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On-line biosensor for the detection of putative toxicity in water contaminants

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ABSTRACT

Potential threat on drinking water requires monitoring solutions, such as the one proposed herein, as a real-time, wide ranged, water monitoring system to detect the presence of toxicants in water. We studied the role of a selected number of parameters affecting performance and, thus, improved the prototype into an optimized next-generation device, resulting in enabling increased measurement duration, coupled with increased sensitivity. The chosen parameters in question were the peristaltic flow system, the fiber probe matrix stability through a re-design of the fiber probe holder and flow unit cell, as well as the modulation of bacterial medium concentration to increase bioreporter performance while keeping biofouling in check. Measurements were made with spiked samples and validated with polluted field-collected samples.

1. Introduction

Increasing population and activities thereof, produce large varieties and quantities of waste chemicals [1], which oftentimes affect drinking water quality [2–4]. In order to safeguard our water supplies, it must be monitored constantly to prevent its contaminated form from reaching the consumers [5–7], while being diverted to processing centers for its remediation.

There are two main forms of water monitoring. One requires the tester to go to the field and make an on-site test or alternatively taking the sample to an accredited test laboratory. The main disadvantage is that the user must go to the place in question. However, the advantage of the first possibility is that the dispatchable equipment includes disposable elements and the answer is quasi-immediate enabling a response. The second form involves use of a continuous monitoring system, with near real-time monitoring, that will give the user continuous feed on the particular point, where the tests are being conducted, such as a chosen river site, which is advantageous as only infrequent human dispatch would then be required. However, the sophisticated mechanics involved complicates the overall system from a number of engineering parameters.

There are two main analytical categories in monitoring water [8], the classical chemical chromatographic ones and the bioassay-based ones. The former (HPLC, GC, GC/MS [9]) is aimed at both identification and quantification. Their disadvantage is their specificity: an unfamiliar toxicant will not be detected. In addition, these are costly and require both sophisticated instrumentation as well as qualified personnel. They are nonetheless very useful and remain today the workhorse of the chemical identification industry. On the other hand, bioassays assess a potential toxic effect on a living organism, however, they lack the ability to measure a specific concentration or even provide most of the times an identification of the monitored toxicant. Despite these disadvantages, those sensing systems have one great advantage: the toxicant monitoring range is very broad [8,10–14]. In this particular area, bacterial-based bioassays, have shown promising applicative results. These consist in using genetically modified bioreporter organisms that generate light as a response to toxic compounds (see Section 2.2).

Bioassays using bacterial bioreporters are used either in their classical liquid-phase bacterial suspension [15–17] or immobilized in biosensor systems [18–20]. In both cases, the tested water

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samples must be disposed of via a septic step according to licensed (MLI or II) laboratory regulations,, so as to prevent contamination of the environment, which is protected by stringent regulations. Disposable bioassays are usually autoclaved, although one may imagine chemical neutralization could also be used, while immobilized-phase on-line biosensors require asceptization of the test effluent, despite the fact that one can create stable immobilized probes with virtual non-leaching of the said bioreporters [10,13,20], while still retaining good levels of sensitivity.

The aim of this study was to improve on some of the limitations found in the earlier system [13]. It was then discovered that the bacterial on-line system required that the user provide growth medium continuously, so as to sustain the immobilized bacteria in a continuous stream of water. Indeed, the signal improved, however, an increase in biofouling occurred, which could affect the overall sensor performance. Therefore, we checked two types of such growth media at different concentrations so as to obtain that concentration of either growth medium, which is really necessary to reduce increased biofouling potential while helping the system increase its performance. We also then noticed that increased sample flow rate had a detrimental mechanical effect on the hydrogel structure that was used to entrap the bacteria, thus we designed herein, a protective proprietary fiber optic probe flow cell. This study therefore looked at improving both sensor sensitivity and increasing measurement time, while retaining simplicity of operation, size, and real-time monitoring capability.

2. Materials and methods

2.1. Chemicals

Tryptone (T7293), Yeast Extract (92144), NaCl (S7653), alginate (B25266), $CaCl_2$ (C1016), p-chlorophenol, copper sulfate (209198), kanamycin (K1377), and ampicillin (A0797) were obtained from Sigma. All stock solutions were diluted with distilled water (dW) and stored at temperatures as suggested by the manufacturers' instructions.

2.2. Bacterial strain

The bioluminescent *Escherichia coli* bioreporter strain TV1061 [21] used in this study is sensitive to metabolic changes, such as with cytotoxic substances. It harbors plasmid-borne fusions with a specific heat-shock *grpE* promoter adjacent to the *luxCDABE* reporter operon, whose activation is detected by light emission (bioluminescence). The *Lux* operon has five promoterless structural genes. These are responsible for both the heterodimeric luciferase units (*lux* A and B) and the synthesis of the luciferase substrate, tetradecanal, by an ATP- and NADPH-dependent multienzyme complex composed of fatty acid reductase, transferase, and a synthetase (*lux* C, D and E) [22]. Strain stocks were stored at −80 °C with 20% (v/v) glycerol as a cell cryoprotectant additive [12]. Stock bioreporter strains were placed on LB-agar plates (NaCl 5 g L^{-1}, yeast extract 5 g L^{-1}, tryptone 10 g L^{-1}, agar 15 g L^{-1}) supplemented with 50 μg mL^{-1} kanamycin and, thereafter, incubated at 37 °C in a rotary thermo-shaker overnight (MaxQ 4450, ThermoScientific, USA). These were then stored at 4 °C for future experimentation.

2.3. Bacterial growth

Bacterial cultivation prior to measurements was performed in 10 mL LB-medium (NaCl 5 g L^{-1}, yeast extract 5 g L^{-1}, tryptone 10 g L^{-1}) supplemented with 50 μg mL^{-1} kanamycin for TV1061. Cells were grown overnight at 37 °C in a rotary thermo-shaker (MaxQ 4450, ThermoScientific, USA) at 120 rpm in the presence of the antibiotic. Cultures were then diluted to approximately 10^7 cells/mL and re-grown in 25 mL LB at 26 °C without shaking and without antibiotics, to an early exponential phase (OD 600_{nm} of 0.2) as determined by an Ultrospec 2100 pro spectrophotometer (Amersham, England).

2.4. Fiber optic probe bioreporter immobilization procedure

The harvested cells were mixed 1:1 (v/v) with a filter-sterilized 2% (w/v) low viscosity sodium alginate solution. Multimode optical fibers, PUV 400 BN (CeramOptec, GmBH, eramOptecBonn), were used in these experiments. They present a pure silica core diameter of 400 μm, with a refractive index of 1.4571 (at 633 nm) and a cladding diameter of 440 μm, with a refractive index of 1.4011 (at 633 nm). Their black nylon jacket was stripped away from a 1-cm long optical fiber proximal tip, which was then used for the immobilization of the bioluminescent bioreporter bacteria [23]. The 1-cm optical fiber tip was first exposed (for a few seconds) to the bacterial alginate suspension, and then dipped (for a few seconds) into a sterile 0.5 M calcium chloride solution [20], thus entrapping the bacteria onto the fiber proximal tip within a hardened calcium alginate matrix. Six to seven layers have been shown to be the optimal number of layers when previously tested [13]. Thereafter, the optical fiber probe, with immobilized bioluminescent bacteria at its tip, was immediately used after preparation for experimentation (Fig. 1C).

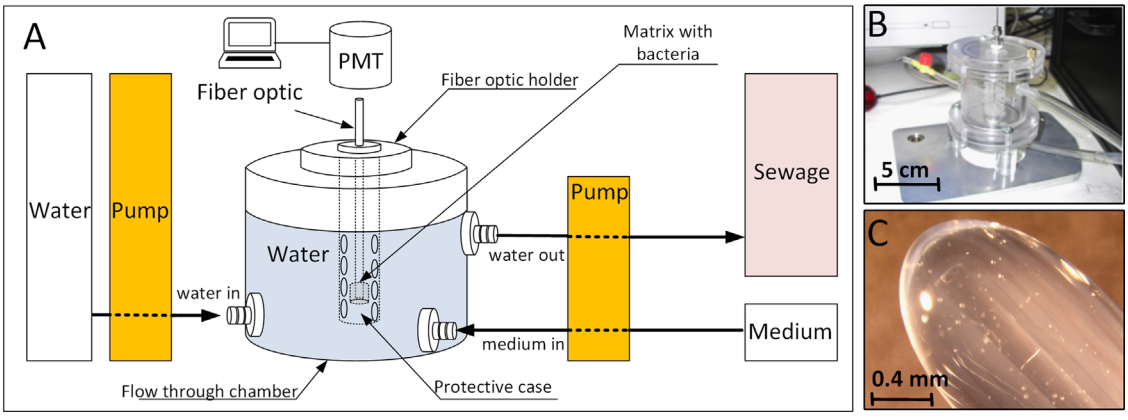

Fig. 1. A. Scheme of the perspex "protective" flow unit holding the fiber optic probe and its connected peristaltic pumps; B. Photo of perspex flow unit; C. Calcium (2% v/v)-polymerized alginate with six adlayers forming the fiber optic probe.

2.5. Instrument setup

A field-operable fiber-optic photodetector device was previously designed [10] and in order to monitor toxicity in a flow-through manner, it was modified in a recent study [13] including improvements, such as an added flow cell, (Fig. 1) which was placed in the light-tight box, so as to prevent any damage to the photon-counting unit by the surrounding light. A Hamamatsu HC135-01 PMT Sensor Module was used for bioluminescence measurements, combining the sensitivity of a photomultiplier tube (PMT) with the intelligence of a microcontroller. The detector was optimized to the blue light region and included a 21-mm diameter active area to gather light radiation without any optical focusing elements.

In order to increase the flow rate of the incoming water sample, while retaining the fiber probe matrix integrity, a new design of its configuration was made (Fig. 1A) due to the fact that a pressure issue was discovered and affected the bacterial performance. In this system, water flows through a workshop-made inner cell, whilst the chamber is made of transparent perspex, thus both protecting the fiber-optic from the water current and also enabling, when needed, a visual supervision of the biosensor probe integrity (Fig. 1B). The cell module design includes two inlets, one for incoming water and the other for supplementary growth medium ($d=3.5$ mm), and one outlet, for the exiting water stream ($d=3.5$ mm), while a separate entrance was made for the fiber optic probe housing the immobilized bacteria. Initially, the water flow in the new setup was conducted with only one peristaltic pump, which brought water into the flow unit; however, we observed a continual underperformance of the immobilized bioluminescent bacteria, which we eventually determined to be due to a pressure build up inside the flow through chamber (Fig. 1A), thus preventing the bioreporters from functioning properly. So as to release the said pressure from developing due to the single peristaltic pump use, an additional pump was connected to the system exit, so as to continually remove the water from the operation unit, with both pumps working at equal flow rates. The upper lid of the fiber optic probe cell includes an inlet for the fiber optic probe, which bathes continually in the water flow, and secured at four different points, so as to prevent it from moving due to the sheer forces caused by the water flow. The said optic fiber probe is thus contained within a porous cylinder. Multiple pores (diameter = 2 mm) were drilled in a regular array over the surface of the cylinder, while the distance between them was 3.5 mm. The main purpose of this structure was to protect the fragile biosensor probe matrix – placed at the proximal end of the fiber – which is exposed to the incoming water sample sheer forces. An added benefit is a decrease in the flow velocity around the matrix. The water flow was measured by a rotometer (1017, Burkert) localized in the inlet water stream. To record and analyze the data, a specific driver was developed using LabView (version 3.1, National Instruments Corporation), which allowed to record and analyze the bioluminescence signal in real time.

2.6. Optimization procedures

2.6.1. Comparative effect of two different growth media and concentrations thereof on bioreporter signal response

Two sterile bacterial media 2 YT (Yeast extract tryptone; composed of 16 g L^{-1} Yeast extract 10 g L^{-1} and 16 g L^{-1} Tryptone) and LB (Luria Bertani broth; composed of Tryptone 10 g L^{-1}, Yeast extract 5 g $L^{-1,}$ and NaCl 10 g L^{-1}) were fed into the on-line system at different concentrations ranging between 0 and 7.5% (v/v), in tap water, through the water flow of a calibrated peristaltic pump (Masterflex® 7754-57, Cole Parmer Instrument Company, USA). The sensor response to the target water pollutant (e.g. 2% (v/v) ethanol) was measured over a period of 4–10 h and any changes or improvements noted.

2.6.2. Effect of the flow system management on sensor activity

As explained above, the original system used one peristaltic pump to bring water into the flow unit, which increased pressure onto the resident immobilized bacteria and therefore prevented the bioreporters from functioning properly. The putative effect of the pressure on bacterial sensor activity was further examined by the addition of another peristaltic pump to the exit of the system, so as to continually remove the water from the operation unit, with both pumps working at equal flow rates. At first, the effect on the bacterial cell activation, when using one working pump, was measured without the addition of any chemicals. Bacterial activity in the flowing tap water (with the addition of medium) was measured with either a working (kinetic mode) or an inactive (static mode) inlet pump. Thereafter, the effect of the addition of a second pump to the flow system was examined. In both approaches (system with one or two pumps), bacteria were exposed (at $t=0$ minutes) to 2% (v/v) ethanol as model toxicant.

2.6.3. Effect of the flow rate on stability of the probe matrix

The stability brought by the protective fiber probe cell module on previously observed matrix erosion to the bacterial immobilization matrix of the fiber probe was examined periodically by visual observation and overall evaluation by light microscopy, before and after a system run of 24 h, occurring at differential flow rates from 0 to 10 L h^{-1}.

2.6.4. Effect of the flow unit design on biosensor sensitivity

Two different architectures of the flow unit (old version (without a flow cell unit) and present version (as described throughout notably in Section 2.5) were tested and compared in this work, so as to evaluate their effect on the sensor probe performance, while being exposed to various concentrations of a positive control (e.g. 0.02, 0.2 and 2% (v/v) of ethanol).

2.7. Increased measurement duration

In order to evaluate the analytical performance of the biosensor device, at a longer measurement duration, bioluminescent bioreporter cells were exposed thrice to water pollutants over a 48-h period (compared to our previous work which was for only a useful 24 h): once at the onset of the experiment, and twice after at 24-h time periods ($t=0$; $t=24$ h; $t=48$ h). Strain TV1061 was exposed to three different model toxicants known to be optimal test concentrations in our previous device (2% (v/v) ethanol, p-chlorophenol (1 mg mL^{-1}) and copper (5 ppm)) in separate experiments.

2.8. Environmental water samples

The 24-h experiments were performed using environmental water from Lakhish River (Israel, Lat.31°48'56.68"N Lon. 34°38'35.66"E) with the TV1061 strain, and the improved experimental prototype setup (Fig. 1B). We only tested 24 h due to the fact that we measured the water in our lab and not on-site so had insufficient amounts of environmental water to increase the monitoring time. Freshly collected Lakhish water was stored at 4 °C and used within five days of collection to reduce field composition changes. In order to determine if any putative effect from water salinity may affect the immobilized cells, bacteria were exposed to synthetic water that was made from deionized water with the addition of 48 mg L^{-1} $NaHCO_3$, 30 mg L^{-1} $CaSO_4 \cdot 2H_2O$, 30 mg L^{-1} $MgSO_4$, and 2 mg L^{-1} KCl, with a hardness of 40 mg L^{-1}.

2.9. Viability measurements

Evaluation of the viability of bioreporter bacterial cells after their exposure to toxicants during each experimental run (24 h) was accomplished via the viable plate counting method [24]. Serial 10-fold dilutions of bacterial samples were made in sterile PBS buffer and 10 μL samples were thereof evenly spread onto non-selective solidified nutrient LB agar surface plates. The number of viable colony forming units (cfu) was enumerated as bacterial colony plaques after 24 h incubation at 37 °C.

3. Results and discussion

3.1. Effect of water pressure on the bacterial response.

The effect of water pressure caused by a single peristaltic pump on the immobilized bacteria was evaluated by observing the bioreporter response in the prototype setup (Fig. 1A), with and without an additional water exit pump (which helps remove the water from the flow unit). In the first mode, we observed that when only one pump (placed before the measuring unit) is used in the sensor system, a high pressure seemed to be created in the measuring unit. In the second mode, where an additional pump was added to the system, and placed after the flow unit, and operated at the same flow rates as those of the input pump, it was observed that bacterial performance increased. Indeed, this step was expected to prevent the generation of pressure inside the measuring unit. Fig. 2 presents the effect of the pressure on the immobilized bacteria. Increased water pressure inside the flow unit housing the immobilized bacteria had an inhibitory effect on the activity of the bioreporter cells (Fig. 2A), exemplified by decreased luminescence in the presence of normally inducing pollutant concentrations in water [25]. Others have shown that increased pressure could affect the membrane integrity [26], fluidity [27], or protein denaturation [28] in bacteria. Fig. 2B shows that the addition of the second pump into the system solved the inhibitory issue. Immobilized bacteria regained their capacity to respond to the toxicant (Fig. 2B) and this dual peristaltic pump mode was adopted in all future experiments.

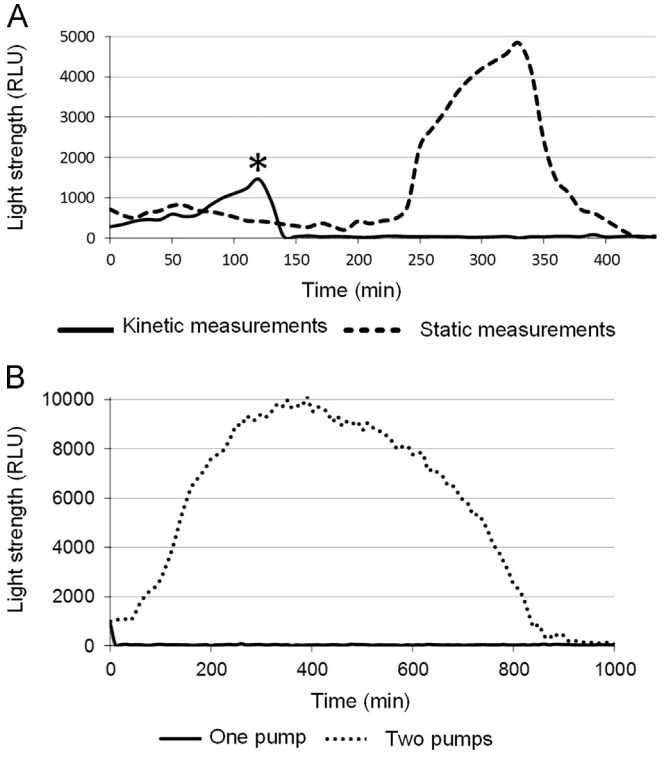

Fig. 2. Effect of pressure on immobilized bacteria in the newly designed instrument. A. Response of the cells once the peristaltic pump is turned on. The solid line represents the flow system with one working pump (*pump operation starts at $t=120$ min) and dash line represents the system without the peristaltic pump. B. Effect of the addition of a second pump to the system, 2% (v/v) ethanol was added at time $t=0$ min.

3.2. Effect of medium type and concentration thereof on bioreporter sensor responsiveness

A living organism needs a continuous source of nutrients to maintain its routine metabolism. The use of bioreporter bacteria as a sensing element raised the issue of the need of supplying any (or enough) nutrients to maintain normal cell functionality when integrated into a continuously operated biosensor device so as to produce reliable measurements. It is known that a sub-minimal amount of nutrients will cause a detrimental effect on natural metabolic processes in the cell, resulting in unreliable responses of the biosensing system [29]. The bioluminescence signals of exposed bio-reporter bacteria to medium-free tap water have already been investigated in previous studies [10,13,23], where exposure to medium-free water resulted in a strong and rapid increase in light induction (without toxicant addition), followed by a sharp decrease which stabilized at a basal level. The proposed flow-through prototype compared two different and popular media (LB and 2 YT) at three different concentrations: 2.5%, 5% and 7.5% (v/v), to which was added 2% (v/v) ethanol as the model toxicant. Ethanol is commonly used as an antimicrobial compound and is known to cause protein damage and to induce a protein damage sensitive *grpE*-dependent bioluminescent response in TV1061 [21].

Fig. 3A represents the different effects on sensor activity, from the nature of the growth medium tested, and this in combination with its various concentrations. The addition of LB to the cells induces the best bioluminescence response. It is important to remember that 2 YT medium differs from LB media in its amino acid concentration (tryptone/yeast extract) (twice lower in LB), while NaCl in LB is twice higher, suggesting that salt may have a more central role in the bacterial bioluminescence response to the toxicants. We will not speculate further here, although this physiological response is worthy of further investigation, just as other parameters would likewise need investigation such as

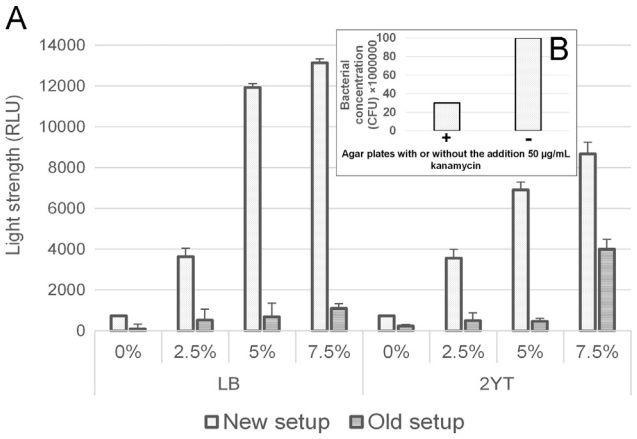

Fig. 3. A. Differential response obtained with TV1061 strain using 2% (v/v) ethanol with the addition of either 2 YT or LB growth media at different concentration. B. Viability count of bacteria (bioreporters versus biofouling bacteria) on agar plates differentiated by the addition or absecence of 50 μg mL^{-1} kanamycin. The previous setup is the tubing mode and new cell unit design.

temperature, pH, incubation time, and matrix composition [30]. The complicated relationship between bioreporter bioluminescence expression on biosensor performance and its bathing medium [31], endogenous aldehyde and internal ions (e.g. potassium, sodium, calcium and magnesium) [32–34] requires careful regulation. Interestingly, there were no significant differences in the cell response, namely in the signal obtained, with the higher concentrations of either 5% or 7.5% (w/v) of LB (Fig. 3A). Possibly the required threshold of essential components are thus satisfied with 5% (w/v). Thus, to help reduce the inevitable bacterial contamination to the system, a lower LB concentration was chosen.

An additional modification brought to the present (dual pump) setup was to add the medium directly into the fiber probe cell unit, unlike previously, when this was done at the onset of the water flow (Fig. 1). This step decreased the contamination rates of the system from biofouling activities on the alginate matrix [35–37], from unwanted bacterial overgrowth, originating in the water tubing and inner cell. These may also cause false positive signals from the inlet stream by reducing toxicant exposure to the immobilized bio-reporter cells, by preventing (or reducing) diffusion of both the nutrients and any putative toxic agents to the immobilized reporter bacteria. This phenomenon is obviously enhanced in systems that are rich with nutrients, thus minimal optimal levels of nutrients need to be carefully controlled. Fig. 3A demonstrates that a good and reliable signal response can still be obtained with a reasonable level of medium (5% (v/v)) maintained in the inlet flow. To determine the ratio between biofouling bacteria (kanamycin sensitive) and those immobilized (genetically engineered bioreporters resistant to kanamycin) in the alginate matrix, a water flow (with the addition of 5% (v/v) LB) was passed for 24 h. Fig. 3B shows the presence of resistant and non-resistant bacteria at the probe matrix. Calcium alginate hydrogel was decomposed and the released cells were grown on agar plates (with or without antibiotic). Indeed, there was an increase in the bacterial concentration on the agar plates without any antibiotic addition (Fig. 3B), suggesting the occurrence of biofouling upon the immobilization matrix. We cannot discard the fact that an additional possible reason for these results is that during long-term exposure some bacteria may remove their plasmid and thus loose their resistance to kanamycin.

3.3. Effect of flow rate on matrix stability

It has been reported that bacteria used in real-time flow experiments cannot afford a flow rate higher than 3 L h^{-1} [17,38] as this may cause an erosion of the probe matrix with a consequent loss of bacterial cells, thus altering the analytical performance of the water-pollutant detection device. Parameters enabling decreased response time and increased sensitivity are important for real-time monitoring of drinking water, using reasonable sampling volumes at a reasonable pace. The ability to work under high flow rates is an elegant solution for this issue. In designing a dual pump device, the effect of the water flow rate on the bacterial probe matrix integrity is evaluated qualitatively by its exposure to a range of different flow rates for 24 h (Table 1). As explained hereabove, the effect of the the water flow was examined by visual observation and overall evaluation by light microscopy, before and after a system run of 24 h. The diameter of the fiber optic was measured before after water exposure. Each (+) logged in the table represents 25% of the initial matrix diameter. It can be clearly seen that the present new design (Fig. 1A) shows higher consistency with a reduced visible damage within the reported range of tested flow rates. The proximal probe matrix in the old setup [13] was partially reduced at 3 and 4.5 L h^{-1}, and vanished totally from 6 L h^{-1}, unlike in the present system, thanks to its new design. In the previously published system, the fiber optic was placed directly into the water flow and was thus exposed to the direct shear stresses made by the passing liquid. In the present configuration, there was no direct pressure from the water flow on the matrix, as the fiber optic was placed in a special protective casing and this reduced the damaging effect of the water flow.

3.4. Effect of the flow unit design on sensor sensitivity

An ideal biosensor has two key properties, i.e. high sensitivity (response to a low concentration of analytes) and high specificity (the ability to discriminate between species according to the immobilized biorecognition elements) [39]. The immobilized bioreporter bacteria confer the biosensor's specificity. Indeed, various strains may be designed to be sensitive to different chemicals and stresses (heavy metals, oxidative compounds, general stresses and antibiotics).

In the present research, the effect of the flow-measurement unit design on sensor sensitivity was determined by exposure of the immobilized bacteria to cytotoxic stress (i.e. ethanol and p-chlorophenol). It was noticed that in the two compared flow units, the immobilized cells responded in different manners (Fig. 4). The possible reasons for this phenomenon are the differences in the design of the water flow unit and, consequently, the important changes in the rate flow. One should mention that in both tested devices, the presence of the toxicants was not constant, i.e. the toxicant was added to the system only for half an hour (as a pulse), while for the rest of the measurement, the immobilized bacteria were exposed to a clear tap water flow, with or without supplementary growth medium. It is assumed that the

Table 1
Effect of water flow rate on the stability of the bacterial matrix. Decrease in the number of (+) symbols implies higher matrix erosion (when (++++) no damage is observed in matrix) and (–) represents the absence of matrix on the fiber.

Flow rate (l/h)	Old setup	New setup
0	++++	++++
1.5	++++	++++
3	++	++++
4.5	+	++++
6	–	+++
8	–	++
10	–	++

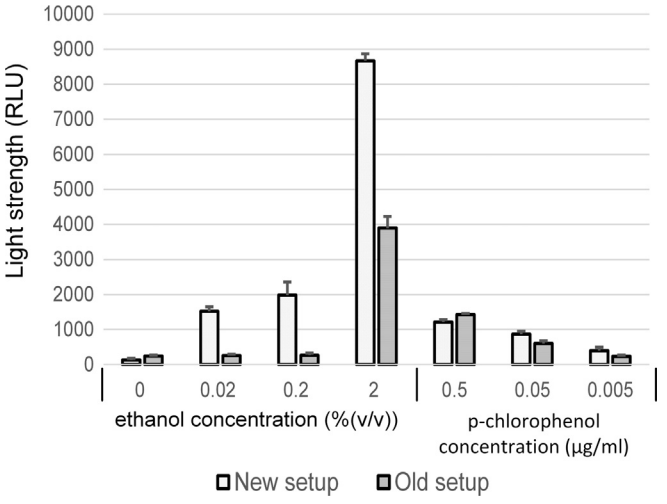

Fig. 4. Improved sensitivity with the use of the new flow unit design tested with different concentrations of ethanol and p-chlorophenol.

diffusion rate of the toxicant into the alginate matrix depended on several main parameters: the concentration of the chemical in the liquid, the time of exposure of the toxicant, as well as, the matrix density. In the tubing-based optical–fiber setup [13], toxicants were passed into the measuring unit much faster and, once simple non-medium containing tap water came as a replacement, they were washed out of the system sooner. In the dual pump setup, the fiber optic is exposed to a reduced toxicant concentration at the beginning, as compared to the previous design. Therefore, it takes longer to reach the same toxicant concentration and the same for nontoxic water to replace it. Thus, immobilized bacteria are exposed to the toxicant for a longer duration, allowing increased diffusion inside the matrix. Our supposition is confirmed by the recent reviews that showed that the longer bacteria are exposed to toxicants, the more material diffuses to its surface and into its cytoplasm [40]. Fig. 4 shows that the new flow cell design allowed an increase in sensor sensitivity (100-fold higher in the case of ethanol) when compared to the previous setup. Ethanol is a model cytotoxic compound that was used as a positive control in many previous publications using TV1061 as the bioreporter bacteria [41–43]. Our probes were also exposed to different concentrations of p-chlorophenol, oftentimes found in wastewaters and sewage [44] and the sensitivity of the previously published setup was roughly comparable to that of the earlier static system [45,46]. However, it was not sensitive enough to detect typical contaminations of surface water in developed countries, which usually range from <1 ng L^{-1} to 1 μg L^{-1}. The new system has, however, demonstrated a higher sensitivity (10-fold higher) compared to the previous setup (Fig. 4) and is now comparable to other methodologies (e.g. electrochemical [47,48]). Obviously solving other engineering issues may help increase further the sensitivity of the system albeit the fact that the bioreporters are themselves limiting. Solving physiological parameters may also help increase their sensitivity.

3.5. Increased on-line measurement duration

The challenge of on-site analysis requires elongated tests [49], during continuous measurements, and this was done here for longer periods of time (two days) using a single optical fiber probe, as compared to previous studies (one day). Experimentally, two setups (previously published one and present one) were operated for 48 h, while the immobilized cells were exposed to different toxicants (copper, ethanol and p-chlorophenol) each for 24 h (at $t=0$, $t=24$ and $t=48$ for 30 min). Indeed, many environmental compounds, such as metal ions, phenols and ethanol, may induce heat shock response in bacteria [50], including toxic copper [51] [52].

It was found that 48-h experiments with the previous setup showed much lower cell response to test controls after 20 h of sensor operation [13], and no activity by 48 h of measurements (data not shown). Many different factors may be responsible for these results, such as biofouling formation on the immobilization matrix, destructive effect of the water flow on the probe matrix, washing out of the matrix calcium, with subsequent loss of alginate polymer and then release of the bacteria from the fiber [13]. In the new setup, on the other hand, bacteria remained active for twice as long (Fig. 5), albeit even longer periods were not yet tested. As immobilized cells respond differently (e.g. time of response, recovery time, strength of the signal) to the pandemonium of possible contaminating compounds due to their available concentration as well as toxic effect, it is suggested that a putative role in the future may be found for the use of data-mining tools to provide analyte detection and identification using panels of bioreporters producing specific fingerprints.

Bioreporter bacteria used in this research were “tailored” to respond in a dose-dependent manner to changes in environmental conditions with luminescence amplitude being proportional to bacterial damage [13,20,53–55]. However, after each exposure (three different times), the responsiveness decreased which could be due to the formation of biofouling (Fig. 3), decrease in their repair mechanism ability or some other reason. An additional and important issue is the recovery capability of the device-based bacteria (i.e. the time required by the system to return back to the unstimulated mode). A faster recovery rate increases the availability of the device for detecting further contaminants, thereby decreasing the possibility of overlooking passing pollutants [49]. In both setups (previous [13] and present), decreasing the toxicant concentration decreases the time needed for recovery, thus lowering the damaging effect of the toxicants on the immobilized cells, which enables faster recovery rate. Furthermore, after the second and third exposure to the same toxicants, there was a further decrease in the recovery time. As explained above, a

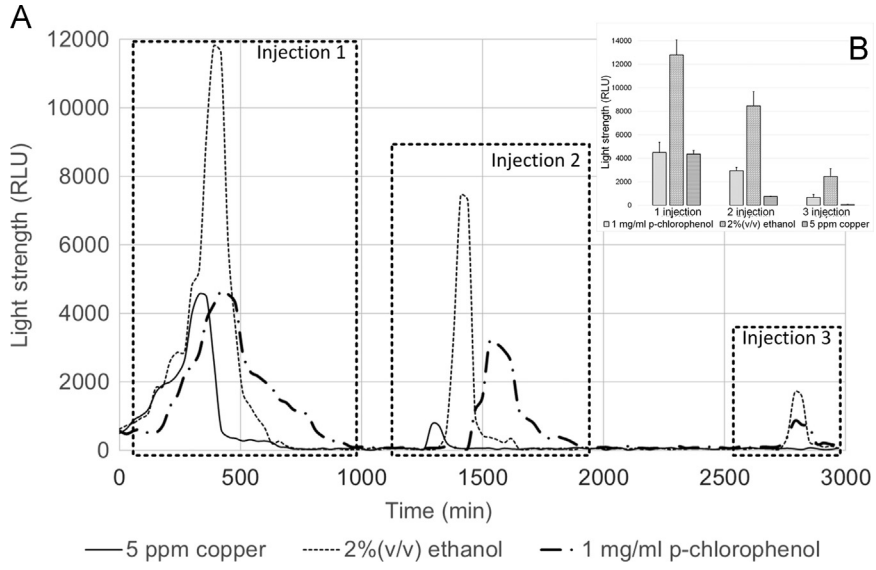

Fig. 5. (A) kinetic response of strain TV1061 to 2% (v/v) ethanol, 5 ppm copper and 1 mg/mL p-chlorophenol. Chemicals were added at $t=0$, $t=1{,}200$ and $t=2{,}680$ min with the addition of 5% (v/v) LB. (B) Summary of results from all experiments ($n=10$) using 2% (v/v) ethanol, 5 ppm copper and 1 mg mL^{-1} p-chlorophenol.

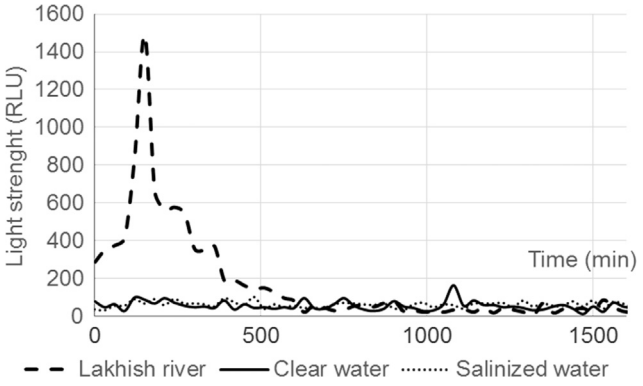

Fig. 6. Response of TV1061 strain to Lachish River water samples with the addition of 5% (v/v) LB.

possible reason is that their defense system may still be active from the first exposure, sensitizing the cells to the next damage.

3.6. *Environmental toxicity*

To evaluate the capability of the newly designed setup (Fig. 1B) to sense the presence of pollutants in environmental samples, bacteria were exposed to Lakhish (Israel) River water. Sewage and waste in the 70-km Lakhish River, which flows through the northern part of Ashdod, have plagued the city's residents over the years. Moreover, rainwater carrying large amounts of contaminations from road surfaces is drawn off into the sea without any treatment and consequently, both the coastal strip and Lachish River are now polluted [56]. Fig. 6 represents a 24-h experiment showing a strong inducing response suggestive of the presence of pollutants. To test the possible salinity effect of river water on immobilized bacteria, cells were exposed to synthetic saline water [57]. In this case the bioreporter bacteria response were similar to clear water, suggesting that the positive signal with the Lakhish samples was indeed due to presence of the contaminating chemicals. Thus, our system can test positive environmental samples, albeit without identification of the actual pollutants, therefore it can only be considered a first line of defense alarm. The kinetics of the response differed from some of the tested chemicals (Fig. 5), surely due to chemical mixtures at various concentrations [58,59].

4. Conclusions

Herein, a further optimized on-line fiber-optic system for monitoring water pollutants is reported after tweaking several engineering parameters. The designed architecture of the fiber-containing measuring unit decreased the shear stress forces, thus preventing its degradation and subsequently, the release of bioreporter bacteria into the tested water sample. Such a system, thus, allows for a higher flow rate of larger volumes of sample to be tested in less time.

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