Biosensors and Bioelectronics 64 (2015) 625–632

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A miniature porous aluminum oxide-based flow-cell for online water quality monitoring using bacterial sensor cells

Sharon Yagur-Kroll [a], Erik Schreuder [b], Colin J. Ingham [c], René Heideman [b], Rachel Rosen [a], Shimshon Belkin [a,*]

[a] Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel
[b] LioniX, Enschede, Netherlands
[c] MicroDish BV, Utrecht, Netherlands

ARTICLE INFO

Article history:
Received 30 June 2014
Received in revised form
23 September 2014
Accepted 25 September 2014
Available online 2 October 2014

Keywords:
On-line monitoring
Whole-cell bioreporters
Fluorescence
Bioluminescence
Porous aluminum oxide
Toxicity

ABSTRACT

The use of live bacterial reporters as sensing entities in whole-cell biosensors allows the investigation of the biological effects of a tested sample, as well as the bioavailability of its components. Here we present a proof of concept for a new design for online continuous water monitoring flow-cell biosensor, incorporating recombinant reporter bacteria, engineered to generate an optical signal (fluorescent or bioluminescent) in the presence of the target compound(s). At the heart of the flow-cell is a disposable chip made of porous aluminum oxide (PAO), which retains the sensor microorganisms on its rigid planar surface, while its high porosity allows an undisturbed access both to the sample and to essential nutrients. The ability of the bacterial reporters to detect model toxic chemicals was first demonstrated using a "naked" PAO chip placed on solid agar, and later in a chip encased in a specially designed flow-through configuration which enables continuous on-line monitoring. The applicability of the PAO chip to simultaneous online detection of diverse groups of chemicals was demonstrated by the incorporation of a 6-member sensor array into the flow-through chip. The selective response of the array was also confirmed in spiked municipal wastewater effluents. Sensing activity was retained by the bacteria after 12-weeks storage of freeze-dried biochips, demonstrating the biochip potential as a simple minimal maintenance "plug-in" cartridge. This low-cost and easy to handle PAO-based flow-cell biosensor may serve as a basis for a future platform for water quality monitoring.

1. Introduction

Traditional approaches for detecting the presence of undesired chemicals in water are mostly based on chemical or physical analyses, which allow highly accurate and sensitive determination of the exact composition of the tested sample. Such methodologies, however, fail to provide information regarding the bioavailability of pollutants, their effects on living systems, and their synergistic or antagonistic behavior in mixtures. They also demand a substantial investment of time, skilled personnel and sophisticated equipment, particularly when there is no preliminary information concerning the sample's composition. A complementary approach is based on the use of living systems in a variety of bioassays: test organisms, ranging in complexity from fish to microbes, are exposed to the sample, and changes in their behavior, morphology, or other biological responses that indicate the presence of toxic substances are monitored (Gerhardt et al., 1998; Shedd et al., 2001; Gerhardt et al., 2006). Unicellular microorganisms, in particular bacteria, are attractive for such purposes due to their large population sizes, rapid growth rates, low cost, easy maintenance and their amenability to genetic engineering (Belkin, 2003; van der Meer and Belkin, 2010).

Bacterial bioreporters have been molecularly engineered to detect specific chemicals, groups of chemicals, or global biological effects such as toxicity or genotoxicity (van der Meer and Belkin, 2010). In most cases, the engineered constructs harbor a sensing element that detects the presence of the target compound(s), fused to a reporter element, the expression of which yields a quantifiable output. The most common sensing elements used are DNA segments harboring gene promoter regions involved in the cellular response to the target chemical(s); of several available reporter elements, most commonly are the *lacZ, gfp* or *lux* genes, yielding colorimetric, fluorescent or bioluminescent signals, respectively. Numerous examples of bacterial sensors for diverse classes of chemicals have been reported, including the detection of genotoxic agents and oxidative stress (Lee et al., 2007; Biran et al.,

* Corresponding author. Fax: +972 2 6585556.
E-mail address: shimshon.belkin@mail.huji.ac.il (S. Belkin).

http://dx.doi.org/10.1016/j.bios.2014.09.076
0956-5663/

2009), toluene and related organic compounds (Applegate et al., 1998; Willardson et al., 1998), antibiotic substances (Melamed et al., 2012), tri- and di-nitrotoluene and related compounds (Yagur-Kroll et al., 2013), heavy metals (Trang et al., 2005; Magrisso et al., 2009) and more.

To turn such bacterial strains into on-line biosensors, they need to be integrated into a hardware platform containing all the components essential for continuous flow, combined with a sensing device (Elad et al., 2008). Elad et al. (2011) described a flow-through biosensor with disposable modular poly(dimethylsiloxane) (PDMS) chips, incorporating agar-immobilized bioluminescent recombinant reporter bacteria, with a continuous water flow for up to 10 days. It was shown that a 4-reporters panel detected all simulated contamination events within 0.5–2.5 h, and its response was indicative of the nature of the contaminating chemicals. Buffi et al. (2011) described a microfluidics biosensor for fluorescent *Escherichia coli* bioreporter cells immobilized in agarose beads for arsenite detection in aqueous samples. The microfluidic cartridge was composed of a PDMS block, containing channels and cages bonded on a microscope glass slide. Charrier et al. (2011) reported an on-line multi-channel biosensor for the detection of heavy metals and general toxicity, in which bioluminescent bacteria were agarose-immobilized in a removable card. The simultaneous on-line detection of one or more heavy metals as well as the measurement of the overall toxicity of the sample were demonstrated. Other researchers have alginate- or agarose-immobilized reporter bacteria on optical fiber tips (Eltzov et al., 2009), or on a multi-well transparent polycarbonate platform (Horry et al., 2007).

Here we describe a proof of concept for a new design for an online continuous water monitoring flow-cell with integrated recombinant reporter bacteria. The heart of the device is a disposable chip made of porous aluminum oxide (PAO), a nanoporous matrix that is a highly suitable for microbial culture support (Ingham et al., 2007): it is exceptionally porous (40% by volume), inert, and retains microorganisms on the rigid planar surface while allowing them full access to nutrients diffusing through the chip. In this study, the PAO-based chip has been employed in two forms. For initial examination of its capacity to support bacterial bioreporters, it was first tested as a "naked" PAO chip with cavities etched on its surface loaded with bacterial reporters. A dose-dependent fluorescent response was observed when the chip was placed on solid agar containing different concentrations of a target analyte. For demonstrating the suitability of the chip for online water quality monitoring, the PAO layer was incorporated into a specially designed flow system, and a concentration-dependent fluorescent response was demonstrated by a hydroquinone sensor strain. The induction of a 6-member bacterial reporter array by different chemicals was demonstrated, both in laboratory media and in wastewater effluents, as well as the detection of different optical outputs (green or red fluorescence and bioluminescence). To simplify future practical deployment of any live-cell biosensor system, it is highly desirable that the sensor cells are stored as an integral component of the device; for this end we have also demonstrated the applicability of on-chip live cell preservation by freeze drying. This easy to handle PAO-based flow-cell may provide a basis for a future online monitoring system for environmental, industrial or security applications.

2. Materials and methods

2.1. Bacterial reporters used in this study and growth conditions

The bacterial reporter strains used in this study, all of them previously characterized, are listed in Table 1. Fluorescent strains were grown in TGA medium (10 g L^{-1} Bacto tryptone, 5 g L^{-1} NaCl, 2 g L^{-1} D-(+)glucose, 11.9 g L^{-1} HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, $C_8H_{18}N_2O_4S$), pH 7.0). The bioluminescent strains were grown in Lysogeny Broth (LB), pH 7.0. Antibiotics for plasmid maintenance were employed according to Table 1.

2.2. Bacteria reporter induction on PAO chips: stationary system

Microbial PAO culture chips (8×36 mm^2; MicroDish model MDCC160.40, Ingham et al., 2007) containing more than 4000 round microwells (40 μm deep, 160 μm in diameter), coated by a 10 nm layer of platinum over the upper surface to limit autofluorescence (Fig. 1A, top), were used. The chips were sterilized by plasma cleaning (10 s, low power setting, Harrick PDC-002 Plasma

Table 1
Bacterial reporter strains used in this study.

	E. coli host	Sensing element (gene promoter)	Reporting element	Chemicals detected	Model chemical, mg/l	Antibiotic resistance[1]	Reference
1	MG1655	*recA*	*gfpmut2'*	DNA damaging agents	Nalidixic acid, 10	Amp	Lab collection
2	MG1655	*recA*	*gfpmut2*	DNA damaging agents	Nalidixic acid, 10	Kan	(Zaslaver et al. 2006)
3	MG1655	*micF*	*gfpmut2*	Oxidative stress agents	Paraquat, 400	Kan	Yagur-Kroll, unpublished
4	MG1655	*zntA*	*gfpmut2*	Heavy metals	Cadmium chloride, 25	Kan	(Zaslaver et al. 2006)
5	MG1655	*yqjF*	*gfpmut2*	2.4-DNT and byproducts	Hydroquinone, 200	Kan	(Zaslaver et al. 2006)
6	DH5α	*yqjFB2A1*	*gfpmut2*	2.4-DNT and byproducts	Hydroquinone, 200	Kan	Yagur-Kroll, unpublished
7	RFM443	*recA*	*DsRed-Express*	DNA damage	Nalidixic acid, 10	Amp	(Hever and Belkin 2006)
8	DH5α	*yqjFB2A1*	*luxCDABE*	2.4-DNT and byproducts	Hydroqinone, 200	Amp	(Yagur-Kroll et al. 2013)
9	MG1655	*CP38*	*gfpmut2*	Constitutive expression (no induction)		Amp	Yagur-Kroll, unpublished

[1]Amp, ampicillin (100 μg/ml); Kan, kanamycin (30 μg/ml).

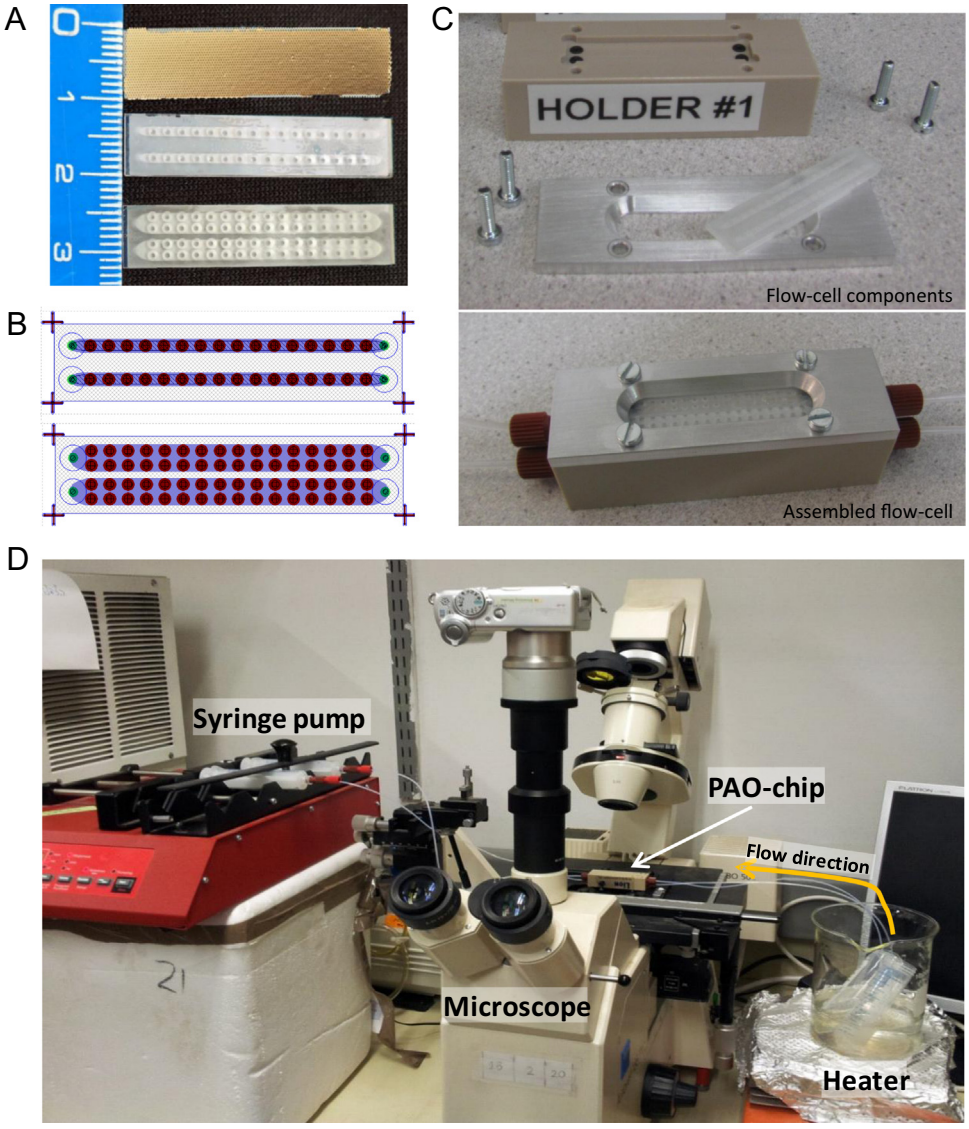

Fig. 1. The PAO-based flow-cell system. (A) A "naked" PAO chip with its 160 μm microwells (top) and disposable PAO-based flow-through chips with 16 × 2 (center) or 32 × 2 (bottom) positions. (B) Technical drawing of the PAO-based flow-through chips. (C) Flow-cell components (top): PAO chip, flow-cell cover, chip holder with tubing, and assembled flow-cell (bottom). (D) Fully assembled flow system with the flow-cell positioned on a microscope stage: the inflow tubes are placed in heated (37 °C) media reservoirs (right), while the outflow tubes are connected to a syringe pump (left).

Cleaner) and then placed on top of LB-agar containing predetermined concentrations of nalidixic acid (NA) in individual wells of a 6-well microtiter plate. A genotoxicity reporter strain harboring a *recA::GFPmut2′* fusion was grown overnight at 37 °C with shaking in TGA medium supplemented with ampicillin (100 μg/ml). The overnight culture was diluted × 1/10 in fresh medium, and 3 μl of the culture was inoculated onto the PAO chip. The plate was incubated at 37 °C and fluorescence was measured using a fluorescent microscope (AXIOVERT 135TV, Zeiss) and quantified using Fiji software (http://fiji.sc/Fiji).

2.3. Flow-cell assembly, operation and bacteria preparation

A disposable PAO-based flow-cell chip was constructed in which a microporous aluminum oxide chip (8 × 36 mm^2, 60 μm thick, trade name Anopore, General Electric, Germany) was positioned between two layers of glass. The top layer harbored 1 mm diameter powder-blasted holes which function as small compartments/wells to hold the report cells. The bottom glass layer contained access holes for sample inlet and effluent outlet, and two microfluidic channels to allow sample flow underneath each compartment. Two types of flow chips were designed: a 16 × 2 chip, in which each set of 16 cell deposition positions can be simultaneously exposed to two independent sample flows (Fig. 1A, center and Fig. 1B, top), and a 32 × 2 chip with twice the number of positions (Fig. 1A, bottom and Fig. 1B, bottom). The flow-cell system includes the PAO chip, a flow-cell cover, a chip holder with tubing and 4 screws (Fig. 1C, top). In the fully assembled system (Fig. 1C, bottom), the chip is placed in the chip holder, the cover of which is tightly sealed by screws. Water flow through the system is driven by a syringe pump (constant flow rate, 0.5 ml/h) connected to the outflow (Fig. 1D, left), with the inflow connected to a heated (37 °C) reservoir (Fig. 1D, right) containing the tested sample. The bacterial sensor strains were grown overnight at 37 °C with shaking in TGA or LB media supplemented with the appropriate antibiotic as listed in Table 1, and then concentrated × 20 in fresh medium. Aliquots (0.5 μl) of the concentrated culture were pipetted onto each well on the surface of the chip, and the bacteria were exposed to the target chemical prepared in TGA or LB media, pH 7.0. When wastewater effluents were used, 10-fold

concentrated TGA medium was added to a final concentration of ×1, and the pH was verified to be 7.0. For online monitoring of changes in fluorescence of the bacteria, the flow-cell was placed under a regular fluorescence (AXIOVERT 135 TV, Zeiss) or a dissecting fluorescence (Olympus) microscope. The magnification of the regular microscope allowed the simultaneous imaging of 2 wells, while the dissecting microscope enabled the imaging of the entire chip. For online monitoring of changes in bioluminescence, the flow-cell was placed in the dark, and images were captured by a digital camera (Nikon D700 using a 50 mm f1.4 lens, a 5 min exposure). Image analysis and processing were carried out with Fiji software (http://fiji.sc/Fiji), by measuring the fluorescence intensity of a 0.7 mm diameter circle at the center of each well and subtracting the background fluorescence of the chip material, as measured in a circle of the same diameter between two adjacent wells.

2.4. Freeze-drying protocol

On-chip long term preservation of the sensor bacteria was tested by freeze-drying of both the "naked" PAO chips and the flow-cells. Following overnight growth at 37 °C in TGA medium containing the appropriate antibiotic (Table 1), the culture was centrifuged and concentrated ×10 in 32% trehalose. Suspension aliquots were either spread on the surface of the bacterial culture chip (3 μl), or pipetted into each of the wells of the flow-cell chip (0.5 μl). Both chips were placed in sterile glass vials and freeze-dried (Advantage, VirTis). The procedure included an initial 2 h freezing (500 mTorr, shelf temp −40 °C) followed by 18 h of drying at 30–50 mTorr. After the lyophilization process, the vials were sealed under vacuum and stored at −20, 4 or 30 °C. Prior to the assay, the preserved vials were allowed to reach room temperature before the vacuum seal was broken and the chips were removed to test for bacterial fluorescence induction as described above.

2.5. Off-chip testing of bacterial activity

Prior to the assay, the bacterial reporter strains were grown overnight at 37 °C in TGA medium supplemented with 30 μg/ml kanamycin. Culture aliquots (10 μl) were transferred into an opaque black 96-well microtiter plate (Greiner Bio-One), each well already containing 90 μl of a predetermined concentration of the chemical tested dissolved in fresh TGA medium. Fluorescence (485/535 nm) was measured at 37 °C at 10 min intervals using a microtiter plate reader (Infiniti M200 PRO, TECAN). Fluorescence is displayed as the instrument's arbitrary relative fluorescence units (RFU), and activity as the difference in the intensity of the signal in the presence and absence of an inducer (ΔRFU).

2.6. Chemicals

The following model toxicants were used in this study: nalidixic acid sodium salt (NA), methyl viologen dichloride hydrate (MV, paraquat), cadmium chloride ($CdCl_2$) and hydroquinone (HQ). All chemicals were of analytical grade and were purchased from Sigma–Aldrich.

3. Results and discussion

3.1. Concept validation: genotoxicant detection by a PAO microbial culture chip

The PAO microbial culture chip is a miniaturized, disposable, highly porous ceramic chip with up to one million growth compartments (Fig. 1A, top). Square (1 cm^2) segments of these chips were tested for their ability to serve as support for bacterial sensors by placing them on LB agar supplemented with a concentration series of a model genotoxic agent, nalidixic acid (NA), a DNA damaging chemical. The model sensor strain employed, a genotoxicity sensor harboring a *recA::gfpmut2′* fusion, was inoculated on top of the chip. Fig. 2 demonstrates a quantified dose-dependent fluorescence output by this sensor strain in response to the presence of NA (2.5, 5, and 20 mg/l) compared to a toxicant-free control.

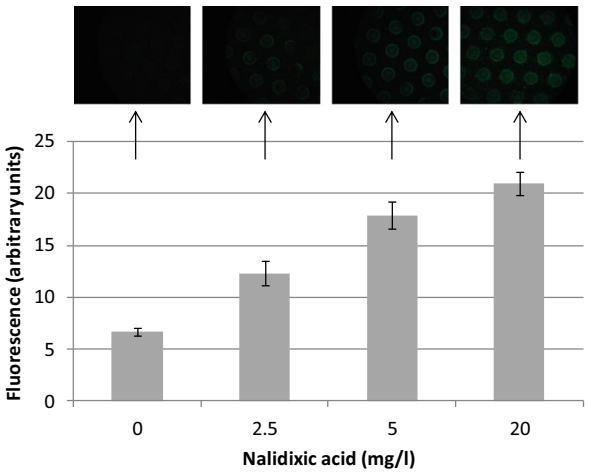

Fig. 2. Detection of model genotoxicant by a *recA::GFP* reporter strain using microbial culture chips. The fluorescent response of the *recA::GFP* genotoxicity reporter to four NA concentrations (0, 2.5, 5 and 20 mg/l) after a 2 h exposure is displayed as microscopic images and as the quantified fluorescence intensity of these images.

When first presented by Ingham et al. (2007), PAO microbial culture chips have been proposed for several complementary microbiological applications, including rapid viability testing, high-throughput fluorescence-based screening, and enzyme (β-galactosidase) assays. Later reports (Ingham et al., 2012) suggested the use of such chips also for microcolony-based antibiotic susceptibility tests (den Hertog et al., 2010), or investigating phenotypic heterogeneity (den Besten et al., 2010). Here we show, for the first time, that such chips can also serve as a viable support for bacterial sensor strains that generate a quantifiable optical signal in response to the presence of a toxicant. In order for this capacity to be of applied relevance for water quality monitoring, the chips need to be integrated into a flow-through device; this is demonstrated in the next section.

3.2. Demonstration of online chemical monitoring with a PAO-based flow-cell

Following the demonstration of the applicability of the PAO layer as a support for active bacterial bioreporters, it was incorporated into a specially designed flow system, rendering it suitable for online water quality monitoring. The fluorescent *E. coli* sensor strain employed was *yqjFB2A1::GFP*, harboring a fusion of a mutated *yqjF* gene promoter to GFP, previously shown to be a sensitive reporter of 2.4-dinitrotoluene (2.4-DNT) and hydroquinone (HQ) (Yagur-Kroll et al., 2013). The cells were deposited onto the chip and online fluorescence development in the presence of HQ was measured. Fig. 3A presents the quantitative concentration-dependent fluorescent response to HQ following a 90 minutes exposure. The linear response displayed allows the calculation of a detection threshold, which we have defined, as previously reported, as the sample concentration exhibiting a fluorescence value two-fold higher than the HQ-free control (EC_{200}; Yagur-Kroll

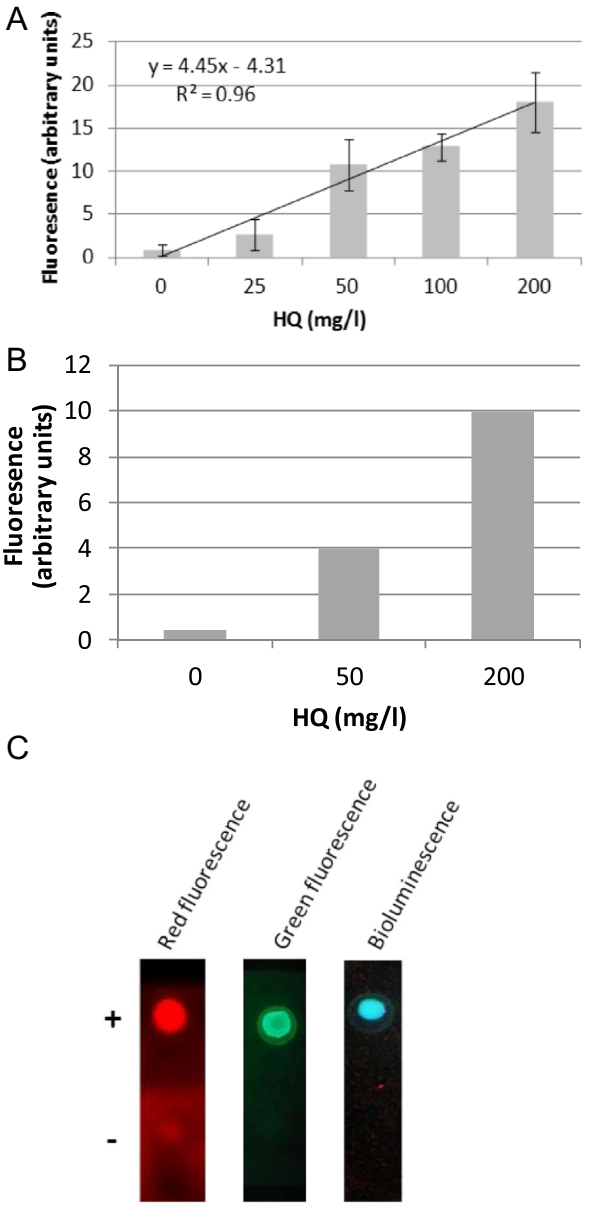

Fig. 3. Online detection of reporter strains activity in the PAO-based flow-cell. (A) A dose-dependent response by a *yqjFB2A1::GFP* reporter strain to HQ following 90 min online exposure. (B) Dose-dependent fluorescence response by a *yqjFB2A1::GFP* reporter strain to HQ in municipal wastewater effluents following a 1 h online exposure. (C) Detection of diverse optical outputs: red fluorescent signal of a *recA::redExp* reporter (left), green fluorescent signal of *recA::GFP* (center), both in the presence and absence of NA (10 mg/l), and the bioluminescent signal of the *yqjFB2A1::luxCDABE* reporter (right), in the presence and absence of HQ (200 mg/l).

et al., 2013). This calculated value, 1.4 mg/l HQ, was very similar to that calculated for induction of suspended cell preparation in a microtiter plate, 1.52 mg/l. Similarly, a concentration-dependent response to municipal wastewater effluents spiked with HQ was observed (Fig. 3B). It should be noted that while a quantifiable output was observed already following a 30 min exposure, some of the dose-dependency of the signal was lost after longer incubation periods (24 h; not shown). This effect is caused by the high stability of the GFP protein; as it accumulates, the differences between the different induction intensities are progressively masked. In order for the system described herein to serve not only as a basis for an early warning system but also as an indicator of toxicants' concentrations, future generations of the device will employ reporter strains harboring destabilized GFP with a short half-life (Miller et al., 2000). The response of such a reporter would be manifested as a distinct peak, as previously shown for on-line bioluminescence reporters (Elad et al., 2011). Such peak data can be used for system calibration and concentration determination, regardless of the lag time before they occur.

To demonstrate that on-chip detection of sensor activity is not limited to green fluorescence, the PAO-based flow chip was also tested using bacterial sensor strains constructed with two additional reporter functions: *recA::DSredExp*, a NA-inducible strain that produces a red fluorescence output, and *yqjFB2A1::luxCDABE*, a reporter strain that luminesces in the presence of HQ. These were exposed to their respective inducers along with the previously described green fluorescent *recA::GFP* reporter, and the results are displayed in Fig. 3C. In each case, a clear signal, compatible with the reporting element of the bacterial reporter, was obtained in the wells exposed to the sample but not those exposed to the toxicant-free control, demonstrating the flexibility of the PAO-based sensor platform. A potential future embodiment of the platform may be envisaged, in which different stress types or toxicant classes would be characterized by different output signals.

A related issue that needs to be noted in this context is the liquid flow rate, in the ml/h range, administered through the system described here. This low value, dictated by the low internal volume of the system as well as by its mechanical configuration, should nevertheless faithfully represent the quality of the source water when continuously withdrawn from even a rapidly flowing stream.

A previous study in which a fluorescein dye was used to assess the kinetics exchange of small molecules between the PAO chip and the bulk medium (Ingham et al., 2007) indicated that an influx of fluorescein equivalent to more than 90% of the concentration of the medium was achieved in less than 10 s, and that a dilution of more than two orders of magnitude occurred in less than 25 s. Thus, in contrast to whole-cell biosensor devices in which the reporter bacteria are immobilized in a polymeric matrix that may hinder diffusion of the target chemical to the immediate vicinity of the cells, the permeable PAO layer presents no permeability barrier to small molecules and exchange is expected within a few seconds, as judged from the stationary system. At the same time, the PAO layer acts as a complete barrier to microbial penetration and does not allow the bacteria to escape through the chip to the environment. This was verified by plating samples of "used" medium, collected following exposure to the bacteria reporters, on nutrient agar. No growth of colonies was observed following 48 h incubation at 37 °C, indicating that no viable cells were able to penetrate the PAO-chip.

3.3. Online detection of chemicals using a 6-member reporter array

The design of the PAO-based flow-cell chip enables the simultaneous exposure to the sample of multiple bioreporters, each designed to detect a different chemical or a group of chemicals, thus providing a broad-spectrum online detection capacity. We have demonstrated this concept by assembling a 6-member bioreporter array, composed of well characterized reporter strains, each "tailored" to detect a different class of toxic chemicals (Table 1): genotoxicants (*recA::GFP*), heavy metals (*zntA::GFP*), 2.4-DNT and related aromatics (*yqjF::GFP* and *yqjFB2A1::GFP)* and oxidative stress (*micF::GFP*). In addition, a constitutively fluorescent strain, *CP38::GFP*, served as a control for bacterial viability. The 6 strains were deposited next to each other as shown in Fig. 4A, in both channels of a 2×32 chip. The toxicant-containing sample was flowed through the lower channel, while the upper channel was continuously exposed to a toxicant-free control medium. As expected, exposure of the array to HQ, $CdCl_2$, paraquat

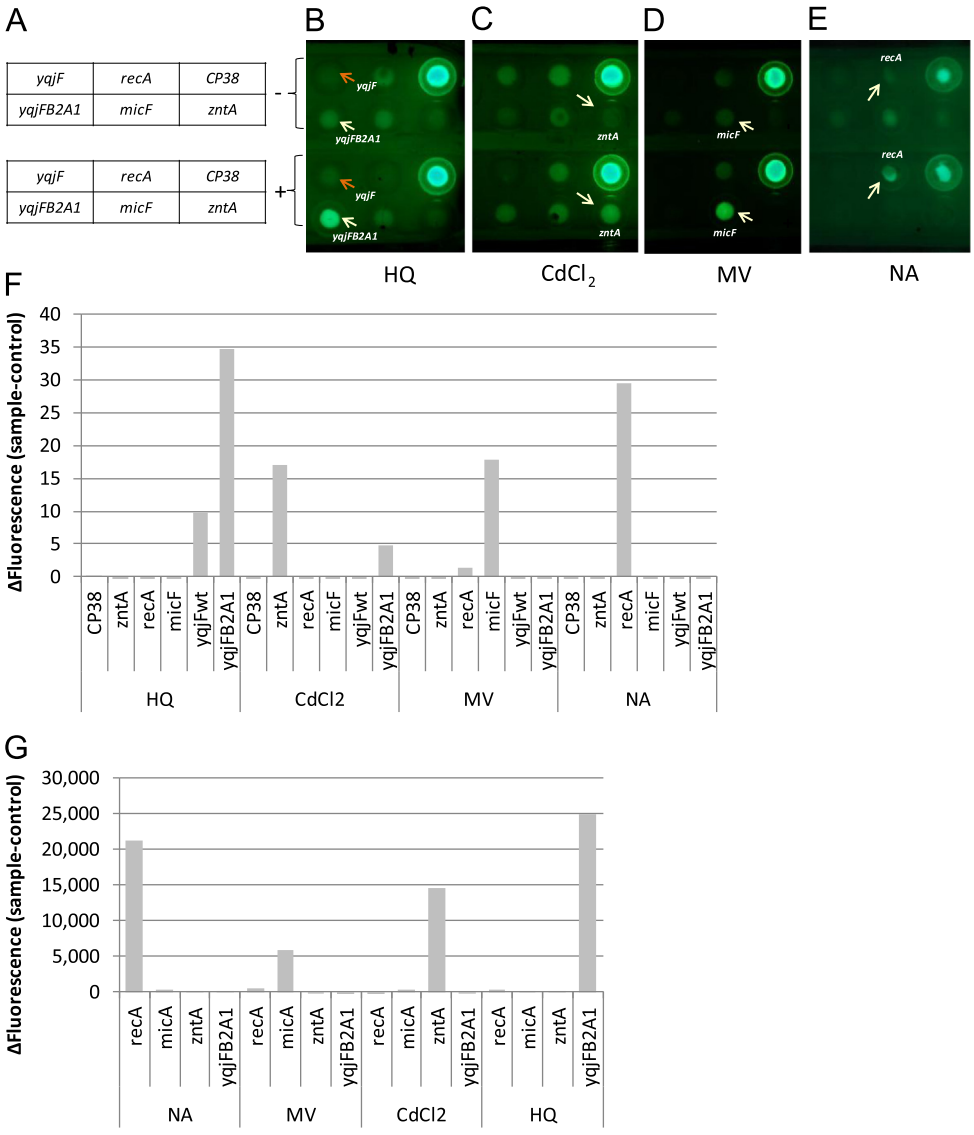

Fig. 4. Simultaneous online detection of chemicals using a 6-member reporter array. (A) A diagram of the 6-member array setup: each sextuplet was deposited on both arms of the 2 × 32 flow-cell, one set exposed to the target chemical and the other to a toxicant-free control. (B). Selective induction of *yqjF::GFP* and *yqjFB2A1::GFP* reporters by HQ. (C) Selective induction of *zntA::GFP* by $CdCl_2$. (D) Selective induction of *micF::GFP* by MV. (E) Selective induction of *recA::GFP* by NA. (F) Induction of the 6-member array by 4 different model chemicals, as calculated from B–E. (G) Induction of a 4-member array by the same chemicals using a microtiter plate. Data in the bar graphs are presented as the difference in fluorescence intensity (in arbitrary units) between the toxicant-exposed wells and the control. The strong fluorescence at the top right corners of all images is that of the constitutively fluorescent "normally on" control (CP38).

(MV) or NA, selectively induced *yqjF::GFP* and *yqjFB2A1::GFP* (Fig. 4B), *zntA::GFP* (Fig. 4C), *micF::GFP* (Fig. 4D) and *recA::GFP* (Fig. 4E), respectively. Fig. 4F summarizes the difference in induction (Δfluorescence) of the 6 array members by each of the model chemicals. Of the two HQ reporters, the improved sensor, *yqjFB2A1::GFP*, previously demonstrated to be stronger, faster and more sensitive to HQ, exhibited a much stronger response than *yqjF::GFP*, in agreement with its previously reported superior

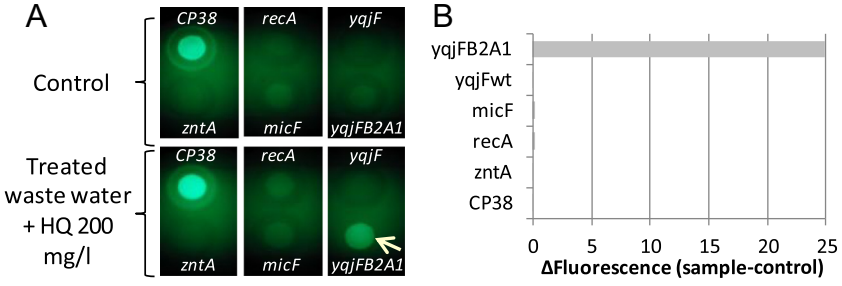

Fig. 5. Online detection of HQ-spiked (200 mg/l) municipal wastewater effluents by a 6-member reporter array. (A) Microscopic images of the array following a 3 h online exposure. (B) Calculated differences in fluorescence intensity between the toxicant-exposed wells and the control.

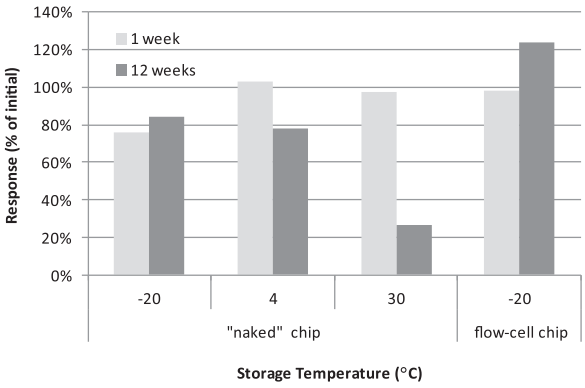

Fig. 6. Activity of revived reporter bacteria following on-chip lyophilization and a 12-week storage. Data in the bar graphs present the activity of the revived bacteria as % of their activity prior to preservation. The reporter strains used were *recA::GFP* ("naked" chip), exposed to 10 mg/l NA for 5 h, and *yqjFB1A1::GFP* (flow cell), exposed to 200 mg/l HQ for 6 h.

performance (Yagur-Kroll et al., 2013). A slight induction of *yqjFB2A1::GFP* upon exposure to $CdCl_2$ could also be detected, possibly due to traces of HQ remaining in the flow system. This effect was not observed for the less sensitive *yqjF::GFP*. MV, a strong oxidant and a redox-cycling agent, also slightly induced the *recA*-based genotoxicity reporter. This specific pattern of induction matched that obtained off-chip by the same reporter strains in a microtiter plate (Fig. 4G). The bright fluorescence of the constitutive CP38 reporter observed in all panels in Fig. 4B–E serves to demonstrate the wellbeing of the sensor cells, indicating that a lack of fluorescence is not due to cell death or inactivation. The weaker fluorescence of a reporter in the presence of the sample, for example *recA* in the presence of HQ, is possibly due to an inhibitory effect of the target chemical on that specific promoter. As expected, on-chip exposure of the array to a mixture of model chemicals (NA, MV, $CdCl_2$ and HQ) caused the simultaneous induction of all reporter strains (data not shown). Finally, the 6-member array was exposed to treated wastewater spiked with HQ; Fig. 5 presents the specific induction of the *yqjFB2A1::GFP* reporter by HQ.

The use of a sensor array, rather than individual reporter strains, can serve to indicate not only that a toxic event has occurred but also the nature of the toxic chemical involved (Elad et al., 2011). A PAO-based device harboring such an array could thus serve both as an early warning tool against the presence of toxic compounds in the monitored water as well as provide an indication as to their chemical or toxic characteristics. The success of such an approach will depend, to a large extent, upon the availability of a broad selection of reporter strains, each member of which is responsive to a different chemical or class of chemicals (Elad and Belkin, 2013), as well as on the availability of a suitable platform incorporating these sensors. This first example of an online flow cell incorporating live sensor cells in 32 or 64 discreet array locations is an important step towards the realization of this concept.

3.4. Long-term on-chip preservation of reporter bacteria

An important parameter in the evaluation of any biosensing device is the shelf life of its biological component, its applicability increasing the longer it can be stored in a "ready to use" format. We have thus examined the possibility of vacuum storage of freeze-dried PAO chips, along with the reporter bacteria deposited onto their surface. This was tested both for the "naked" chip as well as when in its flow-through casing. The NA-inducible *recA::GFP′* strain served for the former and the HQ-inducible *yqjFB2A1::GFP* strain for the latter configuration. The naked chip-containing vials were stored under vacuum at −20, 4 or 30 °C, and the flow-cell chip at −20 °C, both for 12 weeks. Fig. 6 displays the activity of the bacteria revived after 1 and 12 weeks, clearly indicating that bioreporters stored on-chip at −20 °C and at 4 °C, but not at 30 °C, retained significant inducible activity even after 3 months of storage. Induction of the freeze-dried bacteria was slower than that of fresh cells, exhibiting a 1–2 h lag (not shown), but the fact that almost a full response capability was observed after 12 weeks of storage is highly encouraging for a future potential use for such chips for on-line water monitoring.

4. Conclusions

Scientific and technological advances allow the coupling of reporter bacteria and diverse engineering platforms in the design of novel whole-cell biosensing devices (Ben-Yoav et al., 2011). Here we present such an integrated biosensor for continuous online water monitoring, at the heart of which is a disposable PAO-based chip placed in a fluidics system. This detection system is robust, user friendly, and fully amenable to optical imaging. The uniqueness of the system lies in the PAO layer supporting the sensor cells, which enables a constant supply of nutrients and an uninterrupted diffusion of toxicants (Ingham et al., 2012). The non-toxic PAO layer demands no pretreatment of either the support surface or of the bacteria, and polymer encapsulation is not required. Furthermore, as the bacteria cannot escape through the PAO layer into the tested water, the risk of biofouling and the need for effluent disinfection are greatly reduced.

This paper provides a concrete proof of concept of an online flow-through toxicity monitoring biosensor. The PAO-chip is a disposable ready-to-use platform harboring an array of freeze-dried bacterial reporters, easily coupled to the flow device by untrained personnel without any pretreatment. In addition, as the diffusion rate in and out the PAO layer is high, the biochip can be easily washed of earlier samples and be immediately reused. Clearly, however, further research efforts are necessary before such a device can be put into full-scale field tests. These include the establishment of the detection limits for a broader range of toxic chemicals, individually and in mixtures, and the composition of the reporter panel should be adjusted to accommodate an expanded′ spectrum of target chemicals. Additional operational parameters should be investigated in detail, including the effects of bacterial growth history, cell density and cavity dimensions. An automated procedure for the deposition of the reporter strain into the 1 mm wells should also be developed, possibly in a manner similar to that described by Melamed et al. (2011) who printed nanolitre-size bacterial spots on chemically modified glass or by Ingham et al. (2010) who printed bacteria on functionalized PAO surfaces. The availability of a material with a lower fluorescent background should be researched, and flow rates through the monitoring device may need to be optimized so that the water sampled out of the monitored water system faithfully represent the its real-time status. Another desirable future modification will involve replacing the microscopic imaging by a more convenient fluorescence quantification technology, alleviating the requirement for a microscope and enhancing the system's portability potential.

Acknowledgments

The study was supported by EU FW7 project Biomonar; we are grateful to the consortium coordinator, R. Town, for her support. Additional support for HUJI was provided by the Minerva Center

for Bio-hybrid Complex Systems, and for MicroDish by the SaWa grant (sensor and water). We thank Daniel Bar for technical support with the dissecting microscope.

References

Applegate, B.M., Kehrmeyer, S.R., Sayler, G.S., 1998. A chromosomally based tod-luxcdabewhole-cell reporter for benzene, toluene, ethybenzene, and xylene (BTEX) sensing. Appl. Environ. Microbiol. 64 (7), 2730–2735.

Belkin, S., 2003. Microbial whole-cell sensing systems of environmental pollutants. Curr. Opin. Microbiol. 6 (3), 206–212.

Ben-Yoav, H., Melamed, S., Freeman, A., Shacham-Diamand, Y., Belkin, S., 2011. Whole-cell biochips for bio-sensing: integration of live cells and inanimate surfaces. Crit. Rev. Biotechnol. 31 (4), 337–353.

Biran, A., Rami, P., Buchinger, S., Georg, R., Belkin, S., 2009. Genetically Engineered Bacteria for Genotoxicity Assessment. Biosensors for Environmental Monitoring of Aquatic Systems. In: Barceló, D., Hansen, P.-D. (Eds.), D. Barceló and P.-D. Hansen, Springer Berlin Heidelberg., 5J. Springer, Berlin Heidelberg, pp. 161–186.

Buffi, N., Merulla, D., Beutier, J., Barbaud, F., Beggah, S., van Lintel, H., Renaud, P., Roelof van der Meer, J., 2011. Development of a microfluidics biosensor for agarose-bead immobilized *Escherichia coli* bioreporter cells for arsenite detection in aqueous samples. Lab Chip 11 (14), 2369–2377.

Charrier, T., Chapeau, C., Bendria, L., Picart, P., Daniel, P., Thouand, G., 2011. A multi-channel bioluminescent bacterial biosensor for the on-line detection of metals and toxicity. Part II: technical development and proof of concept of the biosensor. Anal. Bioanal. Chem. 400 (4), 1061–1070.

den Besten, H.M.W., Garcia, D., Moezelaar, R., Zwietering, M.H., Abee, T., 2010. Direct-imaging-based quantification of bacillus cereus ATCC 14579 population heterogeneity at a low incubation temperature. Appl. Environ. Microbiol. 76 (3), 927–930.

den Hertog, A.L., Visser, D.W., Ingham, C.J., FHAG, Fey, Klatser, P.R., Anthony, R.M., 2010. Simplified automated image analysis for detection and phenotyping of mycobacterium tuberculosis on porous supports by monitoring growing microcolonies. PLoS ONE 5 (6), e11008.

Elad, T., Almog, R., Yagur-Kroll, S., Levkov, K., Melamed, S., Shacham-Diamand, Y., Belkin, S., 2011. Online Monitoring of Water Toxicity by Use of Bioluminescent Reporter Bacterial Biochips. Environ. Sci. Technol. 45 (19), 8536–8544.

Elad, T., Belkin, S., 2013. Broad spectrum detection and “barcoding” of water pollutants by a genome-wide bacterial sensor array. Water Res. 47 (11), 3782–3790.

Elad, T., Lee, J.H., Belkin, S., Gu, M.B., 2008. Microbial whole-cell arrays. Microb. Biotechnol. 1 (2), 137–148.

Eltzov, E., Marks, R.S., Voost, S., Wullings, B.A., Heringa, M.B., 2009. Flow-through real time bacterial biosensor for toxic compounds in water. Sens. Actuators B: Chem. 142 (1), 11–18.

Gerhardt, A., Carlsson, A., Ressemann, C., Stich, K.P., 1998. New online biomonitoring system for gammarus pulex (L.) (Crustacea): in situ test below a copper effluent in south Sweden. Environ. Sci. Technol. 32 (1), 150–156.

Gerhardt, A., Ingram, M.K., Kang, I.J., Ulitzur, S., 2006. In situ on-line toxicity biomonitoring in water: Recent developments. Environ. Toxicol. Chem. 25 (9), 2263–2271.

Hever, N., Belkin, S., 2006. A Dual-color bacterial reporter strain for the detection of toxic and genotoxic effects. Eng. Life Sci. 6 (3), 319–323.

Horry, H., Charrier, T., Durand, M.-J., Vrignaud, B., Picart, P., Daniel, P., Thouand, G., 2007. Technological conception of an optical biosensor with a disposable card for use with bioluminescent bacteria. Sensors and Actuators B: Chemical 122 (2), 527–534.

Ingham, C., Bomer, J., Sprenkels, A., van den Berg, A., de Vos, W., van Hylckama Vlieg, J., 2010. High-resolution microcontact printing and transfer of massive arrays of microorganisms on planar and compartmentalized nanoporous aluminium oxide. Lab on a Chip 10 (11), 1410–1416.

Ingham, C.J., Sprenkels, A., Bomer, J., Molenaar, D., van den Berg, A., van Hylckama Vlieg, J.E.T., de Vos, W.M., 2007. The micro-Petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms. Proceedings of the National Academy of Sciences 104 (46), 18217–18222.

Ingham, C.J., ter Maat, J., de Vos, W.M., 2012. Where bio meets nano: The many uses for nanoporous aluminum oxide in biotechnology. Biotechnology Advances 30 (5), 1089–1099.

Lee, J.H., Youn, C.H., Kim, B.C., Gu, M.B., 2007. An oxidative stress-specific bacterial cell array chip for toxicity analysis. Biosensors and Bioelectronics 22 (9–10), 2223–2229.

Magrisso, S., Belkin, S., Erel, Y., 2009. Lead Bioavailability in Soil and Soil Components. Water, Air, and Soil Pollution 202 (1-4), 315–323.

Melamed, S., Ceriotti, L., Weigel, W., Rossi, F., Colpo, P., Belkin, S., 2011. A printed nanolitre-scale bacterial sensor array. Lab on a Chip 11 (1), 139–146.

Melamed, S., Lalush, C., Elad, T., Yagur-Kroll, S., Belkin, S., Pedahzur, R., 2012. A bacterial reporter panel for the detection and classification of antibiotic substances. Microbial Biotechnology 5 (4), 536–548.

Miller, W.G., Leveau, J.H.J., Lindow, S.E., 2000. Improved gfp and inaZ Broad-Host-Range Promoter-Probe Vectors. Molecular Plant-Microbe Interactions 13 (11), 1243–1250.

Shedd, T.R., van der Schalie, W.H., Widder, M.W., Burton, D.T., Burrows, E.P., 2001. Long-term operation of an automated fish biomonitoring system for continuous effluent acute toxicity surveillance. Bull. Environ. Contam. Toxicol 66, 392–399.

Trang, P.T.K., Berg, M., Viet, P.H., Mui, N.V., van der Meer, J.R., 2005. Bacterial Bioassay for Rapid and Accurate Analysis of Arsenic in Highly Variable Groundwater Samples. Environmental Science & Technology 39 (19), 7625–7630.

van der Meer, J.R., Belkin, S., 2010. Where microbiology meets microengineering: design and applications of reporter bacteria. Nat Rev Micro 8 (7), 511–522.

Willardson, B.M., Wilkins, J.F., Rand, T.A., Schupp, J.M., Hill, K.K., Keim, P., Jackson, P. J., 1998. Development and Testing of a Bacterial Biosensor for Toluene-Based Environmental Contaminants. Applied and Environmental Microbiology 64 (3), 1006–1012.

Yagur-Kroll, S., Lalush, C., Rosen, R., Bachar, N., Moskovitz, Y., Belkin, S., 2013. Escherichia coli bioreporters for the detection of 2.4-dinitrotoluene and 2.4.6-trinitrotoluene. Applied Microbiology and Biotechnology, 1–11.

Zaslaver, A., Bren, A., Ronen, M., Itzkovitz, S., Kikoin, I., Shavit, S., Liebermeister, W., Surette, M.G., Alon, U., 2006. A comprehensive library of fluorescent transcriptional reporters for Escherichia coli. Nat Meth 3 (8), 623–628.