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Bioreporters and biosensors for arsenic detection. Biotechnological solutions for a world-wide pollution problem

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A wide variety of whole cell bioreporter and biosensor assays for arsenic detection has been developed over the past decade. The assays permit flexible detection instrumentation while maintaining excellent method of detection limits in the environmentally relevant range of 10–50 μg arsenite per L and below. New emerging trends focus on genetic rewiring of reporter cells and/or integration into microdevices for more optimal detection. A number of case studies have shown realistic field applicability of bioreporter assays.

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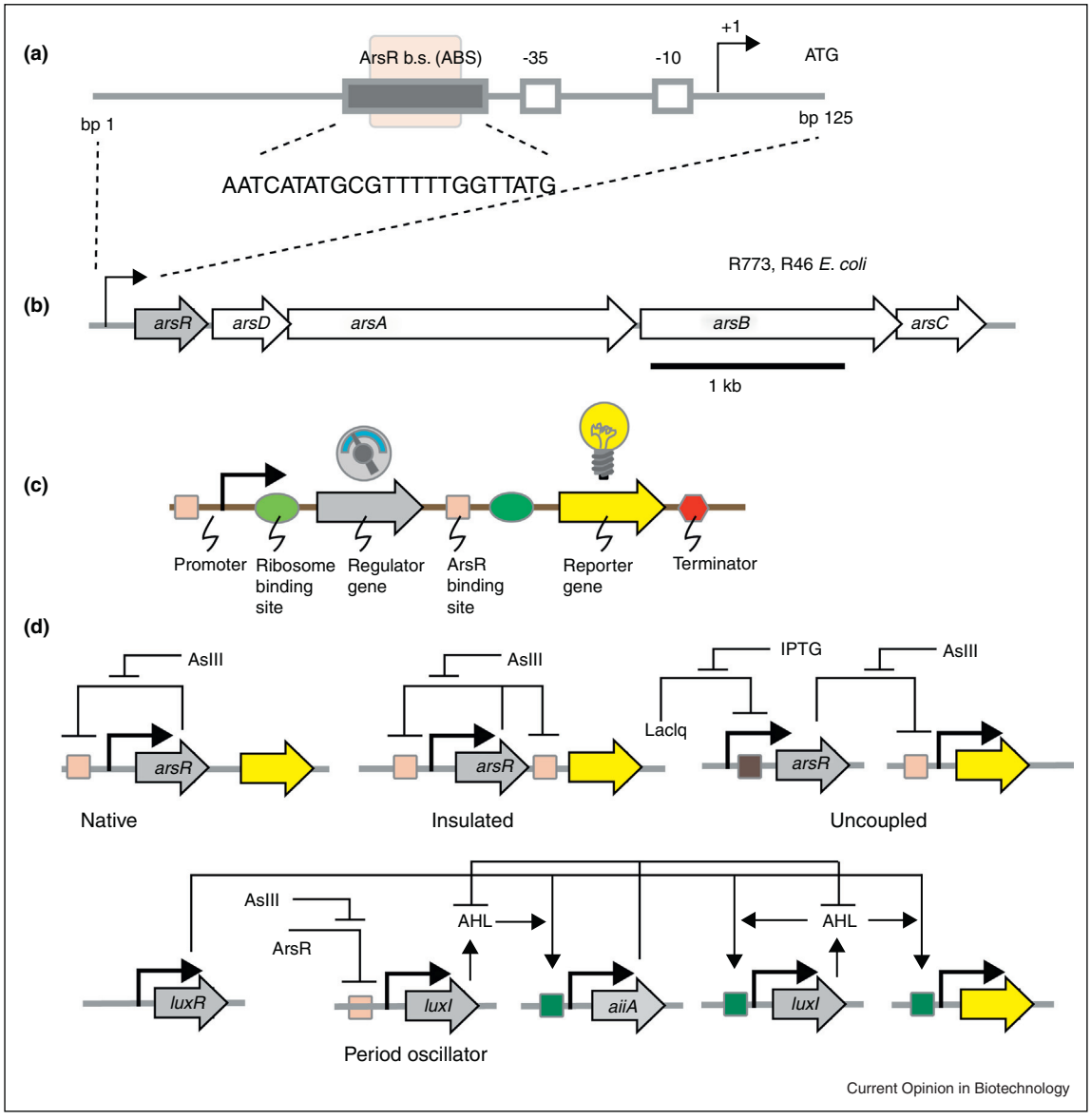
Introduction

Arsenic is an abundant but tightly bound element in the Earth's crust, which, however, under particular hydrogeological conditions and/or as a result of human activities can lead to serious and widespread contamination [1,2,3••]. Arsenic has a complex chemistry and can occur in different inorganic forms, such as trivalent AsIII in As_2O_3, AsO_2^-, AsO_3^{3-} (arsenite) or AsH_3 (arsine), or as five-valent AsV in AsO_4^{3-} (arsenate). In addition arsenic can be bound in organic form, such as in arsenobetaine, trimethylarsine or arsenosugars, often as a result of enzymatic processes [4]. Arsenicals are generally considered as extremely toxic [5], and the provisionally tolerable weekly intake for inorganic arsenic is set at 15 μg/kg of body weight [6] whereas the current WHO recommended drinking water limit is 10 μg/L [1]. The drinking water limit for inorganic arsenic is by far surpassed in potable water sources in large geographical areas such as the deltas of the Ganges, Brahmaputra, and Meghna (India, Bangladesh), Mekong (Loas, Cambodja) or Red River (Vietnam) [3••]. Unfortunately, many of the most affected zones with arsenic groundwater contamination are also those where people have the least access to centralized drinking water systems, and where the logistics of measuring arsenic levels in potable water sources is cumbersome [7]. In addition, accurate quantification of arsenicals requires sophisticated chemical analytics and chemical field-tests are not satisfactory for a variety of reasons [7–9]. Consequently, many research groups have proposed alternative methods for assaying arsenic based on biological systems (e.g. bioreporters and biosensors). The purpose of this current opinion article is thus to review the most recent developments in bioreporters and biosensors for arsenic detection. We will briefly recapitulate the principal concepts of the biological detection of arsenic, then will focus on recent genetic engineering efforts, synthetic biology designs and micro-engineering strategies to incorporate and improve bioreporter assays, and finally will discuss the merits of biological assays under realistic field conditions.

Biological detection of arsenic

Arsenic is not only toxic to higher organisms but also to prokaryotes, and most prokaryotes have evolved exquisite resistance systems against arsenic [10]. Probably the best characterized resistance system to arsenic (and simultaneously to antimonite) was discovered on the plasmid R773 in *Escherichia coli* [11], and is encoded by the *arsRDABC* operon (Figure 1). Expression of the operon is controlled by the ArsR protein, which in absence of arsenic binds as a dimer to an operator DNA called ArsR-binding-site (ABS, Figure 1A) slightly upstream of the −10/−35 promoter [12]. Binding of ArsR to the ABS prevents transcription, but in the presence of arsenic the affinity of the dimer for the ABS decreases, allowing transcription to occur [12]. ArsD is a chaperone protein, which may help to bind and present arsenite to the ArsA ATP-ase part of a specific arsenite efflux pump (ArsAB) [13–15]. Cells can further reduce arsenate to arsenite by means of a reductase (ArsC), which can then again be removed from the cell through the ArsAB efflux pump [15]. Different varieties of the *arsRDABC* operon are found, some of which lack an *arsD* and *arsA* homologue [16], or which carry an additional gene *arsH*, an NADPH-dependent FMN reductase that might prevent re-oxidation of arsenite to arsenate [17]. Arsenic-resistance

Figure 1



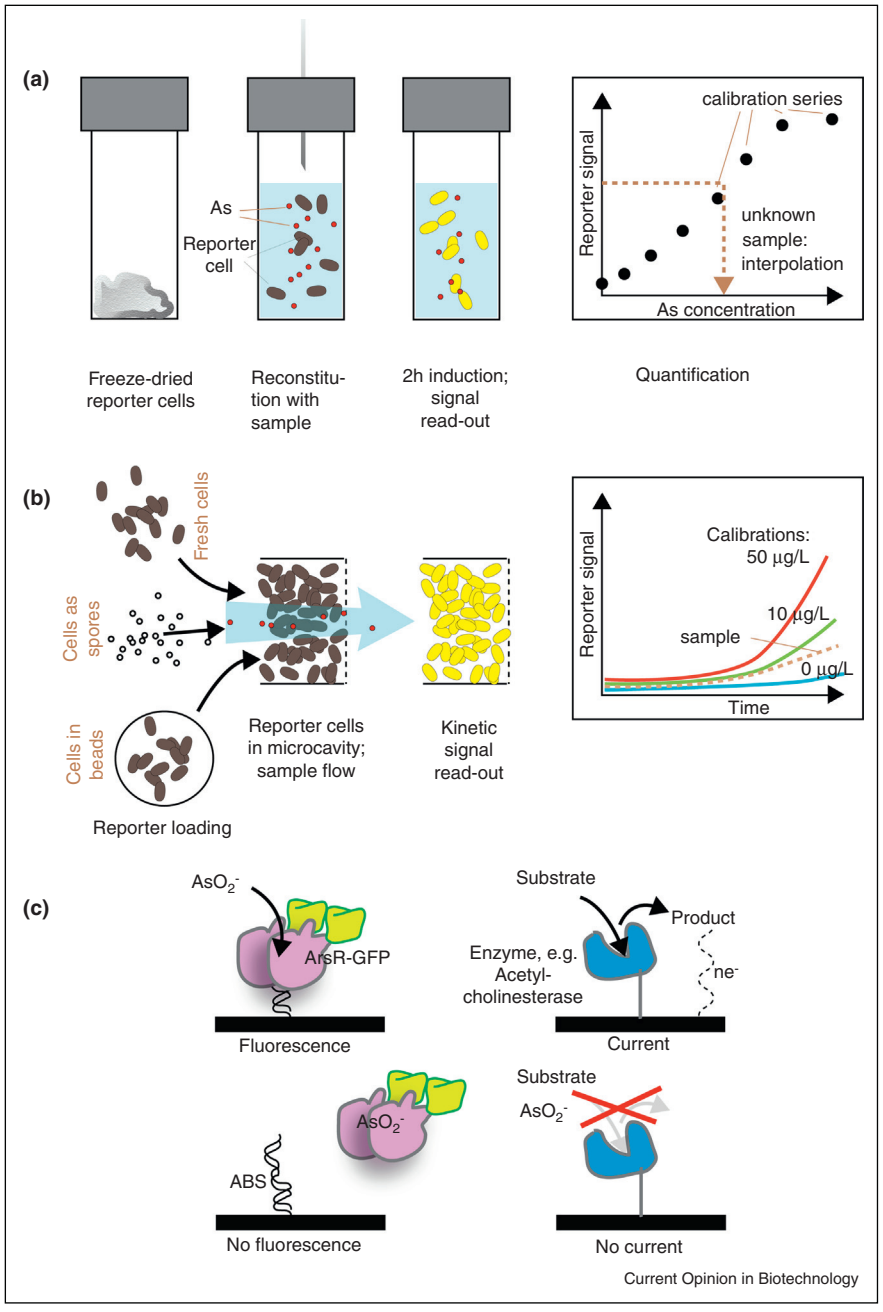
Genes and regulatory elements in the design of arsenic bioreporters. **(A)** Detail of the *ars* promoter region of the *arsRDABC* operon of plasmid R773 **(B)**. **(C)** DNA parts representation of a typical *arsR*–P_{ars} based gene circuitry. **(D)** Circuitry functioning of the native ArsR repressor loop, the insulated version with the extra ArsR binding site [24], the uncoupled loop [37], and the period oscillator [38••]. For explanation, see main text. Thick arrows: promoters; thin arrows: activation; flat lines: repression. AHL, acylhomoserine lactone.

operons are also found in anaerobic arsenate-respiring bacteria, which, in addition to an *arsDABC* resistance operon carry the genes for arsenate respiration (e.g. *arrA* and *arrB*) [18]. Recent data suggest that the genes for arsenate respiration are also under control of an arsenite-dependent ArsR-repressable system, the gene for which is located outside the resistance operon [18].

The discovery of the arsenic-binding *trans*-acting ArsR repressor was the basis to propose the genetic engineering of a bacterium (in the following: a 'bioreporter') that would be capable of producing a specific non-cognate reporter protein in response to contact with arsenite [19–22]. The genetic circuit that permits such arsenite-dependent reporter protein formation typically consists of the DNA fragment encompassing the ABS and the *arsR*-promoter, the *arsR* gene and the gene for the reporter protein (Figure 1). Such circuit was constructed several times independently with some small differences in, for example, host background or source of *ars* and reporter genes but with in essence similar functionality [20–24,25•]. Arsenite (or antimonite) entering the reporter cell is thus detected because

it binds ArsR, which leads to derepression of the *arsR*-promoter and *de novo* transcription of the reporter gene. The amount of produced reporter protein is proportional to the arsenite concentration within a certain range (Figure 2A). The arsenic concentration in unknown samples is thus quantified by interpolating the bioreporter response to a calibration series of known arsenite concentrations under the same assay conditions (Figure 2A) [24,26]. Although the *arsR*–P_{ars} system is specific for arsenite and antimonite, arsenate can be detected by the same reporter strain at one-fifth of the sensitivity of arsenite, probably because it is first reduced to arsenite by the cell [27]. Trimethylarsenoxide is also detectable at one-tenth of the response to arsenite [27]. Interestingly,

Figure 2



Arsenic bioreporter and biosensor assays. **(A)** Classical assay format with reporter cells in solution, here outlined as performed by Siegfried *et al.* [61••]. **(B)** Microfluidics-type bioreporter assays with cells loaded in microcavities through which the sample is perfused, as in [52•]. Spore applications outlined by Daunert and co-workers [48•,49]. **(C)** Concept of the ArsR-GFP/immobilized operator DNA bioassay in 96-well format [30,31], and of the arsenic enzyme inhibition sensors [32–34].

by tuning the intracellular expression level of ArsR the same genetic circuit can selectively differentiate between phenylarsenite and methylarsenite, and arsenite [25•].

Apart from the *arsR*–P_{ars} systems, two alternative inducible eukaryotic promoters have been exploited to construct bioreporters for arsenic detection. One of these consists of the promoter of the *acrA* gene from *Aspergillus niger*, which encodes a putative arsenite efflux pump [28]. Interestingly, an *A. niger* bioreporter carrying an *acrA–egfp* transcriptional fusion is responding to arsenite and arsenate, but not to antimonite [28]. A second alternative gene reporter circuit was based on the *UFO1* promoter of *Saccharomyces cerevisiae*, which is inducible by UV-B and DNA damage, but also by arsenate [29]. When a *UFO1* promoter-*luxAB* fusion was introduced into a *S. cerevisiae pdr5*Δstrain lacking an ABC-type transporter to extrude arsenate, luciferase expression was induced at arsenate concentrations as low as 1 pM [29].

Cell-free biosensors for arsenic

A number of cell-free bioassays and biosensors for arsenic detection have also been recently proposed, which take advantage of either specific As-protein interactions, or of competitive inhibition by As of more general enzymatic reactions. Maeda and co-workers [30,31] developed a bioassay in which the reversible binding of ArsR-GFP protein to its operator DNA is tested in presence or absence of As (Figure 2C). Hereto, the operator DNA is immobilized on microplate well surfaces, to which ArsR-GFP from a cell-free extract is bound, resulting in maximum starting fluorescence levels. Addition of an arsenite-containing sample will cause part of bound ArsR-GFP to dissociate from the operator, which is washed away from the reaction. This leads to a decrease in fluorescence intensity, which can be measured by a plate reader. Method detection limits of between 5 and 10 μg arsenite per L were reported for the *in vitro* ArsR-GFP ABS assay [30,31].

Three biosensors for arsenic detection described so far use surface immobilized or electrode immobilized enzymes. Arsenic is detected through non-specific competitive inhibition of enzyme activity and a decrease in the generated spectroscopic or amperometric signal (Figure 2C). One of those exploits the inhibitive action of arsenite (but not arsenate) on acetylcholinesterase (ACH) activity [32]. ACH activity can be measured amperometrically through the release of thiocholine from the substrate acetylthiocholine iodide in the enzyme reaction. The biosensor consists of ACH bound on screen printed carbon electrodes. Detection starts by the addition of substrate, leading to maximum current output. Subsequent addition of a sample with arsenite will inhibit ACH activity and will thus diminish current production [32].

To develop a specific sensor for arsenate, the same group of researchers turned to the enzyme acid phosphatase (from potato), which was again immobilized on screen printed carbon electrodes [33]. Acid phosphatase can catalyze the conversion of the substrate 2-phospho-L-ascorbic acid, liberating the electrochemically active compound L-ascorbic acid, which can be measured by cyclic voltammetry. Arsenate inhibits the enzyme reaction, therefore, less L-ascorbic acid is released and less current is produced [33].

Finally, also immobilized cytochrome *c* was used for arsenic detection (here in form of As_2O_3) [34]. Cytochrome *c* is an electron-transfer protein, the activity of which can be measured by electrochemistry directly on enzyme-bound boron-doped diamond surfaces. Not only arsenic but also other typical cytochrome *c* inhibitors such as cyanide, will bind to the protein and will reduce electron transfer, which can be measured by a variety of different techniques.

Optimizing gene circuitries for arsenic detection

In particular the *arsR*–P_{ars} systems have been extensively used in approaches to better tune the natural repressor-promoter configuration for more optimal detection possibilities. In the native *arsRDABC* or, for that matter, *arsRBC* resistance operons, *arsR* transcription is under control of its own promoter P_{ars} (Figure 1). In this configuration the promoter is a bit leaky to allow for production of ArsR, which needs to repress the system in absence of arsenite. Early constructions using reporter genes that were placed downstream of the *arsR* gene suffered, therefore, from a relatively high background reporter protein expression even in the absence of any arsenite in the system [24]. To reduce background expression of the reporter gene but to keep transcription of the *arsR* gene itself intact, Stocker *et al.* almost ten years ago successfully used a transcriptional 'insulator' [24]. This insulator consisted of a second copy of the ABS-DNA, placed downstream of the *arsR* gene and upstream of the reporter gene (Figure 1C, D). Probably, this approach worked satisfactorily because the ArsR-dimer bound downstream of the *arsR* gene blocks RNA polymerase from continued transcription even when starting at the P_{ars} promoter. Secondly, having two ABS in the gene circuitry DNA reduces the likelihood of liberating both bound ArsR-dimers simultaneously except for at high arsenite concentrations.

Secondly, a series of optimizations were engineered in the ribosome binding sites of the reporter gene in an *arsR-lacZ* configuration [35]. This resulted in a decrease of the arsenite concentration from 10 to 0.2 μg/L at which the reporter signal became visible [35]. This difference in response range of the various bioreporter cell lines was then used to propose a calibration-independent

(or 'traffic-light') reporter system [35,36]. Tani *et al.* were the first to uncouple the natural *arsR*–P_{ars} configuration by placing the expression of *arsR* in *trans* under control of either P_{T7} or P_{lac} but maintaining *gfp*-expression under control of P_{ars} (and thus ArsR, Figure 1D) [37]. Interestingly, they found that overexpression of ArsR was toxic to the *E. coli* cells, but that 'uninduced' levels of ArsR expression from P_{T7} or P_{lac} were sufficient to control *gfp* transcription from P_{ars}. In addition, they tested the effect of having one, two or three tandems of P_{ars}-*gfp* on the arsenite-dependent GFP signal formation. Three tandem reporter copies indeed improved the signal-to-noise ratio, but only in the uninduced P_{lac} and not the P_{T7} system. Detection of 50 μg As/L after 6 hours incubation was achieved, but that of 10 μg As/L remained problematic and close to the detection limit [37]. Chen *et al.* recently showed that tuning the intracellular expression levels of ArsR from *Acidithiobacillus ferrooxidans* with the help of the arabinose-inducible P_{BAD}-promoter can change the selectivity of detection from arsenite to phenylarsenite and methylarsenite [25•].

A completely different approach was taken by Prindle *et al.*, who used *arsR*–P_{ars} to wire a gene circuitry that produces oscillating amounts of an unstable superfolder variant GFP in *E. coli* reporter cells maintained in microfluidics cavities (so called 'biopixels') [38••]. Arsenite-dependent ArsR control is mediated not on *gfp* directly, but on components of the acyl-homoserine lactone quorum-sensing system (*luxR* or *luxI*), which on their turn control *gfp* expression (Figure 1D). Synchronized oscillation within the cells in a biopixel is further achieved by LuxR-control of expression of *aiiA*, which encodes a lactonase that degrades acyl-homoserine lactone [39]. Synchronized oscillation between biopixels is achieved via gaseous diffusion of hydrogen peroxide, that is produced spontaneously as a result of oxygen radical formation at high GFP fluorescence. The H_2O_2 on its turn derepresses expression from the *lux* promoters via the cognate ArcAB aerobic response control system. Output is recorded from a few thousand biopixels simultaneously, which levels out cell-to-cell variations in expression. Rather than taking the absolute level of GFP expression as measure for arsenite-dependent derepression of the system they used the duration of the oscillation period, which increases at higher arsenite concentrations. Interestingly, the oscillation period is independent of the absolute fluorescence intensity, therefore, making the biopixel system output independent of detector/light source fluctuations [38••].

Another complex rewiring approach in which the *arsR*–P_{ars} system was engaged was carried out during the iGEM 2006 competition by the University of Edinburgh team. Their goal was to create a sensor that would produce pH change through urease activity and lactose fermentation [40]. Hereto the authors modeled what would happen when *arsR* would drive expression of the phage lambda cI repressor, which on its turn repressed urease expression. In addition, this system would have *lacZ* expression under control of *arsD*. Unfortunately, only the outlines of the system have been published but not the workable complete reporter strain. These examples show that the ArsR/P_{ars} system is not only relevant for environmental purposes but is interesting to integrate in more complex synthetic designs, which by now are often limited to such regulatory systems as TetR/P_{tet}, LacI/P_{lac}, cI/P_L or AraC/P_{ara} [41,42,43••,44].

Many research groups working with arsenic bioreporter strains have varied reporter genes in order to obtain different signal outputs, but essentially remained within the regular ArsR/P_{ars} architecture. Already in very early designs, beta-galactosidase (*lacZ*), bacterial luciferase (*luxAB*), or autofluorescent proteins (e.g. *egfp*) were employed [20–22,24]. Presence or activity of those reporter proteins can be detected with a variety of classical means, which have extensively been summarized in other excellent reviews [36,45]. This was more recently repeated by producing the yellow fluorescence protein (phiYFP) from ArsR/P_{ars} [46], or by producing the carotenoid-metabolizing protein CrtIBS from *Rhodopseudomonas palustris* [23]. This somewhat exotic reporter protein results in color changes in cells that can be observed with near-infrared light emitting diodes [47]. Wackwitz *et al.* produced an array of reporter strains each with a different kinetic mutant cytochrome *c* peroxidase from yeast as reporter protein [35]. Because of the difference in enzyme kinetics of the cytochrome *c* peroxidase mutants the individual strains produce visible signal in separate arsenite concentration ranges, which can again be used for a calibration-independent assay [35,36].

Optimizing bioreporter assay detection

In its simplest form, bioreporter assays for arsenic are carried out by incubating suspended reporter cells for a particular period of time in liquid medium with the (aqueous) sample, after or during which the development of the reporter signal is recorded and compared to a simultaneously processed calibration series with known arsenite/arsenate concentrations (Figure 2A, B). Typical incubation times for *E. coli arsR*–P_{ars} based bioreporters range from 30 min to a few hours, and detection limits of below 10 μg As/L can be reproducibly achieved [26]. In comparison, the afore-mentioned *S. cerevisiae UFO1-luxAB* bioreporter has a similar response time but is already induced 3-fold at 1 pM arsenate. However, its dynamic range is very poor, as reporter induction is no different between 1 pM and 1 μM arsenate [29]. By contrast, the *A. niger* fungal bioreporter is significantly slower with 12 hours response time and a method detection limit of ~20 μg As/L [28]. Depending on the detection method, number of replicates and incubation time,

the precision of the *E. coli arsR*–P_{ars} based bioassays (as relative standard deviation between replicates on spiked recovery assays) is in the order of 5% [26], which has not improved much in more recent assay formats or configurations. Various groups have miniaturized assay forms or have parallelized assays in microfluidics formats [38••,48•,49,50,51•,52•]. Miniaturized assays can have the advantage of using less material while performing replicates simultaneously in a single chip, and permitting close integration of reporter cells with electrodes or detectors. For example, Buffi *et al.* fabricated an integrated biosensor with micro-chambers to hold agarose-bead immobilized *E. coli* arsenic reporter cells, which could be stored frozen on chip with maintenance of activation potential [52•,53]. Collecting reporter cells in a specific incubation chamber permitted better capturing of the reporter signal, and both kinetic or end-point measurements showed reproducible and precise detection of 10 or 50 μg As/L (Figure 2B) [52•]. Parallel assays can also implicate multiple reporter strains by which different chemical targets in the same sample can be measured [50,51•,54]. Immobilized and constantly exposed panels of bioreporter strains with different target specificities were successfully used in a microfluidics system during 10 days to detect chemical assaults in water [55]. This and other efforts to grow or maintain bacterial reporter cells 'on chip' [56••,57,58–60] may permit in the future to have automated reporter platforms on which cells can be maintained during long time and detect toxicants in sampled water or air streams.

An alternative interesting approach to maintain reporter cells on a microfluidic platform for prolonged periods of time was to use spore-forming bacteria. This concept was developed and tested by Daunert and co-workers who reconstructed arsenite and zinc-sensing reporter circuits in *Bacillus subtilis* or *B. megaterium* [48•,49]. Spores re-enter into their vegetative state in a period of between 90 min and 2.5 hours, after which the vegetative cells can react to the target compound and produce the reporter protein. The authors embedded the systems in a rotating microfluidic disk, which opens reservoirs at specific rotating speeds and mixes reagents in a set of predefined chambers [48•,49].

Real life measurements with arsenic bioreporters and biosensors

Despite all the promising developments in arsenic bioreporters and biosensors, and demonstrations of proof of principles, there are actually very few serious studies that have critically evaluated arsenic bio-assays under real-life field conditions [61••,62]. Most of the reporter assays summarized above have a reported detection limit (under laboratory conditions) in the range of what is desired from exposure limits (e.g. 10–50 μg As/L), but this is often compromised by other ions present in the water sample or conditions in the field [62]. Arsenic enzyme biosensors have a potentially 10-fold to 50-fold lower detection limit than bioreporter assays and faster response times, but may suffer from interferences with other ions and have not yet been extensively tested in the field [32–34]. Whereas the response time of a single bioreporter assay is not particularly fast (30 min up to a couple of hours), the time needed for induction can be used to prepare multiple sample assays in series. As an example, de Mora *et al.* simultaneously monitored 50 groundwater samples for color formation in an *arsR*–P_{ars}-*lacZ E. coli* bioreporter test [63]. Even though the individual assays took up to 60 hours to have a reliable result, multiple simultaneous sample processing reduces the time needed per sample. Siegfried *et al.* used a faster responding *E. coli* luciferase reporter assay for arsenic (exactly 2 hours incubation per test) in a screening of hundreds of samples from individual contaminated pump wells in villages in Bangladesh [61••]. Tests were performed on freeze-dried reporter cells in small glass vials, which were reconstituted by injection of the water sample and in which also the luciferase measurement was performed, using a portable luminometer (Figure 2A). EDTA was added to reduce possible interference with iron in the water that may complex As to iron-hydroxides. By running multiple samples in series, they could analyze all wells in a village in an afternoon. Simultaneously carried out (laboratory) ICP-MS analysis for arsenic in the same samples showed a very good correlation to the bioreporter assay results. Importantly, the closed glass vial test is considered a closed system and as such not a release of a genetically modified organism, which can otherwise frustrate field applications of bioreporter assays [61••].

Conclusions

Arsenic bioreporters and biosensors form an excellent example of biotechnological developments in response to a very pressing and world-wide contamination problem. Both bioreporter and biosensors make simple but precise assays, and can be carried out in a variety of flexible formats. Important advances have been made to preserve reporter cells and to miniaturize assays, which can increase their throughput and facilitate their integration in stand-alone measurement units. More importantly, simple and inexpensive bioreporter assays have been demonstrated to work accurate and competitively in the field. Although there is certainly room for further new creative developments and test improvements it would be timely for administrators and regulatory agencies to officially accredit the arsenic bioassays, enabling their market introduction and more wide application in areas affected by arsenic contamination.

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