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Online Monitoring of Water Toxicity by Use of Bioluminescent Reporter Bacterial Biochips

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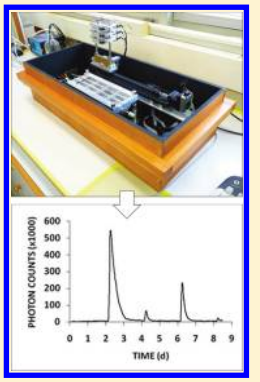
Supporting Information

ABSTRACT: We describe a flow-through biosensor for online continuous water toxicity monitoring. At the heart of the device are disposable modular biochips incorporating agar-immobilized bioluminescent recombinant reporter bacteria, the responses of which are probed by single-photon avalanche diode detectors. To demonstrate the biosensor capabilities, we equipped it with biochips harboring both inducible and constitutive reporter strains and exposed it to a continuous water flow for up to 10 days. During these periods we challenged the biosensor with 2-h pulses of water spiked with model compounds representing different classes of potential water pollutants, as well as with a sample of industrial wastewater. The biosensor reporter panel detected all simulated contamination events within 0.5−2.5 h, and its response was indicative of the nature of the contaminating chemicals. We believe that a biosensor of the proposed design can be integrated into future water safety and security networks, as part of an early warning system against accidental or intentional water pollution by toxic chemicals.



INTRODUCTION

Recent decades have witnessed a rise in water safety awareness. Increasing pollution levels dangerous to human and environmental health, complex water supply systems prone to technical problems and human errors, a potential collapse of infrastructure in the case of natural disasters or wars, and the growing threat of chemical warfare and terrorism have all highlighted the crucial need for online continuous water monitoring devices.

The traditional approach for detecting chemicals in water is based on chemical or physical analysis and allows highly accurate and sensitive determination of the exact composition of any sample. Such methodologies, however, while essential for regulatory purposes, require skilled personnel and sophisticated equipment. They also demand a substantial amount of time, especially when there is no preliminary information concerning the sample's contents. These drawbacks make it hard to integrate analytical techniques into continuous monitoring instrumentation designed to provide an early warning if water quality is compromised. More suitable for this purpose is the bioassay-based approach, which utilizes live organisms in order to monitor water quality. The organisms, ranging from fish to microbes, are continuously exposed to the tested water, and changes in their behavior, morphology, or other bioproperties indicate the presence of toxic substances.[1−3] The use of unicellular microorganisms, in particular bacteria, offers several advantages over animal-based assays. Their low cost, easy maintenance, and rapid response make them an appealing option for pollution monitoring. An additional attractive characteristic of microorganisms is that they can be genetically engineered to respond by a detectable dose-dependent signal to prespecified changes in their environmental conditions.[4]

Numerous recombinant bacterial reporters have been investigated. Most of these harbor a plasmid-borne fusion between a stress- or chemical-specific gene promoter and a reporter gene(s), the expression of which can be readily quantified.[5,6] Common among reporter genes are *luxCDABE*, which encode for bacterial bioluminescence and can be found in either terrestrial or marine bacteria such as *Photorhabdus luminescens* and *Vibrio fischeri*. When the designated environmental conditions are met, the transcription of the *lux* operon is promoted and a light signal is produced that is proportional in intensity to the magnitude of the stimulus. Examples include bacterial reporters responsive to genotoxic agents and oxidative stress,[7,8] as well as heavy metals; benzene, toluene, ethylbenzene, and xylene (BTEX); and polycyclic aromatic hydrocarbons (PAHs).[9−13]

These and many other examples have mostly been restricted to laboratory environments. Other reports have described innovative designs for the integration of such recombinant luminescent

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Table 1. Bacterial Reporter Strains Used in This Study

stress/chemical detected	*E. coli* host	gene promoter	primer sequences[a,b]
DNA damage	RFM443	*recA*	5′-CCGTCGTGGTACCAATGGCGATAG-3′
			5′-CGATGAGCTCTTTTACTCCTGTCATG-3′
oxidative	DH5α	*micF*	5′-CCGAATGCGAGCTCTCCGGTTG-3′
			5′-CCTCATTAATCAGTCGGTACCTCC-3′
heavy metals (As, Sb)	MG1655	*arsR*	5′-CGTAGCCGAGCTCCCAATTG-3′
			5′-CGATTGGTACCGTTGGTTTAAC-3′
cytotoxicity	RFM443	CP38	

[a] Restriction sites are underlined. [b] CP38 was synthesized as double-stranded DNA according to the sequence published by Jensen and Hammer.[23]

reporter bacteria into an online early warning water monitoring system. Gu and co-workers[14,15] have developed a multichannel system featuring stress-responsive reporter strains kept in suspension in a steady physiological state. Each channel was assigned with a reporter strain responsive to a specific kind of stress, and the response profile was used for pollution classification. Avoiding the complexity inherent in maintaining a continuous culture,[16] other researchers have immobilized luminescent reporter cells on the tips of a liquid light guide or an optical fiber.[17,18] Immobilized reporters have also been loaded onto a disposable card.[19] Recently, such a card was employed in the assembly of a multistrain biosensor for the online detection of metal pollution and its classification.[20,21]

Here we describe a new design for online continuous water monitoring with integrated recombinant luminescent reporter bacteria. A panel of bacterial reporter strains, characterized by different toxicant response spectra, is immobilized in specialized polymeric biochips placed in separate flow-through chambers. The biosensor can detect and characterize a range of pollutants with easy installation and minimal maintenance. Light is detected by single-photon avalanche diodes, a technology not previously implemented in similar apparatus. We also demonstrate, for what is to our best knowledge the first time, a recombinant bacteria-based monitoring device that operated successfully under a continuous water flow for several days.

EXPERIMENTAL SECTION

Plasmid and Bacterial Reporter Construction. A promoterless plasmid (pBR2TTS) harboring the *Photorhabdus luminescens luxCDABE* genes served as the basis for all luminescent constructs employed in this study. The construction of this plasmid was described elsewhere.[22] Briefly, a fragment of the *P. luminescens lux* operon was incorporated into a pBR322 derivative and two transcription termination sites were placed upstream of the multiple cloning sites, to reduce background expression of the *lux* genes. The operon encodes for both luciferase (*luxAB*) and the fatty acid reductase complex (*luxCDE*), making the addition of an external substrate unnecessary. The *arsR*, *recA*, and *micF* gene promoter-containing segments were obtained by PCR amplification from the *Escherichia coli* K12 MG1655 chromosome. The synthetic promoter CP38 was synthesized as double-stranded DNA according to the sequence published by Jensen and Hammer.[23] All were ligated into the pBR2TTS promoterless plasmid through *Sac*I and *Kpn*I restriction sites. The plasmids were chemically transformed into *E. coli* K12 strain AG1688, and transformants were selected on Luria−Bertani (LB) agar supplemented with ampicillin. Promoter sequences and orientation were verified by sequencing. The plasmids were then extracted and were chemically transformed into *E. coli* K12 host strains MG1655 (*arsR* and CP38), RFM443 (*recA*), and DH5α (*micF*); these constructs were used in the subsequent experiments. The bacterial reporter strains are listed in Table 1 and are hereafter designated by the *lux*-fused gene promoter they carry.

Reporter Bacteria Storage. The bacterial reporter strains were grown overnight at 37 °C with shaking (200 rpm) in LB medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) supplemented with ampicillin (100 μg/mL). The overnight cultures were mixed with glycerol to a final glycerol concentration of 15% and stored at −80 °C.

Microtiter Plate Assays. Stored bacteria were plated over LB−agar Petri dishes supplemented with ampicillin (100 μg/mL). Fresh colonies were grown overnight in LB medium supplemented with ampicillin at 37 °C with shaking (200 rpm). Bacteria were diluted 100-fold in fresh LB medium and regrown under the same conditions to the midlogarithmic growth phase ($OD_{600nm} \approx 0.3$). The culture was mixed with one volume of either fresh LB medium (for suspended culture assays) or 1.5% LB−agar solution kept at 45 °C (for immobilized bacteria assays). The mixture was pipetted in 60 μL aliquots into an opaque white 96-well microtiter plate (Lumitrac 200; Greiner Bio-One), and the agar was allowed to solidify where relevant. A dilution series of the tested toxicant, including a toxicant-free control, was added in 60 μL aliquots. Luminescence in relative light units (RLU) was measured with a temperature-controlled (37 °C) plate reader (Victor2; Wallac, Turku, Finland) at 10 min intervals.

Biosensor Device Description. The biosensor device, depicted schematically and in photographs in Figure 1, contains four flow-through chambers, each consisting of a glass layer, a poly(dimethylsiloxane) (PDMS) chip, and a poly(methyl methacrylate) (PMMA) cover. The PDMS chip is perforated with a 3 × 4 cylindrical cavities, 4 mm in diameter and 5 mm deep each, giving a total volume of ca. 60 μL. The PMMA cover is carved with a serpentine channel 2 mm in diameter and 2 mm deep. A screw system presses the glass, the perforated PDMS, and the carved PMMA against each other, forming a flow channel with 12 wells in its path. The four flow-through chambers are separately connected to four feeding tubes heated to 40 °C by an elastic heating tape plugged to a digital temperature controller (Eurotherm 3216, Eurotherm). Four other tubes navigate the discharged fluids to a waste container. Three aligned single-photon avalanche diode (SPAD) devices (MPPC C10507-11-100U, Hamamatsu, Japan) detect and quantify the light signal. The detectors are connected to a single-axis linear stepper motor (MDrive, IMS, Marlborough, CT). As the monitored water passes over the immobilized reporters, the detectors scan along the flow-through chambers, stopping above each well to measure the signal. A dedicated LabVIEW program controls the movement of the detectors and records the light signal. The flow-through chambers−detectors

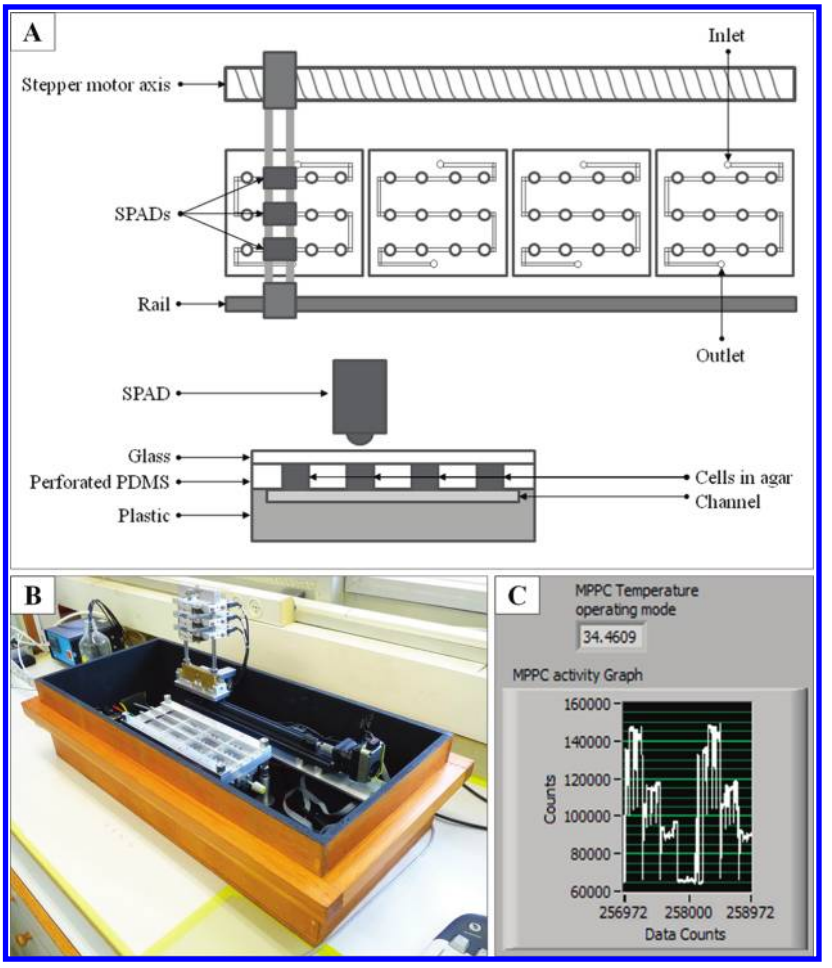

Figure 1. (A) Partial scheme of the biosensor device: a top view of the flow-through chambers, the single photon avalanche diode (SPAD) detectors, and the stepper axis (upper panel) and a cross-section of a flow-through chamber and a SPAD detector (lower panel). Each flow-through chamber is constructed of three layers (glass, PDMS, and PMMA), which form a flow channel with 12 wells in its path. (B) Photograph of the biosensor device. (C) Snapshot of SPAD detector activity as presented on the computer screen. Shown are two scan intervals and the SPAD detector temperature.

complex is kept inside a closed wooden box to ensure the appropriate dark conditions for photon counting.

Device Operational Procedure. The reporter bacteria were grown overnight and then regrown as described under Microtiter Plate Assays above. The bacteria were concentrated to a density of $(2.5-5) \times 10^9$ cells/mL, in order to intensify the light signal, and mixed with one volume of 1.5% LB−agar solution kept at 45 °C. The mixture was immediately pipetted in 60 μL aliquots into the designated PDMS biochip wells and allowed to solidify. Depending upon the experimental requirements, each PDMS biochip was loaded with either a single reporter strain, occupying all of its 12 wells, or a combination of four reporter strains, each occupying a different three-well column. For short-term experiments, toxicants dissolved in 2-fold diluted LB were made to flow through each of the chambers ($1.5\ mL \cdot min^{-1} \cdot chamber^{-1}$) for several hours, immediately after the biosensor device was set, by use of a multichannel peristaltic pump (504S; Watson-Marlow, Wilmington, MA). For long-term operation, tap water was made to flow continuously through the biosensor device for 4−10 days by use of a multichannel peristaltic pump (504S; Watson-Marlow, Wilmington, MA). A second multichannel peristaltic pump (Minipuls 3; Gilson, Middleton, WI) was employed to supply the immobilized reporter bacteria with 10-fold concentrated LB medium supplemented with ampicillin (2 g/L). The tap water and LB medium streams converged upstream of the flow-through chambers. The LB medium was made to flow at a flow rate 39-fold lower than the flow rate of the tap water, resulting in LB and ampicillin final concentrations of 25% and 50 μg/mL, respectively. The total flow rate was set to $1.5\ mL \cdot min^{-1} \cdot chamber^{-1}$. During the operation period, the biosensor was challenged with 2-h pulses of tap water spiked with different toxic chemicals. Luminescence from each well was recorded every 60 s in all experiments. Standard error of the mean of both light intensities and response times did not exceed 20% between experiments.

Signal Processing. All signals were initially processed by a digital low-pass filter to improve signal-to-noise ratio, similarly to what has been described by Daniel et al.[24] Then the average reading of replicate wells was calculated and was smoothed by a simple moving median filter. Finally, the difference between consecutive average readings was calculated and was smoothed by a simple moving average filter. The response time of a specific reporter strain to a specific chemical was determined as the interval between the time of exposure and the time when the smoothed difference exceeded its mean baseline by a predetermined number of standard deviations. The size of the time window used by the filters and the number of standard deviations

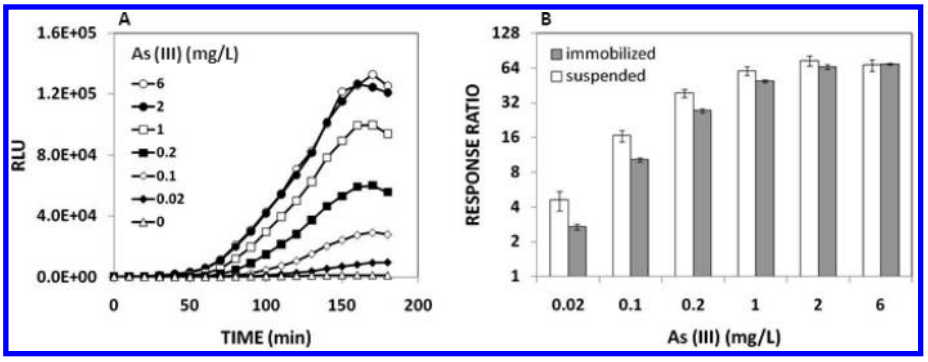

Figure 2. Response of the *arsR* reporter to As(III) in a microtiter plate assay. (A) Response kinetics of immobilized cells. Luminescence is reported in relative light units (RLU). (B) Response ratios (luminescence level of the sample divided by that of the control) of suspended and immobilized cells 120 min after exposure. Experiments were repeated three times. Error bars represent sample standard deviation. Responses of the other reporter strains are presented in Figure S1 in Supporting Information.

by which the difference was to exceed its mean baseline were empirically optimized to ensure no false detections. The selected parameters were a time window of 90 min and a standard score threshold of 5 for both the *recA* and *arsR* reporters, and a time window of 50 min and a standard score threshold of 4 for the *micF* reporter.

Chemicals. The following chemicals were used in this study: nalidixic acid sodium salt, mitomycin C, methylviologendichloride hydrate (paraquat), menadione sodium bisulfite, sodium (meta)arsenite, and potassium antimony(III) tartrate hydrate. All chemicals were of analytical grade and were purchased from Sigma−Aldrich (St. Louis, MO). The chemical industrial wastewater sample tested in the course of this study was a kind gift from A. Brenner, Ben Gurion University of the Negev, Israel. The sample represents the combined wastewater stream of a chemical industrial park in southern Israel and is characterized by high dissolved organic carbon, total dissolved solids, and adsorbable organic halogens concentrations (200, 28,000, and 50 mg/L, respectively) and by neutral pH.

RESULTS

Three newly constructed inducible bacterial reporter strains (Table 1) were used in this study to demonstrate the functions of the water toxicity monitor. The strains incorporate fusions between the *lux* operon and the *recA*, *micF*, and *arsR* gene promoters, respectively activated by DNA damage, oxidative stress, and heavy metals, that have been previously used for the construction of similar bioreporters.[25−27] For an initial characterization of their responses, the three reporter strains were challenged with model toxicants in a microtiter plate assay. The *recA* reporter was challenged with nalidixic acid, a genotoxic antibiotic compound; the *micF* reporter was challenged with paraquat, a superoxide-generating herbicide; and the *arsR* reporter was challenged with arsenic, a metal contaminant of mostly natural origin. The response kinetics of all reporter strains, either in suspension or encapsulated in agar, were characterized by a lag phase, ca. 30 min long, followed by a dose-dependent increase in luminescence. Figure 2A presents, as an example, the responses of the *arsA* strain to arsenic; the responses of the other two reporters to their respective model toxicants may be found in Figure S1A,C in Supporting Information. The response ratios, denoting the ratio between the luminescence level of the sample and that of the control, calculated 2 h from the initiation of the exposure, are displayed in Figure 2B (for *arsR*) and Figure S1B,D (Supporting Information)

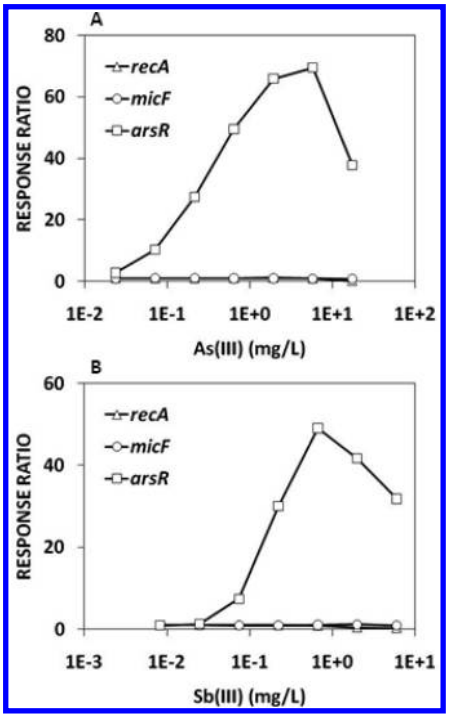

Figure 3. Response of the bioreporter panel following 120 min exposure to (A) As(III) and (B) Sb(III) as measured by a microtiter plate assay with immobilized cells. The experiments were repeated three times and the standard error of the mean did not exceed 15%.

(for *recA* and *micF*). Maximal response ratios obtained were 70 for arsenic (6 mg/L), 80 for nalidixic acid (20 mg/L), and 100 for paraquat (50 mg/L), for both immobilized and suspended cells.

To further assess the range of effector compounds of the reporter strain panel and its ability to distinguish specific contaminants, plate assays were carried out in which each of the three strains, agar-encapsulated, was challenged with all three model toxicants. Trivalent arsenic, which induced the *arsR* reporter, induced neither the *recA* nor the *micF* reporters (Figure 3A). Similarly, paraquat, which induced the *micF* reporter, induced neither the *arsR* nor the *recA* reporters (Figure S2C, Supporting Information). Nalidixic acid, in addition to strongly inducing the *recA* reporter, also induced *micF*, albeit to a much lower extent and at higher concentrations (Figure S2A, Supporting Information).

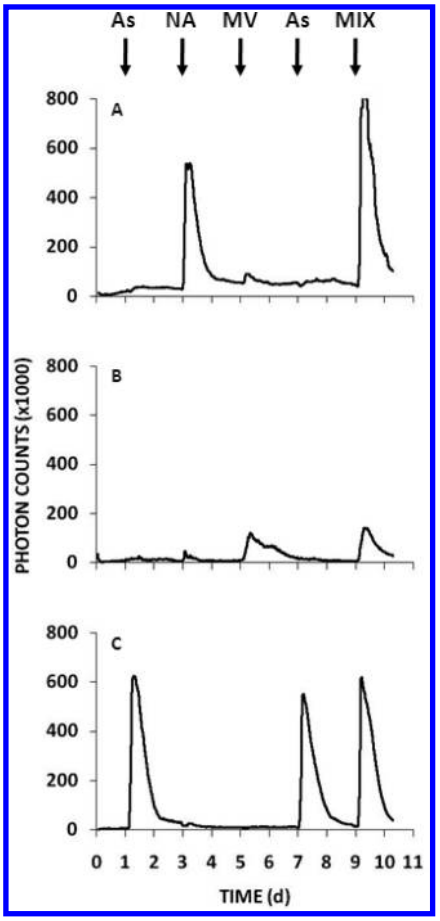

Figure 4. Detection of model toxicants in a 10-day monitoring experiment. Signals emitted by (A) *recA*, (B) *micF*, and (C) *arsR* reporters in the course of a 10-day tap water flow are shown. Arrows mark a 2-h pulse of tap water spiked with nalidixic acid (NA), paraquat (MV), As(III), or a mixture of all three (MIX). Curves represent averages of 12 replicate wells smoothed by a simple moving median filter.

In addition, all three reporter strains were also exposed to three additional substances: mitomycin C, a genotoxicant; antimony, a metal of environmental relevance; and menadione, an oxidant. The same pattern was maintained: trivalent antimony induced only the *arsR* reporter, menadione induced only the *micF* reporter, and mitomycin C induced the *recA* reporter and to a lower extent the *micF* reporter (Figure 3B; Figure S2B,D, Supporting Information). Overall, the different sets of toxicants clearly elicited distinctive response patterns from the reporter panel, which distinguish them from one another.

To validate the capacity of the reporter strains to detect toxic chemicals in water when immobilized in the biosensor device presented in Figure 1, biochips loaded with the three reporter strains were introduced into the device's flow-through chambers and were exposed for a few hours to three concentrations of the relevant model toxicant. The results (Figure S3, Supporting Information) demonstrated a clear dose-dependent response for each of the reporter strains, thus indicating that their activity in the biosensor device is equal to that exhibited in microtiter plates. Once this has been established, the ground has been set for long-term flow-through experiments, in which the biosensor was continuously operated for several days under continuous tap water flow.

Each of the *recA*, *micF*, and *arsR* reporter strains was immobilized in an individual biochip and placed in a different flow-through chamber. Tap water was pumped continuously through the system for 10 days, in the course of which five simulations of pollution events were carried out. In each simulation, the biosensor was challenged with tap water spiked with different toxicants. The signals that were recorded by the biosensor are depicted in Figure 4.

The first simulation was carried out 24 h after the initiation of the tap water flow, as the biosensor was challenged with As(III) (6 mg/L). The presence of the metal was reported by the *arsR* reporter with a response time of 60 min. The second simulation took place on day 3 with a nalidixic acid pulse (20 mg/L), leading to a response of the *recA* reporter after 80 min and also to a weaker response by the *micF* reporter after 106 min. Five days into the experiment, the introduction of paraquat (50 mg/L) induced a response by the *micF* reporter, which indicated the presence of the oxidative herbicide 162 min after exposure. At the same time, the *recA* reporter weakly responded to this stimulus as well, a phenomenon not observed in the microtiter plate assay. After a week of continuous flow, the first simulation event was repeated. The *arsR* reporter maintained its responsiveness and responded within 72 min. The fifth toxic pulse, introduced on day 9, was of a mixture of As(III), nalidixic acid, and paraquat at the above concentrations. The mixture induced all three reporters with response times of 79, 100, and 129 min for the *arsR*, *recA*, and *micF* reporters, respectively. All the responses were characterized by a relatively rapid increase in luminescence followed by a more gradual decrease of the signal back to its basal level (Figure 4). In general, the response patterns were identical to those observed in the plate assays, except for the response of the *recA* reporter to both nalidixic acid and paraquat. However, the ability of the biosensor device to distinguish the two contaminants from one another was not compromised, as the response pattern to the genotoxic agent was dominantly controlled by the *recA* reporter, whereas the oxidative stressor elicited a stronger response from the *micF* reporter (Figure 4).

To demonstrate the validity of the biosensor concept for samples other than standard laboratory solutions, the biosensor was exposed to a spike of a combined wastewater sample from a chemical industrial park in southern Israel. The three reporter strains were immobilized in separate biochips, and tap water was pumped continuously through the system as before. After 24 h, the biosensor was challenged for 2 h with tap water spiked with the wastewater sample at a concentration of 5% (v/v). The biosensor responded within 92 min with a luminescence increase of the *recA* reporter (Figure 5), indicating a potential genotoxic nature of the sample, possibly due to one of its halogenated organic constituents.[28] After an additional 48 h, the biosensor was re-exposed to the mixture of As(III), nalidixic acid, and paraquat, leading to induction of all three reporters (Figure 5) and verifying the specificity of the *recA* reporter's response to the wastewater sample. The data presented in Figure 5 depict the differences between consecutive readings, rather than actual photon counts as in Figure 4. In this form, the data allow the calculation of the response times as explained in the Experimental Section.

Having established the functionality of the biosensor in detecting the presence of toxic chemicals in a flow-through mode, its sensitivity in comparison to existing drinking water standards and guidelines was evaluated. The biosensor was equipped with a biochip housing *arsR* reporter cells and was challenged at 2-day intervals with As(III) at 6 mg/L and then at 0.01 mg/L

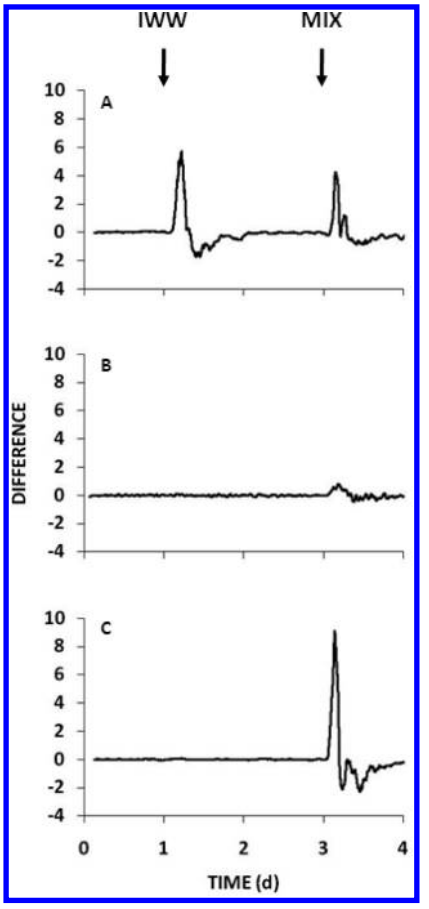

Figure 5. Detection of an industrial wastewater spike. Signals emitted by (A) *recA*, (B) *micF*, and (C) *arsR* reporters in the course of a 4-day tap water flow are shown, during which 2-h pulses of tap water spiked with industrial wastewater (IWW) or a mixture of nalidixic acid, paraquat, and As(III) (MIX) were introduced. Curves represent differences between consecutive readings after smoothing.

(U.S. EPA and EU standards),[29,30] and later with Sb(III), at concentrations of 0.02 (WHO drinking water guideline)[31] and 0.005 (equal to EU standard and lower than U.S. EPA standard)[29,30] mg/L. In all cases, a clear and distinctive response was measured (Figure 6), with no change in response times. The response to the presence of the lower antimony concentration, though significant, was nevertheless close to the lower detection threshold of the current biosensor configuration.

The modularity of the biosensor allows the use of one biochip for the sample while others are used as controls. In this configuration, an array of three reporter strains is immobilized in each of the biochips, each occupying a three-well column; the fourth column harbors the cells of a constantly luminescent strain (Table 1) that carries a fusion between a synthetic constitutive promoter (CP38) and the *luxCDABE* reporter genes.[23,32] This strain serves as an additional control for when a lack of induction of the three "lights on" reporter strains is caused by a sample toxicity that is too high. In such cases, sample toxicity is indicated by a "lights off" decrease in luminescence of the constitutive strain. One such multistrain chip is exposed to the tested water, while another constitutes a positive control and allows for the verification of reporter well-being and responsiveness and is thus exposed to a standard toxicant mixture. The third is used as a negative control, serving as a measure of the background signal and challenged with a buffer solution only.

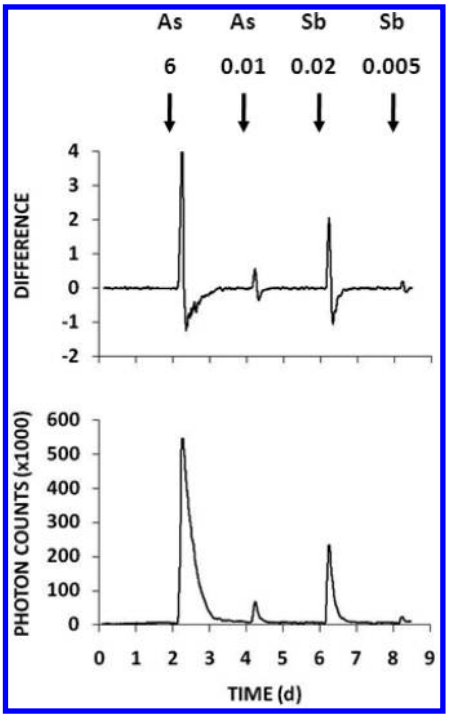

Figure 6. Arsenic and antimony are detected at concentrations relevant for drinking water regulations. The signal emitted by the biosensor device equipped with a biochip harboring *arsR* reporter cells and exposed to 2-h spikes of arsenic and antimony (arrows) at concentrations as noted in the figure (in milligrams per liter) are shown. Lower panel: replicate well average smoothed by a simple moving median filter. Upper panel: difference between consecutive readings after smoothing.

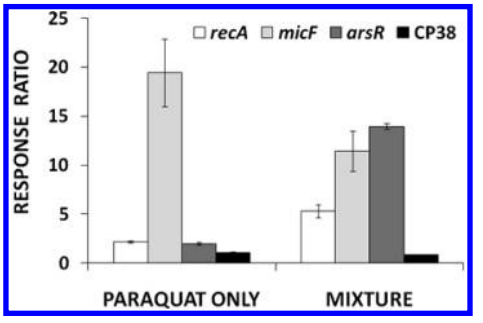

Figure 7. Device operation with positive, negative, and constitutive controls. Columns represent signals of the cells in (left) the "sample biochip" exposed to paraquat and (right) the "positive control biochip" exposed to a mixture of paraquat, nalidixic acid, and As(III), divided by the background signal as emitted by the cells in the negative control biochip, 2 h after exposure. Error bars represent the standard errors of the means of triplicate wells.

This configuration was demonstrated in an experiment the results of which are presented in Figure 7, in which the *recA*, *micF*, and *arsR* reporters as well as the CP38 control were arranged to form a multistrain array in each of three biochips. The biochips were exposed for 2 h to either paraquat; a mixture of As(III), nalidixic acid, and paraquat; or toxicant-free LB buffer. The results (Figure 7) indicate that where only paraquat was present, simulating the "sample chip", an increase in light emission was recorded for the *micF* reporter only. The toxic mixture (positive control)

induced all three inducible reporters, confirming their proper function. No responses were recorded from the negative control biochip, exposed to toxicant-free LB solution; similarly, no change in the signal emitted by the CP38 reporter exposed to the paraquat sample was observed, indicating that the sample content was not highly cytotoxic.

DISCUSSION

Scientific and technological advances now allow the coupling of reporter bacteria and diverse engineering platforms in the design of novel whole-cell biosensing devices.[5,33] Implementing this notion, we have constructed a biosensor for online continuous water monitoring. At the heart of the biosensor are disposable plastic biochips placed in a fluidics system and probed by sensitive light detectors. The biosensor operated for several days under continuous water flow, detecting and classifying all tested chemicals it was challenged with. By its nature, the main use of a device of the type described here, when fully operational, will not be for routine monitoring of water quality compliance with drinking water standards; the low levels of contaminants allowed by regulatory authorities do not require online supervision. In contrast, there is a critical need for such devices for the purposes of early warning against water contamination that may approach acute toxicity levels, and this niche is where the current design is aimed for. Nevertheless, as clearly shown in this paper for arsenic and antimony, the device's detection thresholds can be sufficiently low to also allow real-time testing of compliance with drinking water standards.

The long-term operation of the biosensor was possible owing to the physiological state in which the bacteria are maintained. After a few cycles of cell division, it is predicted that bacterial growth and motility may become limited by the physical constraints imposed by the agar matrix and the biochip well walls. The bacteria then reach a stable state, in which they do not divide but are nevertheless metabolically active. This state allows the bacteria to respond to changes in their environment. While a constant nutrient supply is not necessary for maintaining the viability and responsiveness of immobilized reporters subjected to a continuous flow of saline solution (Belkin, unpublished results), the nutrients are nevertheless essential to allow the reporter cells to respond to the presence of chemical threats. Our flow system thus included a constant supply of organic carbon and additional nutrients in a flow configuration that diluted the online sample by 2.5% only.

This design was capable of indicating not only that a toxic event has occurred but also the nature of the toxic chemical involved. This was accomplished by using a panel of reporter strains, each member of which is responsive to a different class of contaminants. The concept of using a combination of stress-responsive luminous bacteria as an analytical tool was first proposed by Belkin et al.[34] Here it was put into practice by assigning a different biochip in a different flow-through chamber to each reporter strain or by arraying different reporter strains in a single biochip. The capability of characterizing the pollution could prove to be a significant added value over existing biological early warning systems. These systems, the most common of which are based on fish or daphnia, only measure the toxicity of the tested water. Additionally, existing real-time water biomonitoring systems tend to be large and cumbersome. Our chip-based system, in contrast, is easy to install and to operate and has the potential for miniaturization.

Another innovation in the device described herein is the use of SPADs for measuring the signals emitted by reporter bacteria in a continuous monitoring system. Photodiode technology has been scarcely incorporated in bioluminescent biosensors due to its relatively low sensitivity. An exception is the development of the bioluminescent bioreporter integrated circuits (BBICs) that effectively exploited large-area photodiodes for low-level luminescence sensing.[35] SPADs, a unique form of sensitive photodiode technology, have recently been shown to be applicable for measuring bioluminescent signals of low intensities.[24] Unlike other technologies used thus far in similar systems, namely, photomultipliers or cooled charge-coupled device cameras, photodiodes in general and SPADs in particular are expected to decrease in price and size. Therefore, SPAD detectors offer promising prospects in terms of cost reduction and miniaturization, especially when parallel measurements are required.

Full automation of a biological early warning system requires dedicated software that will process and analyze the signal produced by the test organisms. In our case, the software should determine whether an observed increase in the light intensity of the bacterial reporters is significant enough for raising the alarm on a possible contamination event. Here we have laid out and optimized a procedure for signal processing and have used it for the determination of response times. Following further characterization of bioreporter basal light levels, this procedure could be integrated within a stand-alone version of the proposed biosensor. The dedicated software should, ideally, also contain a pattern recognition algorithm for classifying the toxicological nature of the pollutant. Such algorithms have been conceptually proven to provide a tool for identifying toxic chemicals based upon the response pattern of bacterial reporter strain panels.[36−39]

This paper provides a concrete proof of concept of a long-term flow-through toxicity monitoring biosensor by use of a limited range of model toxicants; further research efforts are clearly necessary before such a device can be put into full-scale field tests. In addition to the software development mentioned above, detection limits for a broader range of toxic chemicals should be established and the composition of the reporter panel should be adjusted to accommodate the expanded target chemicals' spectrum. A second challenge is to optimize storage conditions so that the end-user could be provided with ready-to-use biochips that could be easily loaded onto the biosensor device. While agar-immobilized *E. coli* reporters have been successfully stored for 2 weeks,[40] practical applications call for further study of preservation conditions that would allow a longer storage period. Attention should also be given to the potential escape of the reporter bacteria from the agar matrix, in light of the strict regulations meant to prevent the release of genetically modified organisms into the environment. Live cell counts reveal reporter bacteria concentrations of up to 10^8 cells/mL in the stream coming out of the biosensor device (data not shown), stressing the need to disinfect the effluent before it can be discarded.

Once these challenges are overcome, biosensor devices of the type proposed here can play an important role in an early warning scheme against accidental or intentional penetration of toxic chemicals into diverse type of water systems, from surface waters to municipal distribution networks.

ASSOCIATED CONTENT

Supporting Information. Three figures illustrating reporter strain performance as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org/.

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