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IN-LINE MULTIPLEXED BIOSENSING

A bioluminescent arsenite biosensor designed for inline water analyzer

Sandra Prévéral[1,2,3] · **Catherine Brutesco**[1,2,3] · **Elodie C. T. Descamps**[1,2,3] · **Camille Escoffier**[1,2,3] · **David Pignol**[1,2,3] · **Nicolas Ginet**[1,2,3] · **Daniel Garcia**[1,2,3]

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Abstract Whole-cell biosensors based on the reporter gene system can offer rapid detection of trace levels of organic or metallic compounds in water. They are well characterized in laboratory conditions, but their transfer into technological devices for the surveillance of water networks remains at a conceptual level. The development of a semi-autonomous inline water analyzer stumbles across the conservation of the bacterial biosensors over a period of time compatible with the autonomy requested by the end-user while maintaining a satisfactory sensitivity, specificity, and time response. We focused here on assessing the effect of lyophilization on two biosensors based on the reporter gene system and hosted in *Escherichia coli*. The reporter gene used here is the entire bacterial luciferase *lux* operon (*luxCDABE*) for an autonomous bioluminescence emission without the need to add any substrate. In the cell-survival biosensor that is used to determine the overall fitness of the bacteria when mixed with the water sample, *lux* expression is driven by a constitutive *E. coli* promoter P_{rpoD}. In the arsenite biosensor, the arsenite-inducible promoter P_{ars} involved in arsenite resistance in *E. coli* controls *lux* expression. Evaluation of the shelf life of these lyophilized biosensors kept at 4 °C over a year evidenced that about 40 % of the lyophilized cells can be revived in such storage conditions. The performances of the lyophilized biosensor after 7 months in storage are maintained, with a detection limit of 0.2 μM arsenite for a response in about an hour with good reproducibility. These results pave the way to the use in tandem of both biosensors (one for general toxicity and one for arsenite contamination) as consumables of an autonomous analyzer in the field.

Keywords Biosensors · Bioluminescence · Lyophilization

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Sandra Prévéral and Catherine Brutesco contributed equally to this work.

✉ Nicolas Ginet
nicolas.ginet@cea.fr

[1] CEA, DSV, IBEB, Lab Bioenerget Cellulaire, Saint-Paul-lez-Durance F-13108, France

[2] CNRS, UMR 7265 Biol Veget and Microbiol Environ, Saint-Paul-lez-Durance F-13108, France

[3] Aix-Marseille Université, BVME UMR7265, Marseille F-13284, France

Introduction

The negative impacts on ecosystems of environmental pollutants (chemicals, hormones, toxins, etc.) are widely recognized and justify the development of accurate and rapid methods for their detection within the framework of automatic environment monitoring. Biosensors are not intended to fully replace chemical methods but can offer rapid and on-site monitoring of even trace levels of targeted compounds in comparison with non-portable analytical chromatographic methodologies (Sun et al. 2015). Biosensors provide quantitative or semi-quantitative measurements of the target concentrations, thanks to a biological recognition element (whole cells, antibodies, DNA, RNA, enzymes, or receptor proteins) coupled to a physical transducer. Thanks to their high selectivity and specificity, enzymes are widely used for biological recognition but enzyme-based biosensor often requires tedious and costly purification steps representing a limitation for their commercial development. Whole living cells expressing a sensitive element represent good alternatives to enzymes because of their lower fabrication cost and higher stability. One of the most widely used intracellular sensing mechanisms is based on the coupling between a transcriptional regulator and a

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promoter that control the expression of a reporter gene (Daunert et al. 2000); (Van der Meer and Belkin 2010). The variable expression of the reporter gene results in a measurable phenotype variation. Various reporter genes have been described in the literature, resulting in the expression of, e.g., fluorescent, bioluminescent, or chromogenic proteins (Xiong et al. 2012; Hakkila et al. 2002). One can classify whole-cell biosensors into two groups depending on the promoter driving the reporter gene expression: (i) systems based on a constitutive promoter resulting in decreased expression under toxic conditions, and (ii) systems based on an inducible promoter responding to a specific target (Woutersen et al. 2011). In the latter, the interaction between the target molecules and transcriptional regulators activates or represses the reporter gene expression, resulting in a measurable signal change (e.g., fluorescence, luminescence, or colorimetric readouts) in a concentration-dependent manner. Most examples reported in the literature derive from natural resistance mechanisms to compounds such as heavy metals or antibiotics (Ivask et al. 2009). Among the various signals the reporter gene system gives access to, luminescence is an optimized readout signal exhibiting a high sensitivity, a low background signal, and a high signal-to-noise ratio. Among the biological systems that emit light, bacterial luciferase catalyzes the oxidation of a reduced flavin mononucleotide ($FMNH_2$) and a long-chain fatty aldehyde in the presence of molecular oxygen and results in a blue-green light emission. The primary advantages of bacterial luciferase are sensitivity, broad dynamic range, and simplicity. Moreover, expression of the whole bacterial *lux* operon (*luxCDABE*) simplifies the whole-cell biosensor operation as it suppresses the necessity of adding a substrate because it encodes the necessary enzymes to produce it (Baldwin et al. 1995).

A wide variety of whole-cell biosensor assays has been developed over the past decade for the specific detection of various heavy metals or organic compounds (for a recent review, see (Xu et al. 2013)). Efforts have also been made to screen promoters that have evolved greater efficiency or to genetically modify some elements of the regulator protein/promoter pair for increased sensitivity and specificity (Peng et al. 2010); (Behzadian et al. 2011). An example of an in-depth study for improving the efficiency of a luminescent bacterial biosensor for nickel is described in this volume (cf. Cayron et al. in this Special Issue). Crucial for a portable on-field analyzer, immobilization and/or conservation techniques maintaining long-term cell viability in a rapid responsive state have also been investigated (Bjerketorp et al. 2006). Different alternatives show promises, such as lyophilization (Siegfried et al. 2012); (Camanzi et al. 2011), or the use of sporulating bacteria as the biosensor scaffold, which allows the biodetector to be stored as spores for long-time storage (Date et al. 2010). In spite of all these developments, few studies have gone a step beyond this and validated biosensors under real-life field conditions.

Arsenic contamination in groundwater is a serious threat to human health in many parts of the world where people have the least access to centralized drinking water systems, resulting in limited control of arsenic levels in potable water sources. As a consequence, we focus here on the design of a low-cost bioluminescent arsenite biosensor usable in the field. The resistance system to arsenite (and simultaneously to antimonite) is one of the best characterized in *Escherichia coli* (Hedges and Baumberg 1973) and is encoded by the *arsRDABC* operon. Its expression is controlled by the ArsR regulator protein, which in absence of arsenic binds as a dimer to an operator region on the DNA and prevents transcription of the *ars* genes. In the presence of arsenite, the ArsR dimer affinity for the operator decreases, allowing transcription of the genes coding for a specific arsenite efflux pump to proceed. To date, numerous arsenite biosensors based on the ArsR repressor were built (Chen and Rosen 2014). Here, we investigate the response of one of these whole cell bacterial luminescent biosensors in terms of specificity and sensitivity over a period of 7 months following cell lyophilization, paving the way to the use of whole cell reporters in the field using an easy-to-use automated inline analyzer.

Materials and methods

In the following, P_{ars} and P_{mer} refer to the arsenite- and mercury-inducible promoters, respectively, including the *arsR* and *merR* genes encoding the regulatory proteins.

Bacterial strains

E. coli strains used in this study are W3110 (Hayashi et al. 2006) and Life Technologies-branded Dh10beta.

Culture conditions for *E. coli*

Kanamycin and ampicillin were used at 50 μg/ml when needed. *E. coli* strains were aerobically cultivated in Lysogeny Broth (LB) medium with the required antibiotics at 37 °C in agitated Erlenmeyer flasks (150 rpm).

Construction of the bioluminescent cell-survival and arsenite biosensors in *E. coli*

In this study, whole-cell biosensors are constituted by *E. coli* cells transformed with different replicative plasmids using the bacterial luciferase from *Photorhabdus luminescens* as the reporter system (*luxCDABE* operon). In the cell-survival biosensor (plasmid pRpoD-*lux*), the expression of *lux* operon is driven by the promoter P_{rpoD} from the constitutively expressed RNA polymerase sigma factor gene *rpoD* from *E. coli*. In the mercury (plasmid pMer-*lux*) and arsenite

(plasmid pArs-*lux*) biosensors, the expression of the *lux* operon is controlled by the metal-inducible P_{mer} and P_{ars} promoters, respectively. The three plasmids cited above derive from a prototype bioluminescent mercury biosensor encoded in the pCC306 plasmid (Condee and Summers 1992). The *luxAB* genes encoding the *Vibrio harvei* luciferase were replaced in pCC306 (between BamHI and EcoRI restriction sites) by the *P. luminescens* luciferase *luxCDABE* operon obtained from pUTmini-Tn5-*lux*CDABE-Tc (Winson et al. 1998). The resulting intermediary plasmid was termed pMer-*lux*. The P_{rpoD} promoter region from *E. coli* K12 was then amplified by PCR with the following forward (caat**ccatgg**gcatacttgatgacagccagc, *Nco*I restriction site in bold) and reverse (gccacccttgaaaaactgtc**gggccc**atat, *Apa*I restriction site in bold) primers. The fragment containing P_{mer} in pMer-*lux* was removed by restriction (*Nco*I and *Apa*I) and replaced by the 321 pb PCR fragment containing P_{rpoD} by ligation at the corresponding restriction sites, yielding the pRpoD-*lux* plasmid. After verification, the pRpoD-*lux* plasmid was introduced into *E. coli* Dh10beta (Life Technologies). To construct the arsenite biosensor pArs-*lux*, the *lacZ* region of pMV-*arsR*-ABS (Stocker et al. 2003) was removed and substituted by the *luxCDABE* operon using a classical digestion EcoRI/SacI. After verification, the pArs-*lux* plasmid was introduced into *E. coli* Dh10beta (Life Technologies) and W3110 (Hayashi et al. 2006).

Lyophilization of *E. coli* cells

E. coli cells were grown aerobically in LB medium at 37 °C under shaking (150 rpm) until early exponential phase ($OD_{600\ nm}$ = 0.2–0.3). The cultures were then centrifuged and the pellet resuspended in LB + 12 % sucrose to an $OD_{600\ nm}$ equal to 0.5 (or 1.0 for the metal specificity experiment described in Fig. 2); 500-μl aliquots in 2 ml Eppendorf tubes of these bacterial cultures were then frozen at −80 °C then lyophilized for 24 h in a Christ Alpha 1–2 LD Freeze Dryer. The lyophilized cells were stored under nitrogen atmosphere at 4 °C or room temperature until use.

Viability assay

E. coli cells viability after lyophilization was assessed by measuring the intracellular ATP content, which is an indicator of metabolically active cells, with the BacTiter-Glo™ Microbial Cell Viability Assay from Promega. For each data point, three aliquots of lyophilized cells were revived by addition of 500 μl of water and incubated for 20 min at 30 °C. The cells were further diluted 10 and 100 times in water and 100 μl aliquots incubated for 10 min at room temperature with 100 μl of the commercial reagent. Luminescence was read in Nunc™ Microwell™ 96-well optical-bottom black plates with the plate reader TECAN infinity M200, each measure was performed in triplicates. The luminescence expressed in relative light units (RLU) is linearly correlated to the number of colony forming units (CFU) on LB-agar plates (data not shown).

Absorbance and bioluminescence emission measurements

The bioluminescence of the cell-survival pRpoD-*lux* biosensor was continuously monitored with a GloMax® 20/20 Luminometer (Promega) for one hour at 30 °C. For each sampling time, three aliquots of lyophilized samples were resuspended in 500 μl of water in 2 ml Eppendorf tubes. The bioluminescence and absorbance at 600 nm ($OD_{600\ nm}$) of the arsenic biosensor pArs-*lux*, both with fresh and lyophilized cultures, were monitored in sterilized Greiner 96-well black plate and clear bottom, with a multimode microplate reader Infinite® 200 PRO (TECAN) set at 450 nm for bioluminescence and 600 nm for absorbance. The measurements were performed in triplicate, every 10 min during 3 h at 30 °C. For fresh cultures, exponential phase cultures ($OD_{600\ nm}$ = 0.3) were collected and concentrated twofold. For lyophilized cells, each aliquot was resuspended in 500 μl of water and incubated 20 min at 30 °C. In the microplate, each well contained 100 μl of culture and 100 μl of LB medium supplemented with different sodium arsenite ($AsNaO_2$) concentrations. In the case of the metal specificity experiment (Fig. 2), measurements were performed with 180 μl of lyophilized culture resuspended in 500 μl of water with 20 μl of the different metal solutions added. The metal salts used for this experiment are $AlK(SO_4)_2{\cdot}12H_2O$, $NiCl_2{\cdot}6H_2O$, $HgCl_2$, $BiCl_3$, $Ba(CH_3COO)_2$, $K_2Sb_2(C_4H_2O_6)_2{\cdot}3H_2O$, $Pb(CH_3COO)_2{\cdot}3H_2O$, $CoCl_2{\cdot}6H_2O$, $CuCl_2{\cdot}2H_2O$, $FeCl_2{\cdot}4H_2O$, $AgNO_3$, $AsNaO_2$, $Na_2MoO_4{\cdot}2H_2O$, and $ZnCl_2$.

Results

Conservation of bacterial biosensors by lyophilization

Long-term conservation of *E. coli* whole-cell luminescent biosensors by lyophilization is liable to affect both cell survival after rehydration and bioluminescence emission. Lyophilized *E. coli* cells harboring the cell-survival luminescent biosensor (pRpoD-*lux*) were used to evaluate these two parameters. This whole-cell biosensor continuously emits light as the *lux* operon expression is driven by the constitutive *E. coli* promoter P_{rpoD}. A series of identical, ready-to-use aliquots of the lyophilized cells were stored at room temperature and 4 °C over a year, under neutral atmosphere. Cells were regularly revived from this stock by simply adding water to the aliquots. Cell-survival rate was evaluated by measuring the available pool of ATP before and after lyophilization and at different time during storage; in the meantime bioluminescence emission

kinetics were also recorded. Figure 1 depicts the evolution of both parameters over a year of storage, at two different conservation temperatures.

Fairly good levels for cell survival and bioluminescence emission are measured right after lyophilization, with respectively 74 and 98 % of the initial values (before lyophilization) retained. For both storage temperatures tested here and in accordance with the cell-survival decrease, bioluminescence emission drops down after 15 days in storage. As evidenced in Fig. 1a, the cell-survival rate drops down after 4 months in storage at room temperature and after 1 year no reagent aliquot can be revived. When stored at 4 °C, the cell-survival rate remains fairly constant after the initial decrease, with a final score around 50–60 % after 1 year on the shelf (Fig. 1b). The room temperature would have remained a viable option for storage at less than 3 months notwithstanding its impact on bioluminescence emission. Whereas bioluminescence emission is close to nothing after 3 months in storage at room temperature (Fig. 1a), it remains fairly constant at 4 °C over a year, with approximately 20 % of the initial bioluminescence level (Fig. 1b). One can conclude that revived cells can produce a functional bacterial luciferase even after one year on the shelf at 4 °C. On a first impression, an 80 % drop in bioluminescence emission sounds enough to fail the measure even in the best case at 4 °C. Measurement of the light signal essentially depends on the sensor used in the analyzer, namely its gain, sensitivity and signal-to-noise ratio. The results here were obtained with the GloMax® 20/20 luminometer which is 100 times less sensitive than the custom-made light sensor developed for the inline water analyzer prototype described in this Special Issue (cf. Descamps et al. in this Special Issue). Despite these non-optimized light collection conditions, a mean bioluminescence emission level of 18 455 ± 4 394 RLU was measured using lyophilized reagent samples kept at 4 °C for 6 to 12 months. This is about 30 times higher than the bioluminescence baseline estimated from the data

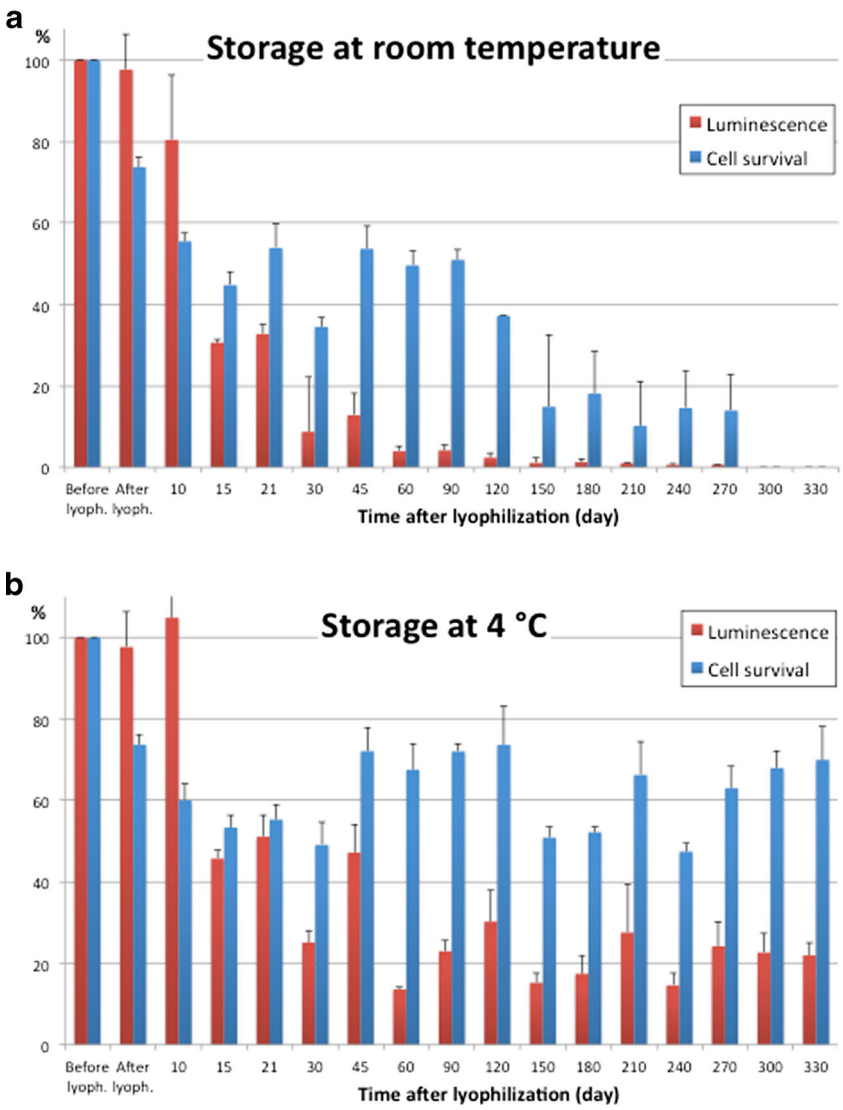

Fig. 1 Impact of lyophilization, storage temperature and shelf life on the pRpoD-*lux* cell-survival biosensor. *Blue*, cell survival measured by ATPmetry. *Red*, bioluminescence emission measured 45 min after rehydration. **a** Room temperature (22 °C) storage; **b** 4 °C storage. We used *E. coli* Dh10beta strain to harbor the biosensor in this section

obtained after 9 months in storage at room temperature. In laboratory conditions, the bioluminescence emission level of the cell-survival biosensor is more than high enough to be measured with sensitivity after 12 months of conservation at 4 °C.

As a conclusion for this section, we validated lyophilization as a robust and efficient technique to conserve luminescent bacterial biosensors, provided that reagents aliquots are stored at 4 °C under nitrogen atmosphere.

Bioluminescent metal biosensors

A model metal biosensor was constructed by the transcriptional fusion between the bacterial luciferase operon *lux* and an arsenite-inducible promoter P_{ars} controlled by the transcriptional repressor ArsR. This construct derives from a mercury biosensor published by Condee and Summers. The genetic construct pArs-*lux* was hosted in *E. coli*. Metal specificity and dose response for this arsenite bioluminescent biosensor was first characterized, prior to its conditioning as a lyophilized reagent for usage in a semi-autonomous inline water analyzer.

Response of the arsenite biosensor

The bioluminescence emission was measured 60 min after the addition of different metals in a broad range of concentrations (10 nM to 10 mM range) (Fig. 2). The pArs-*lux* biosensor exhibits a very narrow specificity, with no or very little response for all the common toxic metals tested here but arsenite and antimony with a 15 times more sensitive response for arsenite than for antimony. This dual response is not surprising and is well documented in the literature (Wang et al. 2015) for the arsenite resistance system in *E. coli*.

Sensitivity towards arsenite was further investigated using living cells before lyophilization. Figure 3 shows the absorbance and bioluminescence emission kinetics over a range of arsenite concentrations in growing cultures of *E. coli*. An increase of bioluminescence emission upon addition of increasing concentration of arsenite in the 0–3 μM range was observed. It is all the more important than the metal concentration is high (Fig. 3b). This is not due to differential cellular growth as $OD_{600\ nm}$ kinetics measured—reflecting the bacterial growth during the same time window—are essentially stable and superimposed (Fig. 3a). We thus clearly evidenced a dose response to arsenite of the whole-cell P_{ars} bioluminescent sensor, an example of which is given in Fig. 4 recorded 60 min after the addition of arsenite.

In fresh cultures, one can detect as low as 0.1–0.2 μM of arsenite (Fig. 4). The dynamics extends up to 3 μM; higher concentrations resulted in a rapid decrease of the living bacterial content (evidenced by the decrease of $OD_{600\ nm}$ kinetics) due to arsenite toxicity for the cells (data not shown). At the time of metal addition, the bioluminescence level is not null, even without arsenite. Furthermore without arsenite, there was a slight increase of bioluminescence over time (Fig. 3b), which was interpreted as a "leakage" of the P_{ars} promoter, i.e., incomplete binding of the ArsR repressor in the absence of metal. Although the dose response to arsenite is very good (Fig. 4), quantitative results can be given if a negative control is provided together with the unknown sample measurements. It is fairly easy to do in laboratory conditions but in most cases not achievable for on-field measurements, as it would require an uncontaminated environmental water sample. As a result, this arsenite biosensor will be used semi-quantitatively in the autonomous inline water analyzer.

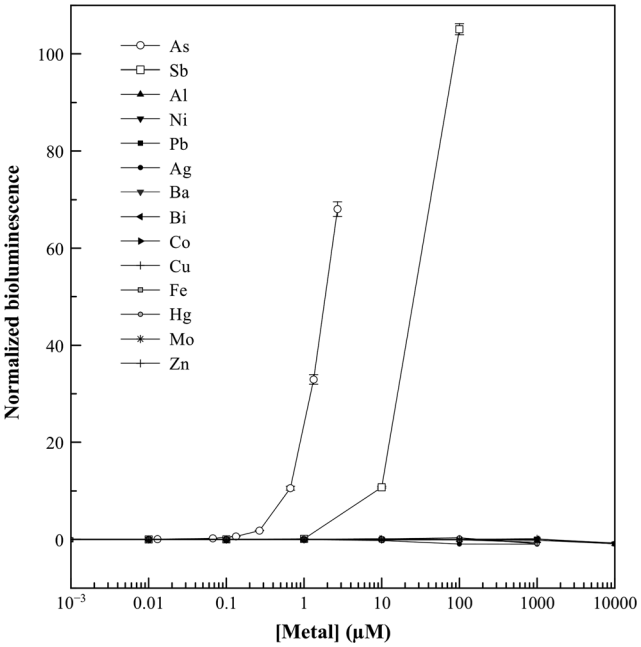

Fig. 2 Metal specificity of the arsenite biosensor pArs-*lux* hosted in *E. coli*. Metals are given in the legend by their atomic symbols and the metallic salts used here are listed in the "Materials and methods." Bioluminescence emission levels were recorded 60 min after metal addition. Normalized bioluminescence (*NB*) is calculated by the following equation: $NB = (B - B_0)/B_0$ where B is the bioluminescence measured at $t = 60$ min for a given metal concentration and B_0 the bioluminescence measured at $t = 60$ min without metal addition

Lyophilized arsenite luminescent biosensors as a reagent for a semi-autonomous inline water analyzer

The impact of lyophilization upon the bioluminescence response of the pArs-*lux* arsenite biosensor was examined: the dose response of the arsenite biosensor was measured with lyophilized aliquots stored for 7 months at 4 °C. To assess the reproducibility, three different pools were used, each one constituted by four lyophilized bioreagent aliquots resuspended in water. Figure 5 displays the average bioluminescence emission kinetics recorded in the presence of increasing arsenite concentrations.

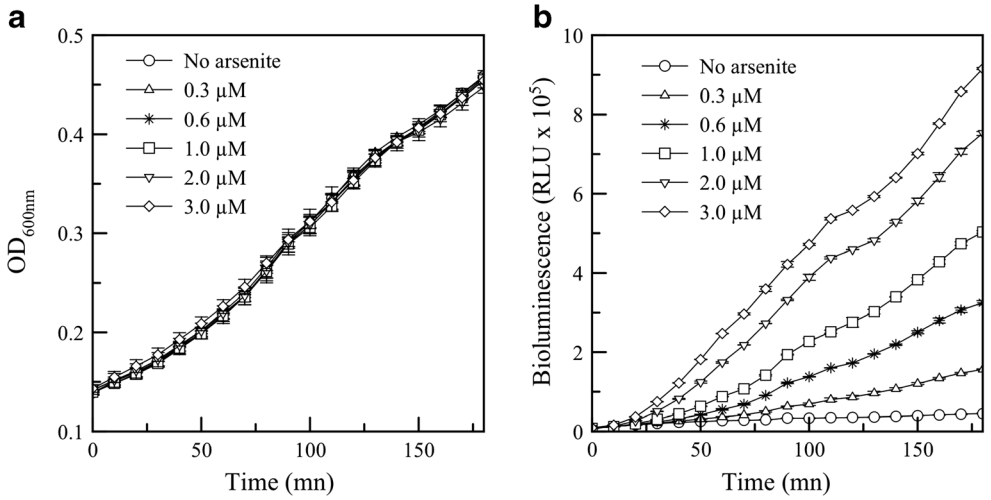

Fig. 3 Cellular growth and bioluminescence emission kinetics in the presence of arsenite of the pArs-*lux* biosensor hosted in fresh *E. coli* cells. **a** Optical density kinetics recorded at 600 nm. **b** Bioluminescence emission kinetics (in RLU × 10^5). For this experiment, *E. coli* W3110 cells hosting the arsenite biosensor were harvested during the exponential phase at $OD_{600nm} = 0.3$ and concentrated two times in LB medium. Each well contained 100 µl of culture supplemented with 100 µl of different arsenite concentrations. Final concentrations were 0, 0.15, 0.3, 0.6, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 2.75, and 3 µM but only the results for 0, 0.3, 0.6, 1.0, 2.0, and 3.0 µM are displayed here for clarity. Each condition was measured in triplicate. OD_{600nm} and bioluminescence emission values were averaged and standard errors are displayed in (**a**) and (**b**) (most of time are undistinguishable from the data symbols)

We observed in all cases an increase of bioluminescence over time when arsenite is added, all the more important when the arsenite concentration is high (Fig. 5a). We also observed bioluminescence emission without arsenite added, probably due to P_{ars} promoter leakage as hypothesized above. Similarly to the results obtained with living cells before lyophilization (see above), the bioluminescence increase is not due to differential bacterial growth as $OD_{600\ nm}$ kinetics are essentially stable and superimposed (data not shown). The light signal remains fairly high with values around 45,000 RLU for 3 µM arsenite (Fig. 5b). Thus as observed for the pRpoD-*lux* cell-survival biosensor, the pArs-*lux* arsenite biosensor remains perfectly functional after lyophilization and storage for at least 7 months at 4 °C. The theoretical detection limit of the biosensor can be defined as the arsenite concentration for which the bioluminescence emission level *minus* five times its standard error is superior to the bioluminescence emission when no arsenite is added *plus* five times its standard error, i.e., 167 nM. To summarize, the lyophilized arsenite biosensor described in this study is functional after 7 months in storage at 4 °C with a detection limit of 0.2 µM.

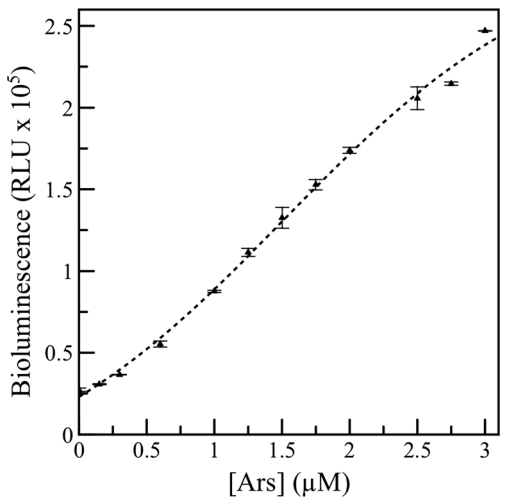

Fig. 4 Dose response of the pArs-*lux* arsenite bacterial biosensor hosted in fresh *E. coli* cells. Raw bioluminescence emission values (in RLU × 10^5) measured 60 min after arsenite addition. Arsenite concentrations are 0, 0.15, 0.3, 0.6, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 2.75, and 3 µM

Conclusion

We aimed at investigating the performances of two bioluminescent sensors hosted in lyophilized *E. coli* cells for

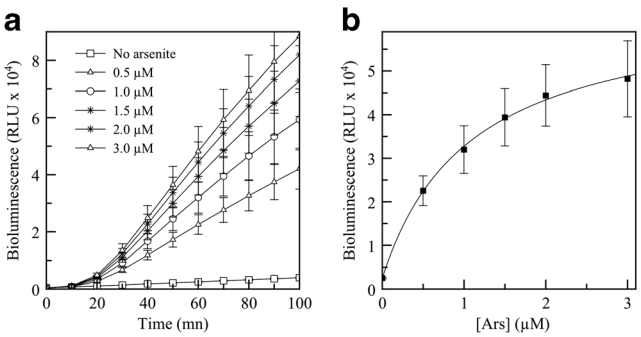

Fig. 5 Dose response of the pArs-*lux* arsenite biosensor with 7 months lyophilized bioreagent aliquots. **a** Mean bioluminescence emission kinetics (in RLU × 10^4) after arsenite addition. **b** Standard curves for arsenite (in RLU × 10^4) at $t = 60$ min

water network monitoring. The constitutive emission of light by the cell-survival pRpoD-*lux* biosensor evaluates the global toxicity of the water samples, while the arsenite-responsive pArs-*lux* biosensor is intended to raise the alarm when a contamination threshold is reached. We selected lyophilization as a simple and robust process for long-term storage of these whole-cell biosensors, enabling their utilization as bioreagents in a prototype of semi-autonomous inline water analyzer. We evidenced here that such a technology is well suited for the intended purpose, provided that the lyophilized cells are stored under neutral atmosphere at 4 °C. The cell-survival biosensor light response remains stable after 12 months and sufficient for sensitive detection with commercial luminometers. In the future prospect of rapid screening for improved lyophilization conditions, this study allowed us to validate ATPmetry as a rapid and reliable alternative to quantify cell survival instead of cell counting on agar plates.

The pArs-*lux* arsenite biosensor displays a very narrow specificity and responds to arsenite concentration in the 0.15–3 μM range, in less than 1 h using growing cultures of *E. coli*. Antimony Sb(III) and arsenite As(III) sharing the same redox chemistry, they both can interact with ArsR to lift the repression of the *ars* operon and both can be effluxed by ArsB (Wang et al. 2015; as a consequence the pArs-*lux* biosensor is also able to respond to antimony, although it remains 15 times more sensitive towards arsenite. After lyophilization and storage over different periods of time, the arsenite biosensor retains a detection limit of 0.15 μM in a similar time window while maintaining a very good reproducibility. Combined with the stable signal obtained with the cell-survival luminescent biosensor, the lyophilized arsenite biosensor can be used for detection in water samples after 7 months in storage. This stability over time allows the design of inline analyzers with an operating autonomy compatible with the semi-autonomous surveillance of water networks. The analytical spectra can be easily broadened with the inclusion for simultaneous detection of other metal biosensors, *e.g.* the pMer-*lux* for mercury detection we adapted from Condee and Summers, for nickel by Cayron et al. (This Special Issue), and more generally speaking for any *E. coli*-based whole cell biosensor.

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