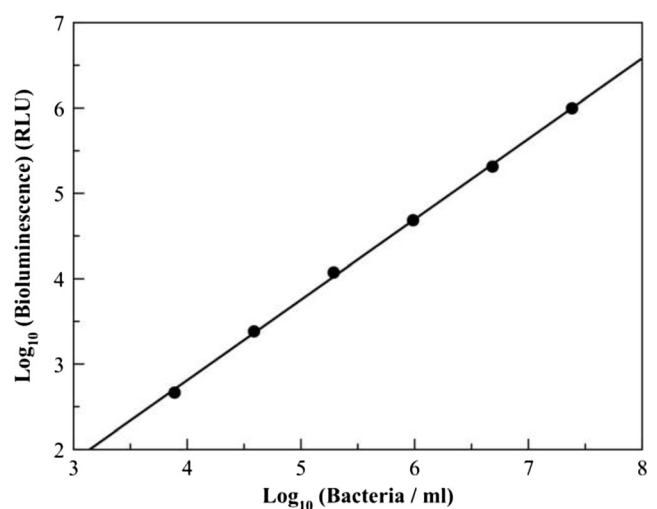


Fig. 1 Detection limit observed with the bacteriophage biosensor with the GloMax® 20/20. These data were obtained by measuring light emission 60 min after infection of a known number of *E. coli* TD2158 live cells by the bacteriophage biosensor

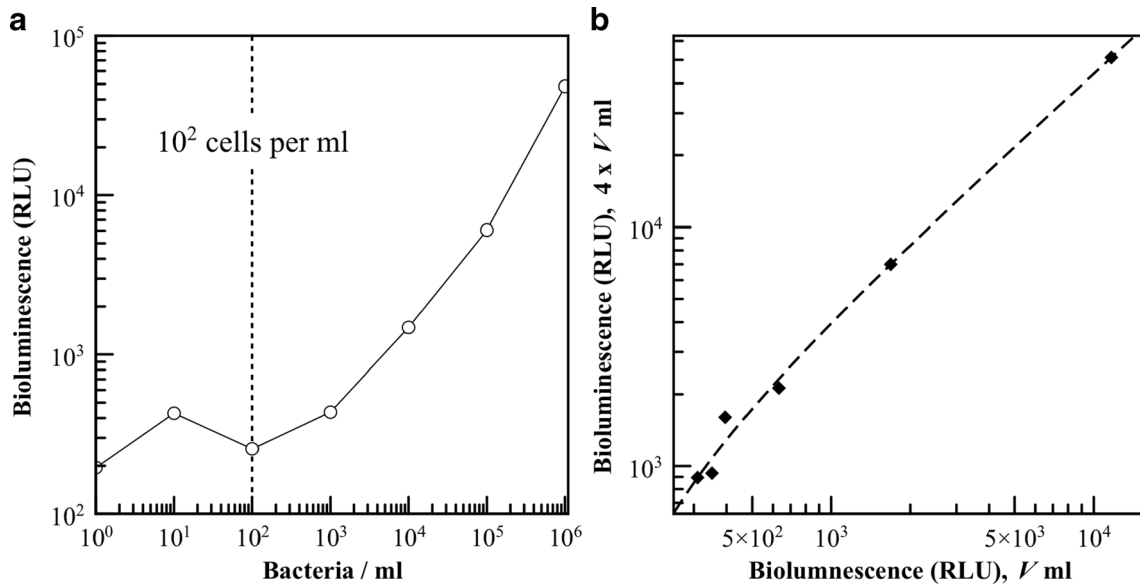


Fig. 2 Qualification of the Hamamatsu laboratory-made luminometer for light detection after membrane filtration. **a** Titration curve with the bacteriophage biosensor obtained with the Hamamatsu laboratory-made

luminometer after membrane filtration. **b** Relationship between the light signal intensity and the volume of bioluminescent *E. coli* cells. The slope of the linear fit is 4.4, with R^2 equal to 0.9999

prototype are based on these reference measurements to match at the very least the laboratory instruments and expand the sensitivity to meet the statutory detection limits. The most critical situation in this respect is the phage biosensor where we set a low detection limit— 10^2 enteric bacteria per ml—which is the limit imposed by the EU regulation (European directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing water quality and repealing Directive 76/160/EEC). Measurements with the commercial luminometer shown in

Fig. 1 indicate that we can detect at most 10^4 infected bacteria per ml with the bacteriophage-based biosensor with the whereas the statutory limit is set at 10^2 cells per ml. Our main objective with the COMBITOX analyzer prototype is to gain at least two logs of sensitivity; such an improvement will also be profitable for the other biosensors (bacterial and immunological sensors). To do so, we count on the Hamamatsu PMT and the concentration of greater water sample volumes on a membrane prior to light measurement. To assess the detection limit of our laboratory-made luminometer, we repeated the

Fig. 3 Disposable reaction cell for bacterial pathogens, metals, and toxin biodetection in semi-autonomous water analyzers. **a** Incubation. **b** Filtration. **c** Light detection. The example displayed here depicts the phage or bacterial biosensor. The chitosan foam loaded with the anti-toxins antibodies is supported by the membrane in the case of the immunological biosensor

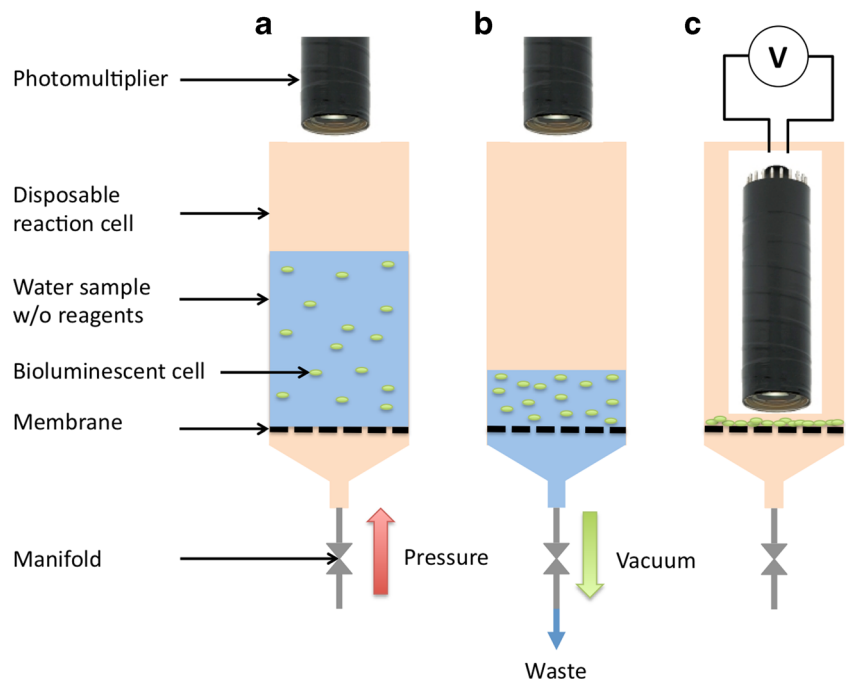
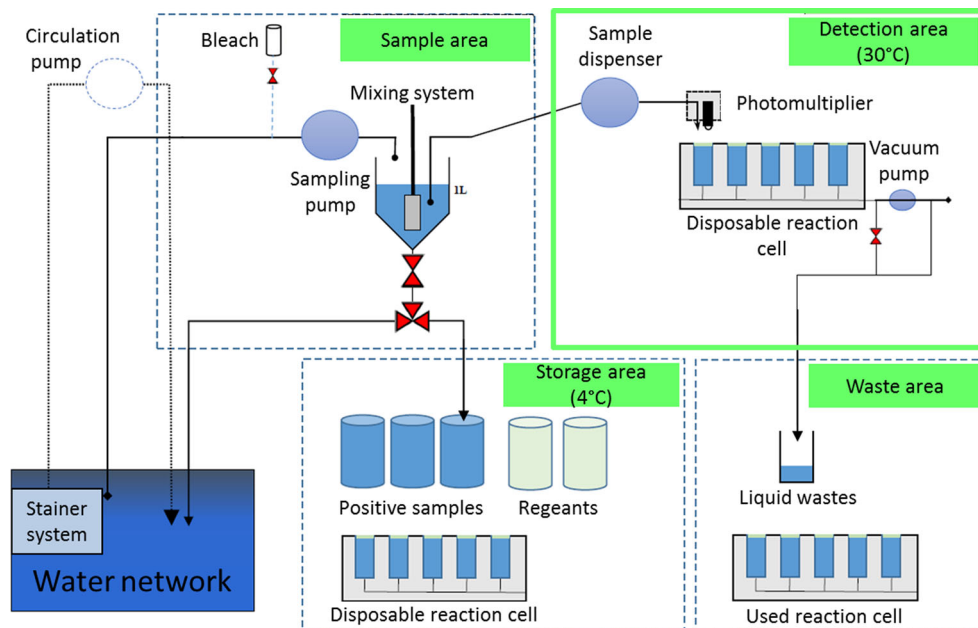


Fig. 4 Schematic of the COMBITOX semi-autonomous inline water analyzer using luminescent biosensors

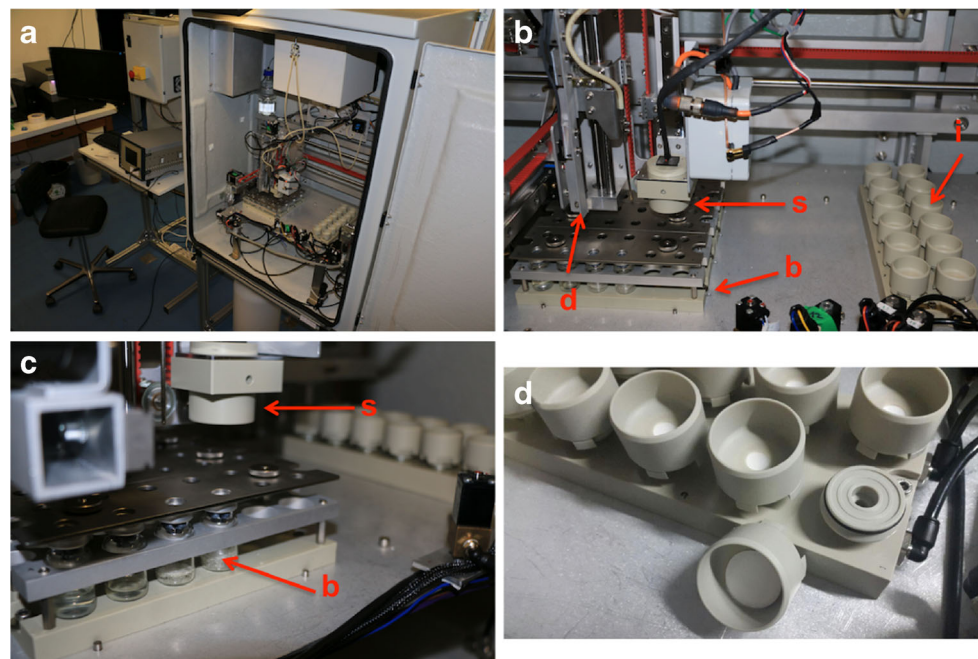


experiment described in Fig. 1 by first filtering the bacterial sample after 1-h incubation with the phages on the nitrocellulose membrane and then reading the light signal at the surface of the membrane (distance from PMT is 5 mm). The resulting titration curve is presented in Fig. 2a. With our experimental setup, we could achieve the detection limit of 10^2 infected bacteria per ml. We also evidenced that the sensitivity of the measurements can be increased by simply varying the volume filtered on the nitrocellulose membrane, as shown in Fig. 2b where multiplying the volume filtered by a factor 4 resulted in more than a fourfold light signal increase.

Guidelines for a disposable reaction cell for all biosensors

The validation of the Hamamatsu PMT setup together with the filtration protocol allowed us to propose the design of a disposable reaction cell able to accommodate either the phage or the bacterial or the immunological biosensor, coupled to a unique light detection system. Basically, it is a 20-mL cylinder closed at one end by a filtration membrane that can be connected to a manifold vacuum system. The membrane serves both as a support for the chitosan foam (immunological biosensor) and as a reaction cell for all three types of biosensors.

Fig. 5 Pictures of the COMBITOX prototype. **a** A general view of the water analyzer. The cabinet includes the interface with the water network, the sampling module, the bioreagent array, the disposable incubation/filtration units, the pipetting and dispensing system, and the light sensor. **b, c** A closer view of the bioreagent array (*b*), the disposable incubation/filtration units (*i*), the pipetting and dispensing system (*p*), and the light sensor (*s*). **d** The disposable incubation/filtration unit



When incubation is required (the water sample with the phage solution, the lyophilized bacteria or the chitosan foam; the chitosan foam with the washing solutions or chemical reagents), a counter pressure is applied to avoid liquid leakage through the membrane pores. When filtration is required, the disposable cell is connected to a vacuum pump. When all the necessary steps are finished, the light detector is lowered by the open end of the cylinder as close as possible to the membrane/chitosan foam surface and the light emission intensity recorded. Figure 3 displays a blueprint of such a disposable incubation/filtration unit.

This system is simple and flexible. The length of the cylinder can be adjusted to accommodate greater volumes if need be for improved sensitivity; this reaction cell can be cheaply produced with 3D-printers in plastics compatible with water analysis and only need to be equipped with simple, calibrated nitrocellulose membranes. This consumable part of the water analyzer can be further processed to accommodate the chitosan foam loaded with the specific anti-toxin antibodies. It is well suited to the various incubation/washing steps required to fabricate the chitosan foam as well as operate the immunological biosensor, thanks to the connection to the vacuum pumping system. Figure 5d shows an example of a disposable incubation/filtration unit obtained with a 3D-printer and equipped with a filtration membrane.

Conclusion

The scientific literature, including this Special Issue, offers various and original detection possibilities using biological transducers, most of them being validated and optimized in research laboratories. Moving now from laboratory bench with manual operator interventions to semi-autonomous processes compatible with on-site measurements represents one of the major bottlenecks for industrial applications of biosensors. The design of a universal device that can house various types of biosensors whose functioning require different actions (addition of substrate, washing steps...) is a key challenge for implementation in the automate. In this article, we contributed to this field with the development of a flexible and original light detector/reaction cell suited for bacterial (nickel, arsenite, mercury detection), immunological (microcystin-LR toxin detection) and bacteriophage (live enteric bacteria detection) biosensors without losing sight of the technical and economical constraints. Our disposable reaction cell, coupled to a unique light detection system, is composed of a 20-mL cylinder close at one end by a filtration membrane that can be connected to a vacuum system. The water sample is dispensed in the chamber and incubated with phage biosensor or whole cell biosensor concentrated solutions. After 60 min incubation, the mixture can be filtrated before lowering the light detector as close as possible to the membrane for light

emission intensity measurements. For immunological biosensors, the membrane supports chitosan foams loaded with the anti-toxins antibodies and the different washing/incubation steps required to obtain the chemiluminescence reaction can be performed via filtration prior to light measurements. These procedures used for the different biosensors can be easily automated to develop a semi-autonomous inline water analyzer where consumables are arrays of disposable reaction cells (with antibodies-loaded chitosan foams for the immunological biosensors), bioreagents for bacterial pathogens and metal detection (phage solutions and lyophilized bacterial cells) and chemical reagents for toxins detection (labeled toxins, luminol, washing solutions). Thus, the results presented here serve as a basis to develop a complete prototype of inline water analyzer prototype using biosensors (Fig. 4). In this prototype, the core unit light detector/reaction cell is connected to a sampling system plugged in the water network, a storage chamber for the reagents and the disposable reaction cells arrays and a collector for wastes coupled to a sterilization process to avoid downstream contamination by the biosensors after usage. Figure 5 presents pictures of the final COMBITOX analyzer prototype working in laboratory conditions. This machine reaches now TRL 4 and will be upgraded to TRL 6 in a following project with the development of a fully functional analyzer working in real conditions plugged to water networks.

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