First tests with a prototype flow-through real-time bacterial toxicity sensor for water contaminants

BTO 2010.030
December 2010

KWR Watercycle Research Institute

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Title
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Project number
11.1508.037 / B111658

Research programs
Chemical Waterquality and Health

Project manager
Annemarie van Wezel

Client
BTO

Quality Assurance
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Sent to
This report has been distributed among BTO-participants and is publicly available.

Preface

The experiments described in this report have been performed by Evgeni Eltzov both at the Ben-Gurion University (BGU) of the Negev in Beer-Sheva, Israel, under supervision of prof.dr. Robert Marks or at KWR Watercycle Research Institute under supervision of dr. Minne Heringa. The report itself was written by Evgeni Eltzov under supervision of both prof. Robert Marks and dr. Minne Heringa. The work was commissioned to BGU and supervised by dr. Minne Heringa in the framework of the BTO project "Supersensor for toxicity" (11.1508.037).

Summary

In the Netherlands, the chemical quality of drinking water is currently guarded by biomonitors at inlets of raw surface water, and chemical analysis of raw and drinking water. Although this is quite an advanced system already, it is still not optimal in view of protecting the health of consumers. What lacks is a sensory system that can rapidly (in real-time) detect the presence of all important human toxicants in water used for the preparation of drinking water.
In the past decade or so, bacterial strains have been developed that can detect the total effect of contaminants causing human toxicity, e.g. genotoxicity, membrane damage, oxidative damage and protein damage, providing results within 1-2 h. These bacteria are genetically modified organisms (GMOs), engineered to luminesce after exposure to certain toxic compounds. The suitability of employing these bacteria for testing static water samples on the presence of contaminants has been demonstrated previously.
In this study, a flow-through fiber-optic-based bacterial monitoring system for online monitoring of toxic pollutants in water has been developed. Two bacterial strains containing fusions of *recA* (DNA damage) and *grpE* (heat-shock) promoters to the *lux* operon (CDABE) were immobilized on a fiber optic and tested for their ability to detect pollutants in flowing tap water and surface water in this new system. Conditions for running the system for 24 h were optimized and first experiments with the system show (1 h-)response times and response heights similar to the previous static systems. Responses were related to the doses and the sensitivity is good (comparable to static systems), but needs to be increased to be able to monitor whether or not the low existing guideline values are exceeded by actual levels of pollutants. 24-h measurements in tap water demonstrate the ability of the device to run for such a time period, but in river water loss of functionality of the bacteria was observed. This flow-through fiber-optic-based monitoring system has proven to be a useful next step in the development of a simple on-line real time sensor for relevant human toxicants in flowing water.
Additionally, studies were performed to obtain an indication on how prepared fibers (with bacteria) can be stored for long periods, so that maintenance of the monitor by regular replacement of the fibers can be facilitated. Different techniques to conserve such bacteria have been investigated: lyophilization and storage at room temperature, 4 ^{0}C, -20 ^{0}C and -80 ^{0}C with either glycerol or sucrose as cryoprotectant. The most viable storage condition was found at -80^{o}C with the addition of 20% of glycerol. Fibers with bacteria can be stored for at least 3 months in this condition. Lyophilization according to the adapted protocol for a liquid solution was not successful at all. As lyophilization may in theory still be a useful method (and more convenient than storage at -80 ^{o}C), a different protocol may be studied in future research.

Contents

1 Introduction

In modern society thousands of chemicals are produced and used, which may eventually reach water systems [1; 2; 3] and threaten the quality of potable water prepared from these sources. For example, the US Environmental Protection Agency (EPA) reported that 141 unregulated chemicals were found in tap water in 45 states in the US, of which 40 chemicals were served to at least one million people [4]. In The Netherlands, the chemical quality of drinking water is currently guarded by biomonitors installed at inlets of raw surface water, and by chemical analysis of raw and drinking water. Although this is quite an advanced system itself, it is still not optimal in view of protecting the health of consumers. The chemical analyses are very sensitive, but fail to detect non target compounds and do not give information on the biological or physiological effects of the contamination. For many chemicals, toxicity data are not available. The biomonitors - employing, for example, mussels, *Daphnia*, and algae - are excellent for detecting the overall effects of contaminants in real time, providing fast alarms when a contamination peak occurs [5; 6; 7]. Detection of such a peak leads to the temporary closure of the water inlet, thus preventing the contaminants to enter the drinking water treatment process. However, the effects observed in these organisms has no clear link with the hazard for humans, and the biomonitors fail to detect some toxic contaminants important for humans (e.g. genotoxicants and endocrine disruptors), because they only detect acute toxicity and not chronic toxicity. Contaminants eliciting chronic effects can be identified with laboratory-based *in vitro* assays [8; 9], but the latter take at least 1-2 weeks to complete. Hence, there is a strong need for a sensory system that can rapidly (in real-time) detect the presence of all important human toxicants in water used for the preparation of drinking water.
In the past decade or so, bacterial strains have been developed that can detect the total effect of a mixture of contaminants in water that cause human toxicity, e.g. genotoxicity, membrane damage, oxidative damage and protein damage, providing results within 1-2 h. These bacteria are genetically modified organisms (GMOs), engineered to luminesce after exposure to certain toxic compounds. In contrast to some other luminescent organisms, these bacteria do not need addition of a substrate for the luminescence enzyme, such as luciferine, facilitating their employment in continuous monitoring. Various strains have been created, bearing plasmids with different promoters linked to a *luxCDABE* cassette, responding to different types of toxic action. For example, strains with a *recA* promoter react to DNA damage caused by genotoxic compounds [10] and strains with a *grpE* promoter react to general toxicity or stress, through the induction of heat-shock protein [11]. The luminescence can easily be measured using a photodetector and does not suffer from a variable background signal from water.
In general, there are two main types of early warning systems using these bacteria: liquid-phase biomonitors that exploit the bacteria in suspension [12; 13; 14], and immobilized-phase biosensors [15; 16; 17; 18; 19]. In the biomonitors, the bacteria are added to the test water, and also discarded with the water. The application of GMOs in the European Union is, however, bound to strict regulations. When a monitoring device with these bacteria is used outside a licensed (MLI or II) laboratory, the user must guarantee that no GMOs can escape to the environment, e.g., through the waste water of a device. The biosensor with the bacteria immobilized to an optic fiber [17; 18] has an advantage with respect to these regulations, as the bacteria in principle do not escape from the sensor device. Optical fiber bioluminescent whole-cell microbial biosensors have thus far only been tested in static setups [17; 18]. Initial work has shown that these systems may be used as sensors that can rapidly detect the presence of many human toxicants in water used for the preparation of drinking water. For this purpose, however, a flow-through setup is necessary.
The aim of this study was to develop a flow-through real time monitoring system, a next step in the ultimate goal of developing a simple field monitor for relevant toxic compounds in water. We have optimized the conditions for this device, tested it with two bacterial strains separately, tested several chemical compounds, and tested it in both tap and river water for up to 24 h. The observed simplicity of usage and maintenance make this application an attractive system for close to real time monitoring in various applications in the water quality monitoring field.
Additionally, studies were performed to obtain an indication on how prepared fibers (with bacteria) can be stored for long periods, so that maintenance of the monitor by regular replacement of the fibers can be facilitated. Although numerous reports describe the genetic engineering of bacterial sensor strains, very few address the largely unresolved concern of the 'shelf-life' of such constructs. Indeed, storage of

reporter bacteria at ambient temperatures for prolonged periods, with maintained response characteristics and without the need to re-grow the cells, is still a major challenge [20]. To meet these demands, various techniques to conserve such bacteria have been investigated, including lyophilization and other freezing methods.

2 Experimental

2.1 Materials

Tryptone, Sucrose, Glycerol, Yeast Extract, NaCl, Ethanol, 4-Chlorophenol, Mitomycin C, alginate, Hydroxylamine-O-sulfonic acid, Magnesium sulfate, Sodium citrate, Ferrous Sulfate heptahydrate, Thiamine hydrochloride, Potassium hydrogenphosphate trihydrate, Sodium phosphate monobasic, Biotin Casamino acid, Glucose, Uracil, Sodium chloride, Zinc sulfate heptahydrate, Copper(II) sulfate pentahydrate, Manganese sulfate monohydrate, Boric Acid, Ammonium molybdate tetrahydrate and Cobalt(II) chloride hexahydrate were of analytical grade and purchased from Sigma. All stock solutions were diluted with double distilled water (ddW), and stored at the temperature suggested by the manufacturer's instructions.

2.2 Bacterial strains

Two different *Escherichia coli* strains were used in this study: DPD2794 [10, 21, 22] and TV1061 [11], both obtained as a kind gift from S. Belkin (Hebrew University, Jerusalem, Israel). Each strain harbors plasmid-borne fusions of the specific promoters to a reporter gene. The *recA* [10] promoter in strain DPD2794 activates DNA repair systems due to DNA damage. The heat-shock *grpE* [23] promoter in the strain TV1061 is sensitive to metabolic changes, such as with cytotoxic substances. All these promoters are chromosomally integrated to the lux CDABE reporter operon, which has five promotorless structural genes 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 multi-enzyme complex composed of fatty acid reductase, transferase, and a synthetase (lux C, D and E) [24[. The strain stocks were stored at -80°C with 20% (v/v) of glycerol as a cell cryoprotectant additive [25]. The bioreporter strains from stock solution were placed on Lutania-Bertani (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 kanamycin and after growth overnight at 37°C in a rotary thermo-shaker (Gerhardt, Germany), they were stored at 4°C for future experiments.

2.3 Growth Conditions

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}) [26] supplemented with 50 µg/mL kanamycin for strains TV1061 and DPD2794. Cells were grown overnight at 37°C in a rotary thermo-shaker (Gerhardt, Germany) 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} of 0.2) as determined by an Ultrospec 2100 pro spectrophotometer (Amersham, England).

2.4 Immobilization procedures

The harvested cells were mixed 1:1 with a filter-sterilized 2% (w/v) low viscosity sodium alginate solution. Multimode optical fibers, PUV 400 BN (CeramOptec, GmBH, Bonn), 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 tip which was then used for the immobilization of bioluminescent cells [27]. 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, thus entrapping the bacteria onto the fiber within a hardened calcium alginate matrix. Repeating these steps thickened the adlayer, thus, increasing the number of bacterial sensor cells attached to the optical fiber transducer. Our lab has found over the years that six to seven layers have shown to be the optimal number of layers [17]. The optical fiber, with immobilized bioluminescent bacteria at the end tip, was then ready for monitoring of the various contaminants in water and was used immediately after preparation.

2.5 Instrument set-up

2.5.1 Setup at the Ben-Gurion University

We have previously designed a field-operable fiber-optic photodetector device [17]. In order to monitor toxicity in a flow-through manner, the device was modified by Neobionics Ltd, Israel, so as to include the components allowing such a feature (figure 1). The original instrument set-up was placed in a light-tight box. To prevent damage to the photon-counting unit by environmental light, a manual shutter (71430, Oriel) was placed in front of the detector (figure 1 B3). To move the slide shutter, a custom-made lever was placed outside the box (figure 1C). The output signal, in analog measurements, was the mean value of the signals that included AC components (pulses) generated after multi-anode magnification in the photomultiplier tube. A Hamamatsu HC135-01 PMT Sensor Module was used for bioluminescence measurements, combining the sensitivity of a photomultiplier tube with the intelligence of a microcontroller (figure 1 B1). The detector was optimized to the blue light region and included a 21-mm diameter active area convenient to gather light radiation without any optical focusing elements [17]. Several components exhibiting various features were then added to this device as follows. Water was flown through polyvinyl (PVC) tubes, where a specific mechanism was installed for the entry of the fiber probe to the water flow system (figure 1A). The flow system contained two taps (30529-04, Cole Parmer instrument company, USA) for controlling the water flow. The water flow was controlled with a tap that controls the incoming and out-coming water flow in the device (figure 1D), while the water flow was measured with a rotometer (1017, Burkert) localized on the exit of the device (figure 1F). The peristaltic pump (Masterflex® 7754-57, Cole Parmer instrument company, USA) allowed the water to flow in a closed circle. To receive and treat data, a specific driver was developed using LabView (version 3.1, National Instruments Corporation), which allowed monitoring of the bioluminescent signal and data handling in real-time.
The first design included a system of PVC tubes to prevent light interference, in order to decrease the background signal. However, the first experiments showed that there was no need for this system, because the levels of the background signal were similar in both cases.

2.5.2 Setup at KWR

In order to test the system with Dutch surface water, the device was moved to the laboratory of KWR Watercycle Research Institute in the Netherlands and placed in a setup as shown in figure 2. Due to Dutch regulations and the fact that it was not certain whether absolutely no bacteria would escape from the fiber, all wastewater from the system had to be collected and sterilized. A 50L tank was filled with tap water or surface water. The sensor device was connected to a tubing through which the tank water was pumped via a valve. The positive controls were 50 µg/L Mitomycin C for strain DPD2794 and 2% (v/v) ethanol or 50 µg/L p-Chlorophenol for strain TV1061. The pH of the positive controls was adjusted to that of the monitored water, to prevent a pH-shock for the bacteria. The water flow of the tested water was constant at approximately 25 mL/min. To maintain a stable signal, the water stream was connected to a stream of LB-medium to bring it to a final concentration of 8% (v/v) LB-medium. The water temperature was monitored through a temperature sensor, and kept at 26°C by adjusting the temperature of the bath (figure 2N). An empty 50L barrel containing 500 mL of household chlorine bleach was placed at the water outlet of the device. This amount of bleach provided a final concentration of 400 mg NaHClO per L when the barrel was full, which is at a much higher level than required for total disinfection. There was no mixing in the collection barrel but in case of a spill, the contents would be mixed in the box in which the barrel was purposely placed.

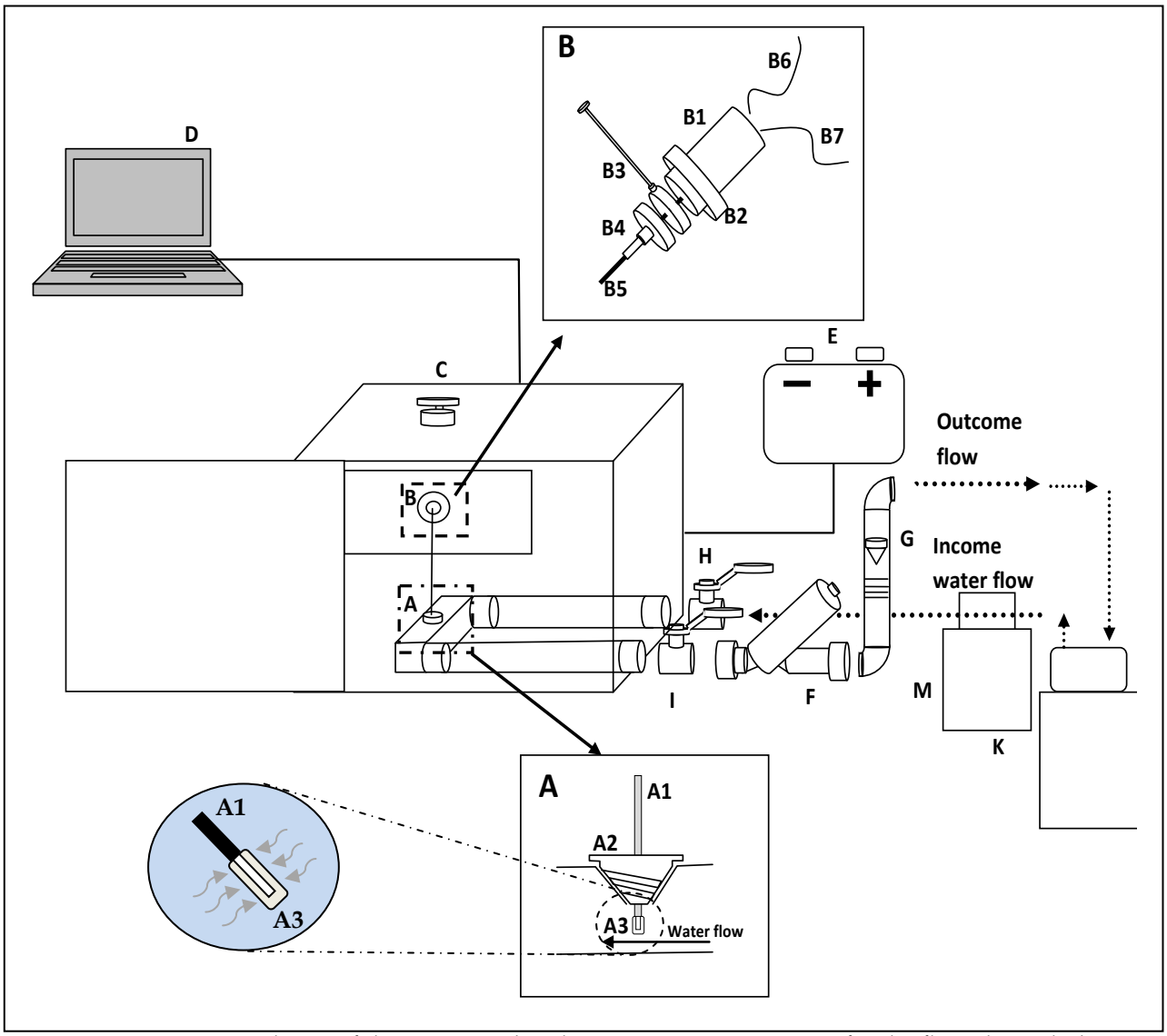

Figure 1: Descriptive scheme of the setup used at the Ben-Gurion University for the flow-through device for real-time monitoring of the toxicity of a water stream. A. Fiber optic device holder with fixed bacteria subjected to the water stream. A1. Fiber optic, A2. Fiber optic holder. A3. Alginate matrix with bacteria immobilized on the fiber optic core. B. Photon counting unit. B1. Hamamatsu HC135-01 PMT Sensor Module. B2. PMT fixation ring B3. Manual shutter (71430, Oriel). B4. Fiber holder that prevents the movement of the fiber inside the photon counting unit. B5. Fiber optic. B6. Connection wire of PMT to computer. B7. Electricity cable. C. The outside handle of the manual shutter that enables light access to the PMT. H. Incoming water flow valve (30529-04, Col Parmer instrument company, USA). I. Outcome water flow valve (30529-04, Cole Parmer instrument company, USA). F. Filter. G. Rotometer (1017, Burkert). D. Computer. E. Electricity source. K. 5-L water bottle. M. Peristaltic pump (Masterflex® 7754-57, Col Parmer instrument company, USA).

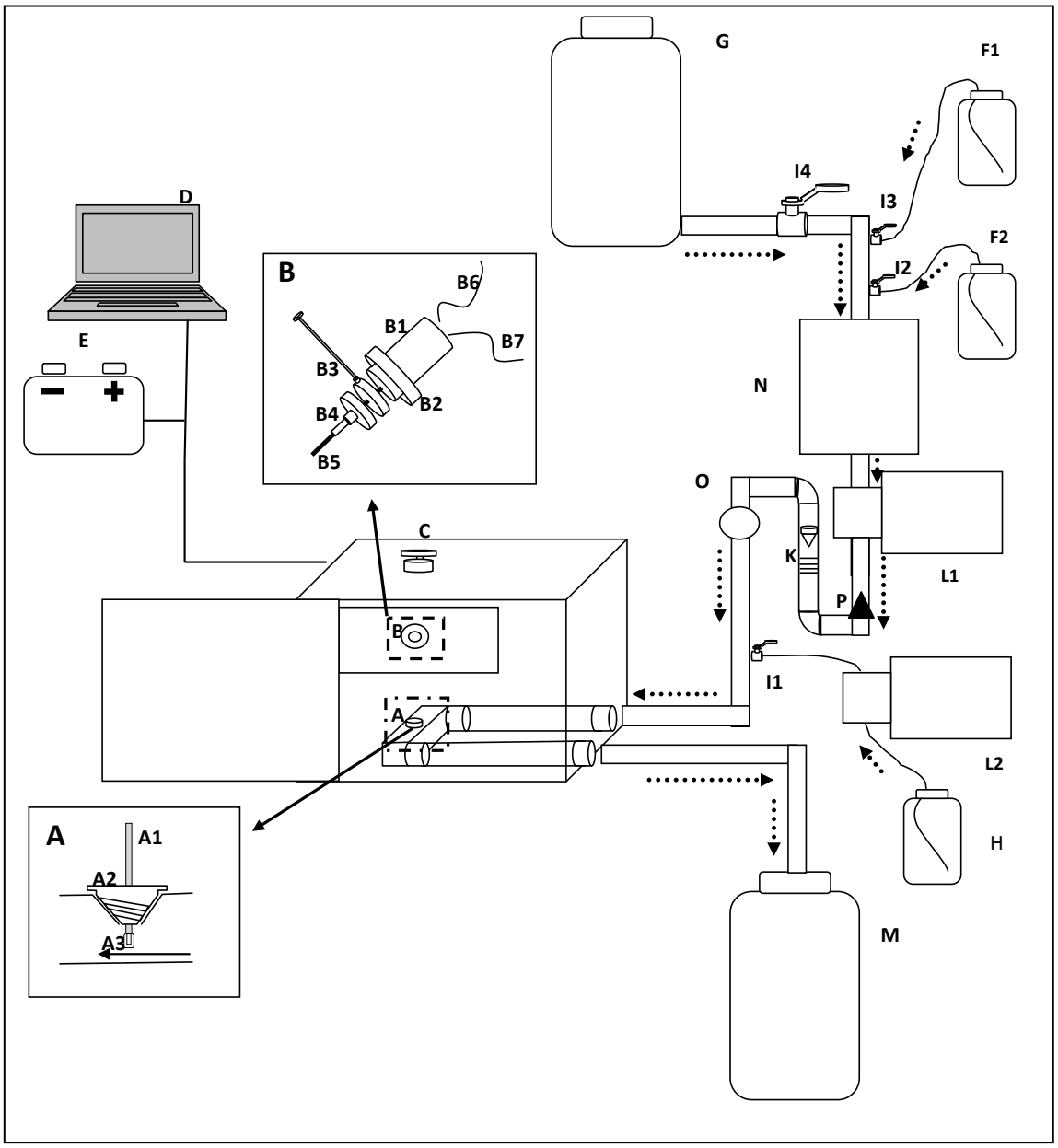

Figure 2: Descriptive scheme of the setup used at KWR for the flow through device for real-time monitoring of the toxicity of a water stream. Dashed arrows indicate the direction of the liquid flow. A. Mechanism of the entrance of fiber optic with fixed bacteria to the water stream. A1. Fiber optic, A2. Fiber optic holder. A3. Alginate matrix with bacteria immobilized on the fiber optic core. B. Photon counting unit. B1. Hamamatsu HC135-01 PMT Sensor Module. B2. PMT fixation ring. B3. Manual shutter (71430, Oriel). B4. Fiber holder that prevents the movement of the fiber inside the photon counting unit. B5. Fiber optic. B6. Connection wire of PMT to computer. B7. Electricity cable. C. Outside handle of manual shutter that enables light access to the PMT. D. Computer. E. Electricity source. H. -L bottle with medium. G. Incoming water container. I. Flow valves (30529-04, Cole Parmer instrument company, USA) I1. Valve for medium flow. I2 and I3. Valves for positive control. I4. Valve for outcome water flow. K. Rotometer (1017, Burkert). L. Peristaltic pump. L1. Pump for tested water and positive control. L2. Pump for medium. M. Container for outcome water. N. Bath. O. Temperature sensor. P. Backflow preventer.

2.6 Determination of the minimum required LB concentration in the water flow

To optimize the survival of the immobilized bacteria during a 24-h exposure to natural water, it was studied whether and how much growth medium would be needed to be added to the water flow. The minimum required LB concentration in the water flow was determined by adding different concentrations 0-20% (v/v) of sterile LB-medium to the water flow. Four-h experiments were performed with tap water and strain TV1061 in the Ben-Gurion setup. To verify whether the reporter strain was functional, after sixty minutes from the beginning of the experiment, bioreporter bacteria were exposed to the positive control, 2% (v/v) ethanol, during 1 h.

2.7 Response of the reporter strains to positive controls

The functionality of the reporter strains in the new flow-through system was studied by exposing them to various concentrations of positive controls. The strain TV1061 was exposed to 50 and 500 µg/L of p-Chlorophenol and 2% (v/v) of ethanol in the Ben-Gurion setup, while DPD2794 was exposed to 100 and 800 µg/L of Mitomycin C in the KWR setup. All solutions were prepared with the same clear tap water that was dosed to the bacteria during experiments.

2.8 Disinfection test

Due to the strict EU regulations for GMO waste, it was investigated how the waste water from the experimental setup at KWR was best disinfected, by measuring how much household chlorine bleach is necessary to disinfect the investigated field water. Five bottles were prepared with 1 L of Meuse water and 8% (v/v) of LB-medium (table 1). Household chlorine bleach (approximately 40 g active chlorine/L) was added at 0, 0.35, 1.5, 3.5 and 14 mL/L of field water. The samples were left to stand for 24 h. The next day 0.5 L of the samples was transferred to specially cleaned glass bottles, ensuring that there was no air left in the bottle. In addition to these samples, 0.5 L was taken from the approximately 36 L of waste water from a 24-h experiment with the same batch of Meuse water and transferred to the special glassware. To the collection tank of this waste water, 500 mL of household chlorine bleach had been added beforehand (final concentration 14 mL of bleach per L of water). The final sample was 1 L of an 8000-fold dilution of the household bleach (0.125 µL of bleach in 1 L of milliQ water), from which 0.5 L was transferred to the special glassware. The free chlorine concentration in these samples was determined according to the KWR protocol LAM-072, which is in conformity with NEN and ISO 7393-1. Three samples of 100 mL of the waste water collected from the 24-h experiment were filtered and analyzed for *E. coli* according to the protocol of ISO 9308-1 at KWR. This analysis was meant to prove that the disinfection procedure of the wastewater was sufficiently effective.

2.9 Test with Meuse river and Lekkanaal water

Two 24-h experiments were performed with water from the river Meuse (The Netherlands) using strain TV1061 in the KWR setup (fig. 2) and one with Lekkanaal water using strain DPD2794. Freshly collected Meuse and Lekkanaal water was stored at 4 °C and used within five days to prevent quality loss as much as possible. To verify whether the reporter strain was functional during the entire duration of the test, the bioreporter bacteria were twice exposed to the positive control: at the beginning of the test and at the end. Strain TV1061 was exposed to 50 µg/mL of p-Chlorophenol or 2% (v/v) of ethanol, while DPD2794 was exposed to 800 ppb of Mitomycin C.

2.10 Storage behavior of reporter cells immobilized in calcium alginate on the fiber optic.

Storage studies were performed using reporter cells (*E. coli* TV1061) immobilized within the calcium alginate matrix on the fiber optic (media condition, immobilization steps described in section 2.2, 2.3, and 2.4). There were two storage strategies (figure 3). The first consisted of storage of immobilized reporter cells on the fiber optic with two well known cryoprotectants (20% (v/v) glycerol or 24% (w/v) sucrose) at various temperatures [25]. The second strategy consisted of freeze-drying (lyophilization)of immobilized reporter cells on the fiber optic and storage at +4°C.

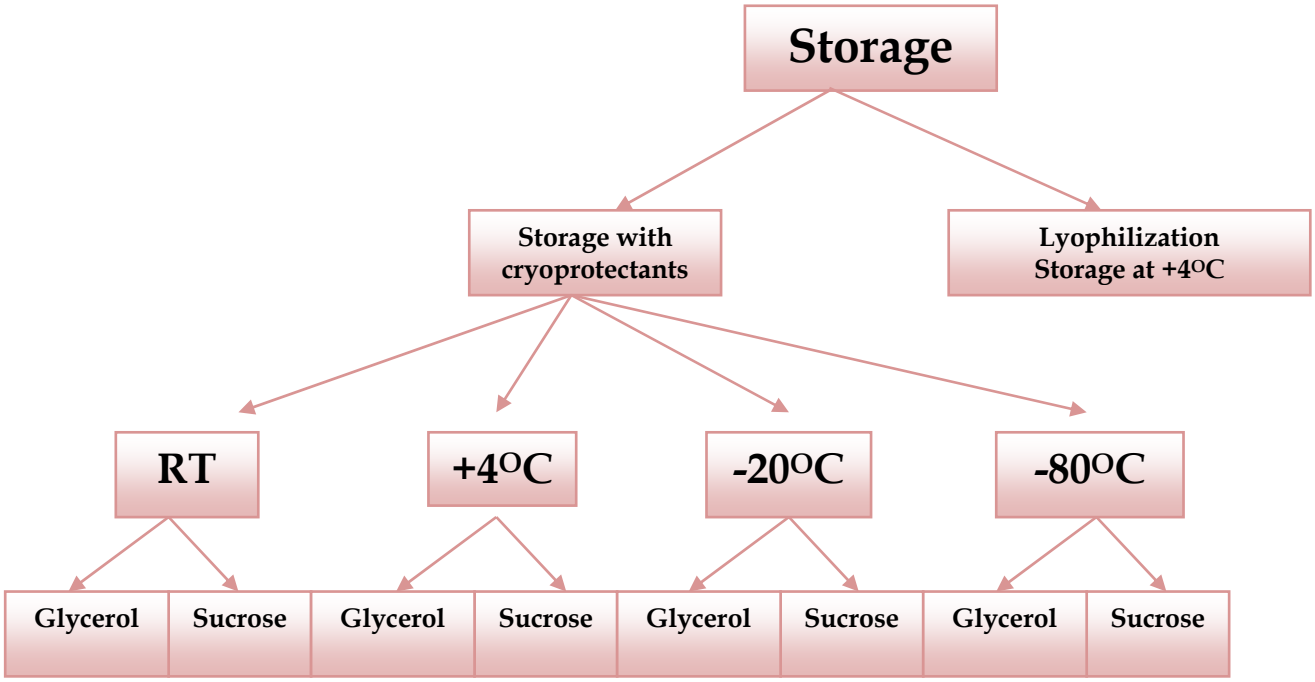

Figure 3: Scheme of investigated storage methods of reporter cells immobilized on the fiber optic with a calcium alginate matrix (RT is room temperature).

2.10.1 Freezing

Prior to immobilization, bacteria were grown and re-grown as described in section 2.3 until the desired concentration (OD_{600} = 0.2) was obtained. Thereafter, bacteria were immobilized on the fiber optic as described in section 2.4. The fiber optic with immobilized bacteria was entered into an eppendorf vial (with a special holder for the fiber optic) with 1.55 mL of one of the storage solutions (figure 4). The storage solutions were either 20% (v/v) glycerol or 24 % (w/v) sucrose in modified LB-medium, where the glycerol and sucrose acted to prevent lysis of bacteria during deep freezing. The alginate gel can be easily redissolved by immersing in a solution containing a high concentration of sodium. Therefore, all bacteria fixed on the fiber optic were placed in modified LB-medium, where the NaCl content was exchanged for an equimolar concentration of $CaCl_2$, to prevent disruption of the hydrogel from fiber tip. To the final solution, 50 mg/L of kanamycin was added for plasmid maintenance in bacteria. The final storage product (FSP) (holder for fiber optic + fiber optic) was incubated for 1 h at +10°C for better freezing resistance and for better glycerol penetration into the cells [25, 28-30]. After this pre-storage, all FSPs were cooled to the investigated storage temperatures (-80°C,-20°C, room temperature (RT) and +4°C) for longer storage periods of two to three months.

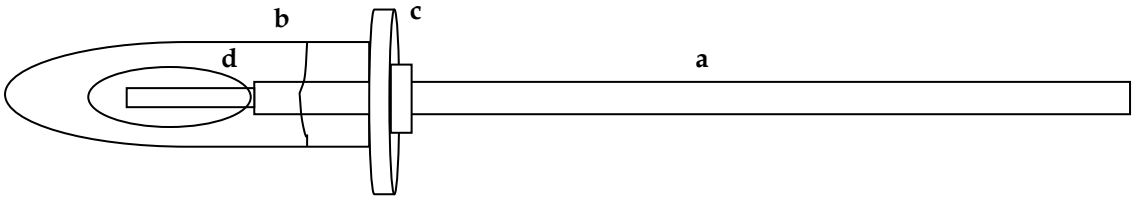

Figure 4: Fiber optic setup for storage. a. fiber optic. b. eppendorf tube c. fiber optic holder, to prevent the movement of the fiber end with fixed bacteria in the tube. d. Immobilized bacteria on the fiber optic end.

2.10.2 Lyophilization

Freeze drying (also known as lyophilization) was performed according to a published protocol [31]. Four different concentrations of alginate (2%, 3%, 4% and 6% (w/v)) were tested, while each concentration

was tested in culture medium with two different concentrations of $CaCl_2$ (an equimolar concentration to NaCl in normal LB-medium and a concentration twice as high). The fibers were placed in the eppendorf storage units (FSPs) containing the modified LB-medium + lyophilization additives: 0.3 g/L NH_3SO_4, 0.45 g/L $MgSO_4$, 0 047 g/L NaCitrate-2×H_2O, 0.025 g/L FeSO4×7H_2O, 0.06 g/L thiamine-HCl, 1.95 g/L K_2HPO_4×2H_2O, 0.9 g/L NaH_2PO_4, 0 005 g/L biotin, 20 g/L casamino acids, and 1 mL trace element solution. The trace element solution consisted of: 8 g/L $ZnS0_4$×7H_20, 3 g/L CuSO, ×5H_2O, 2.5 g/L MnS04×H20, 0.15 g/L boric acid, 0.1 g/L NH4MoO4×4H20, and 0.06 g/L CoCl×6H2O). Prior to freeze drying, FSPs were frozen at -180°C with liquid nitrogen. Frozen FSPs were then placed in a lyophilizer (Beta 1-8, Christ, Germany) and processed overnight at 120 mtorr at -100°C. At the end of the freeze drying process, all FSPs were moved to +4°C for one week. Resuspension of freeze dried fibers was performed with sterile water equal to the volume of the medium prior to the lyophilization process. The alginate matrix integrity was visibly studied and a viability test was performed.

2.10.3 Viability measurements

The evaluation of the viability of stored cells was accomplished by the viable plate counting method [32]. Frozen samples were thawed at room temperature for a few minutes. The alginate gel of each fiber was mechanically removed from each fiber and collected in a separate eppendorf tube, containing 1 mL of sterile 50 mM sodium citrate water solution (citration is used to break down the gel structure containing the entrapped bacteria [33]. The tubes were gently shaken for 20 min on a Heidolph® Polymax wave platform shaker (Brinkmann Instruments, Canada), with the tubes placed horizontally (parallel to the shaker board). Serial 10–fold dilutions were made in sterile PBS buffer, and 100-µL samples plated onto LB agar containing 50 µg/mL of kanamycin. The number of colony forming units (cfu) was counted following a 24-h incubation at 37°C. The bacterial concentration (C) was calculated by equation 1, where D_f is the dilution factor:

$$C = \frac{cfu}{0.1mL} * D_f \qquad \text{eq. 1}$$

3 Results and discussion

3.1 Determination of the minimum required LB-medium concentration in the water flow

When bioreporter strains were exposed to tap water without LB-medium ("clear water"), bacteria did not exhibit any signal response to the positive controls (figure 5). They did show a typical response pattern at the beginning of the exposure to clear tap water only (no positive control added yet): a fast increase of light production at the beginning and then a decrease with a lower rate, until reaching a stable low level (figure 6). This observation can be explained by the fact that for a normal functionality (bacterial metabolism, damage repair and luciferase production), bacteria require a continuous supplement of nutrients. The lack of nutrients in the clear water stream probably "shocked" the bacteria both through cellular damage and DNA-damage pathways, resulting in an initial, rapid increase of the response signal in both bioreporter strains. Subsequent signal decrease and the lack of response to positive controls seem to demonstrate bacterial mortality or metabolic silencing during the experiment with clear water. The observations after addition of LB-medium to the sample water stream support this finding. At low LB-medium concentration (5% v/v), the signal pattern was similar to the clear water responses, while at higher LB-medium concentrations (7.5%, 10% and 20% v/v), the overall picture changed drastically. Increase of LB-medium concentration then increased the background signal (figure 7) and bacteria started to respond to the positive controls (figure 5; for sake of figure clarity only the data for 0% and 7.5% LB-medium are shown;).

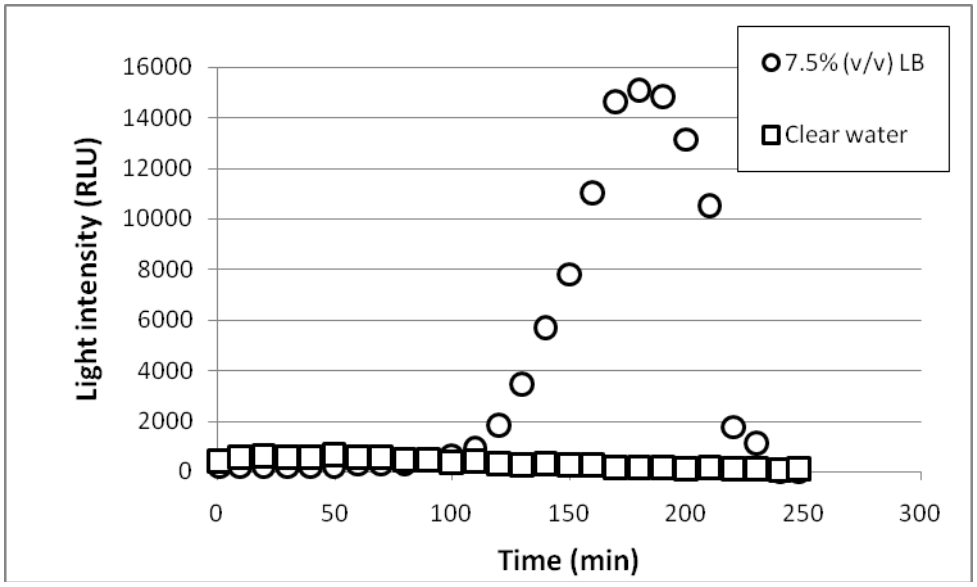

Figure 5: Signal comparison of the TV1061 strain response with (O) and without (□)LB-medium added to tapwater, with addition of 2% (v/v) ethanol at t=60-120 min).

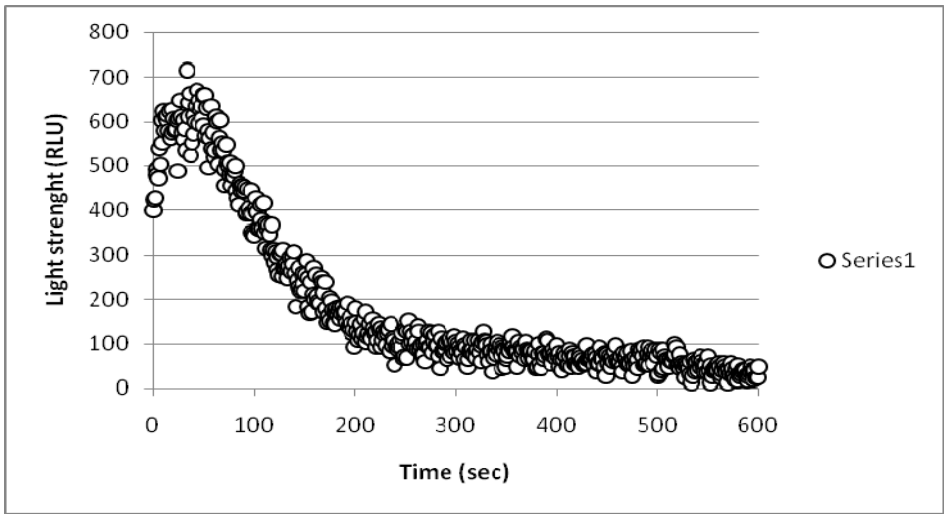

Figure 6: Response of the TV1061 strain to the addition of 2% (v/v) ethanol in tap water at t=0 sec , without LB addition to water stream.

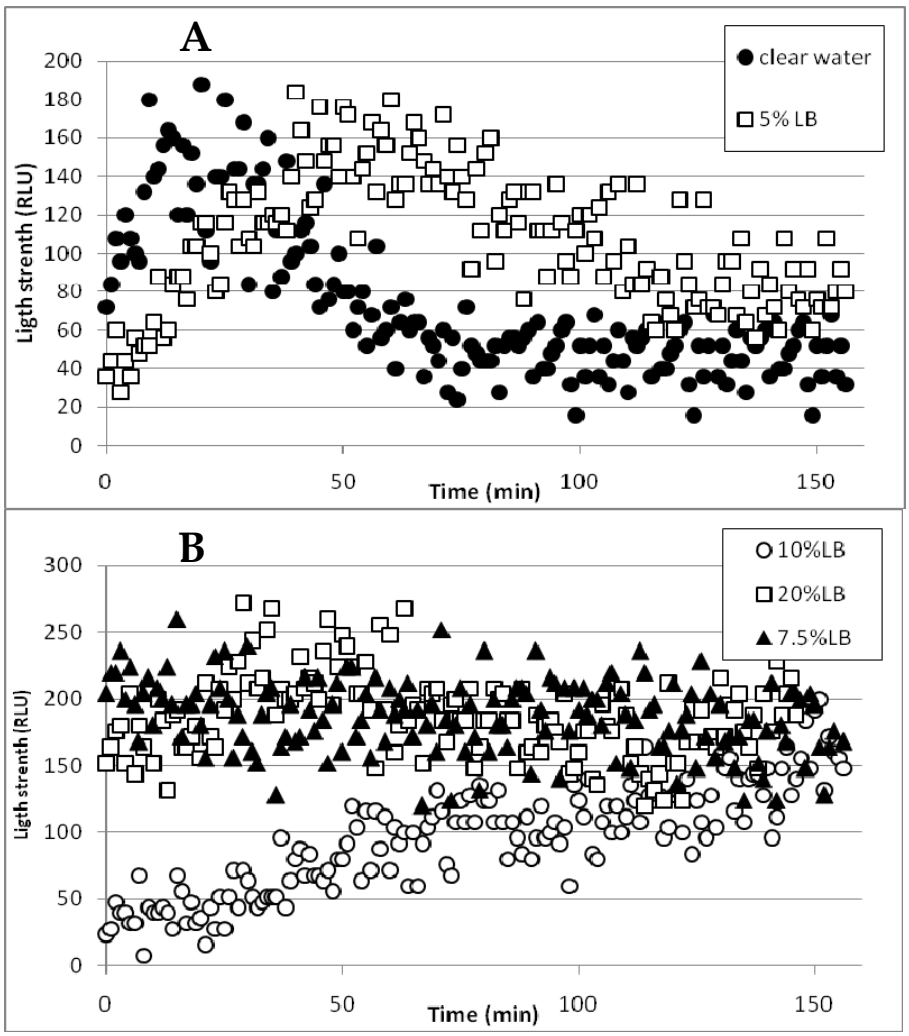

Figure 7: Signal comparison of the TV1061 strain response to different concentrations of LB (A. 0% and 5% (v/v) B. 7.5%, 10% and 20% (v/v)) with addition of 2% (v/v) ethanol at t=100.

For the determination of the optimal LB concentration, the potential for biofouling was also taken into account. Biofouling is the process of the overgrowth of bacteria on a specific matrix [34], in this case the alginate gel. Biofouling may prevent diffusion of the nutrients to the reporter bacteria and of any toxic agents. To prevent biofouling as much as possible, the lowest LB-concentration of 7.5% was chosen with which still a good and reliable signal response could be obtained.

3.2 Response of the reporter strains to positive controls

After the determination of a minimal LB concentration required for the maintenance of the system, bioreporter bacteria were exposed to toxic compounds to demonstrate their dose dependency, in water containing 7.5% LB-medium. The TV1061 strain was exposed to various concentrations of p-chlorophenol, while DPD2794 was exposed to various Mitomycin C concentrations. Mitomycin C agents cause DNA-damage by covalent binding to the minor groove of DNA, thus preventing the separation needed for DNA replication [35, 36]. The DPD2794 strain responses to the two mitomycin C concentrations are shown in figure 8. As it can be seen, increase in mitomycin C concentration caused an increase in the signal response, starting about 1 h after the exposure started. A lower concentration (i.e. <100 µg/L) of mitomycin C can probably be used to still obtain a positive response, as lower signals would probably still be distinct from the background noise.

Phenols, contrary to Mitomycin C, result in damage to the membrane and proteins of the cell [37]. As in the previous case, the increase of the TV1061-signal started 1h after exposure of the bacteria to the p-chlorophenol control and the height of the signal increased with increasing concentration (figure 9). As in the case of DPD2794, a lower concentration than 50 µg/L of p-Chlorophenol can probably be used to still show a positive response. Exposure of TV1061 to cytotoxic compounds showed high responses in both positive control cases (ethanol and p-chlorophenol), with an increase of the signal 1h after exposure to damage agents.

These results show that the sensitivity of this system is roughly comparable to that of the static system [17, 18, 38] where responses were found at 10–5000 µg/L p-chlorophenol and 32–2000 µg/L mitomycin C, while the liquid-culture system [39, 40] was reported to have a detection limit of e.g. 10-50 µg/L mitomycin C. This is not low enough to detect typical levels of contaminations in surface waters, which usually range from < 1 ng/L to 1 µg/L. Some toxicants can have effects at these very low concentrations, some even in the Lng/L range [41]. Guideline values for several compounds also fall in this range of concentrations (e.g. aldrin, chlordane, and NDMA [42, 43]). In The Netherlands, recently indicative drinking water quality objectives have been proposed at 10 ng/L for genotoxic compounds and 0.1 µg/L for other non-regulated compounds (publication in preparation). In order for the sensor to be able too detect if contaminants exceed these levels, either its sensitivity must be increased, or (on-line) preconcentration of the water is necessary.

Another important issue for biosensors is the recovery rate, i.e. the time required by the system to return to the un-stimulated mode. This type of sensor is mainly meant to detect sudden contamination, for example chemical spills in a river, which leads to a peak in the concentration of this chemical. A faster recovery rate increases the availability of the device for detecting consecutive contamination-peaks, thereby decreasing the possibility to overlook pollutants in the water. As can be seen in our setup, a maximum of 10 h is required for both reporter strains to get back to the un-induced state. This rate is three times faster than the recovery rate of the liquid culture system with the same bacteria [39]. Lower doses of toxicants are expected to further decrease the time of recovery, as lighter damage should theoretically require less time to repair. This expectation is confirmed by figure 9, but not by figure 8, however. Therefore, the occurrence of shorter recovery times by lower doses remains to be proven experimentally.

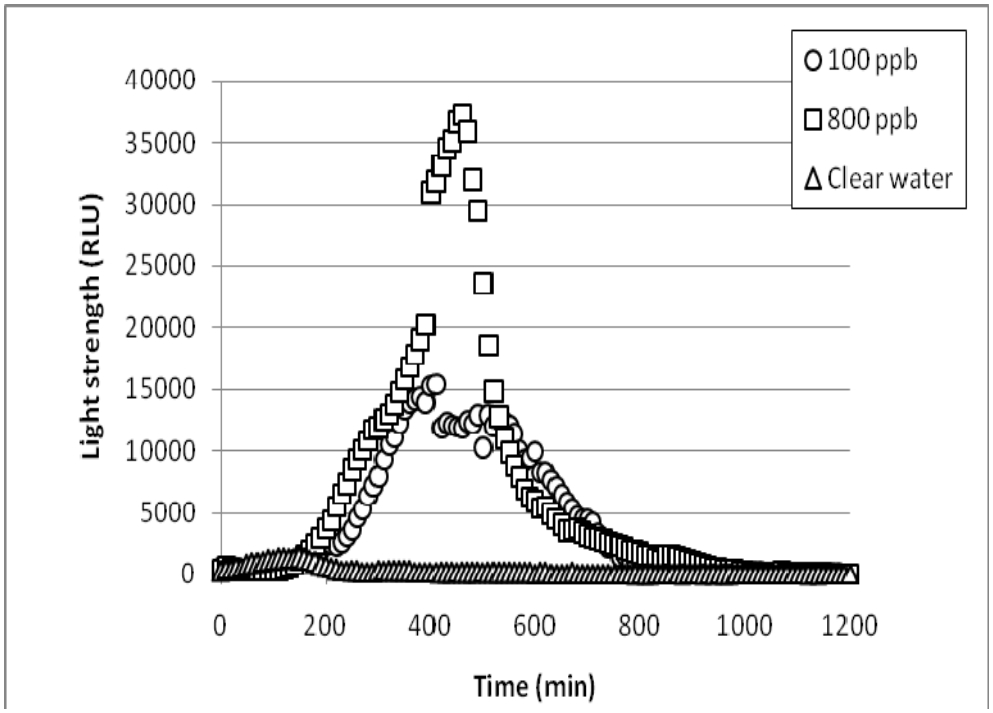

Figure 8: Response of the DPD2794 strain to exposure of a pulse of mitomycin C at different concentrations in tap water.

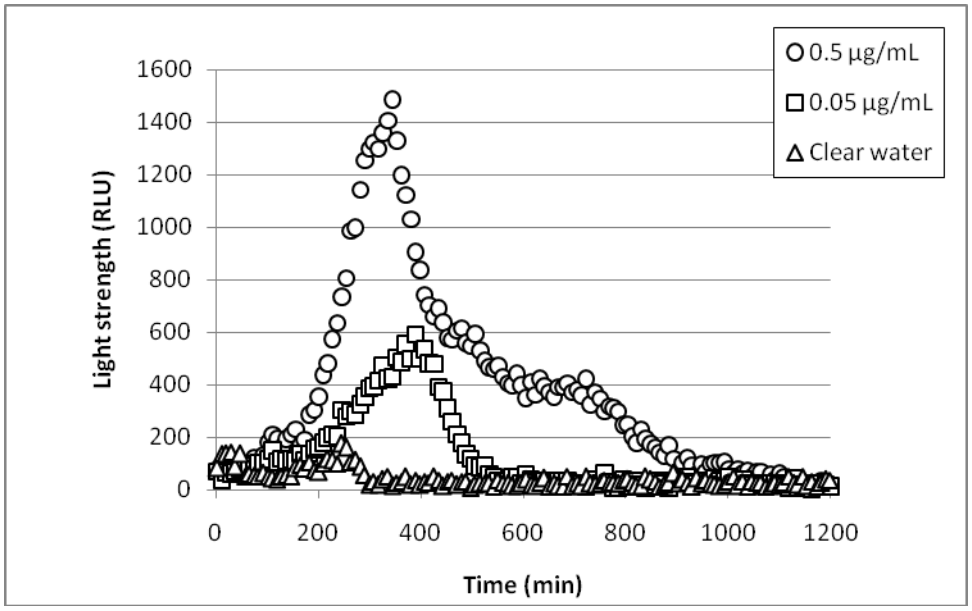

Figure 9: Response of the TV1061 strain to exposure of a pulse of p-chlorophenol at different concentrations in tap water.

3.3 Verification of bleach disinfection of tested water sample

Table 1 summarizes the results of the bleach disinfection experiment. Household bleach was found to have about the same level of free, active chlorine as claimed by the manufacturer (37 g/L versus 40 g/L). The samples used up much of the free chlorine, however, with the prepared samples needing at least 140 mg/L for an overdose. Strikingly, the sample from the 24-h set-up run had less free chlorine left over (0.21 mg/L) than the prepared sample (1.1 mg/L left over), while the same concentration of bleach was added to both samples (560 mg/L). There are two possible causes for this difference. Firstly, the chlorine from the bleach may have evaporated for a larger part in the barrel than in the bottle of the prepared

sample, as the barrel had a lot of headspace. Secondly, the lack of mixing in the waste barrel of the 24-h experiment may have led to an incomplete instantaneous sterilization, leaving some microorganisms to grow during the 24 h. The resulting higher content of organisms would then have used up more of the free chlorine when the barrel content was mixed at the end of the experiment. Clearly, 0.5 L of bleach was necessary per barrel with a 36 L river water collection, i.e. 560 mg chlorine per L. This was confirmed with *E.coli* cultures of the sample from the 24-h experiment, which showed no growth of bacteria, proving that the addition of 560 mg/L of chlorine was indeed effective. The common NaHClO concentration used for the total disinfection of natural water is 10 mg/L, (personal communication Gertjan Medema, KWR). The reason for this difference in bleach concentration may be due to the specific device setup that was used, with a higher water temperature (26 °C), a higher nutrient level from the added LB-medium, and the lack of mixing in the waste barrel.

Table 1: Test results for free chlorine determination.

Sample number	% LB	Volume (mL) of bleach added per L	Corresponding ratio in waste barrel	Resulting conc. active chlorine in barrel/ sample[1] (mg/L)	Free chlorine found (mg/L)
1	7.5	0	0 : 36	0	<0.03
2	7.5	0.350	0.0125 : 36	14	<0.03
3	7.5	1, 5	0,054 : 36	60	<0.03
4	7.5	3,5	0.125 : 36	140	0.16
5	7.5	14	0.5 : 36	560	1.1
6	0	8000-fold dilution of bleach		5	4.6
7	7.5	24-h sample	0.5 : 36	560	0.21

[1] Bleach claimed to contain 40 g active chlorine per L = 40 mg/mL

3.4 Test with Meuse river and Lekkanaal water

The results of the 24-h experiment using river Meuse water are shown in figure 10A. Exposure of the bacteria to the positive control after a 20 h experiment produced a signal response, which demonstrated that part of the bacteria were still functional. However, this response was much lower than the day before. A possible reason for the signal intensity decrease may be that the bacteria were unable to totally recover after the initial positive control exposure. If the bacteria are damaged too much by the positive control, they will lose their viability, causing the sensor to function less well. If this is the case, this could be prevented by applying the lowest concentration of positive control possible, that still elicits a clear response, but that does not significantly harm the bacteria. Future research must determine these minimal positive control concentrations. Another possible reason for the decrease of the bacterial sensitivity is the creation of environmental bacterial layers on the alginate matrix, i.e. biofouling. Biofouling may prevent diffusion of the nutrients into the matrix or even produce bioactive compounds, which could damage the bioreporter bacteria after diffusion into the matrix and decrease their sensitivity.

Figure 10A also shows an increased signal at around 600 minutes runtime, which was during the night. We have no explanation for this increase. It cannot be caused by the emergence of water contaminants, as the water came from a tank and is therefore expected to have had a consistent composition. Possibly, new compounds, formed by growing microorganisms, may have caused this temporary increase in the signal. In other 24-h runs, we have seen inexplicable oscillations in the signal in the same overnight time period, reaching up to 2000 RLU (figure 10B). In contrast, figure 10C (where the bacteria were exposed to tap water instead of river water) does not show an increased signal during the night. These increases in response and oscillations may cause undesirable false alarms when this system would be used for monitoring, therefore the cause must be found and removed.

In figure 10C, the response to the second dose of the positive control seems better than in the river Meuse water experiment, reaching higher response values. Unfortunately, we were not able to continue the measurements to obtain the full response peak to the second dose of the positive control in this

experiment, as the water stock became depleted. It seems that the bacteria have more trouble surviving and functioning in river water than in tap water. Many of the studies published on the survival of *E.coli* in environmental waters relied on laboratory microcosm experiments [44]. In environmental water, bacteria may find themselves battling for survival, facing large variations in a complex array of physical, chemical, and biological factors [45, 46]. Therefore one or a group of these negative factors may explain survival differences between river and tap water. The precise cause of this must be elucidated and solved in future research before this type of sensor can be effectively used for monitoring natural water.

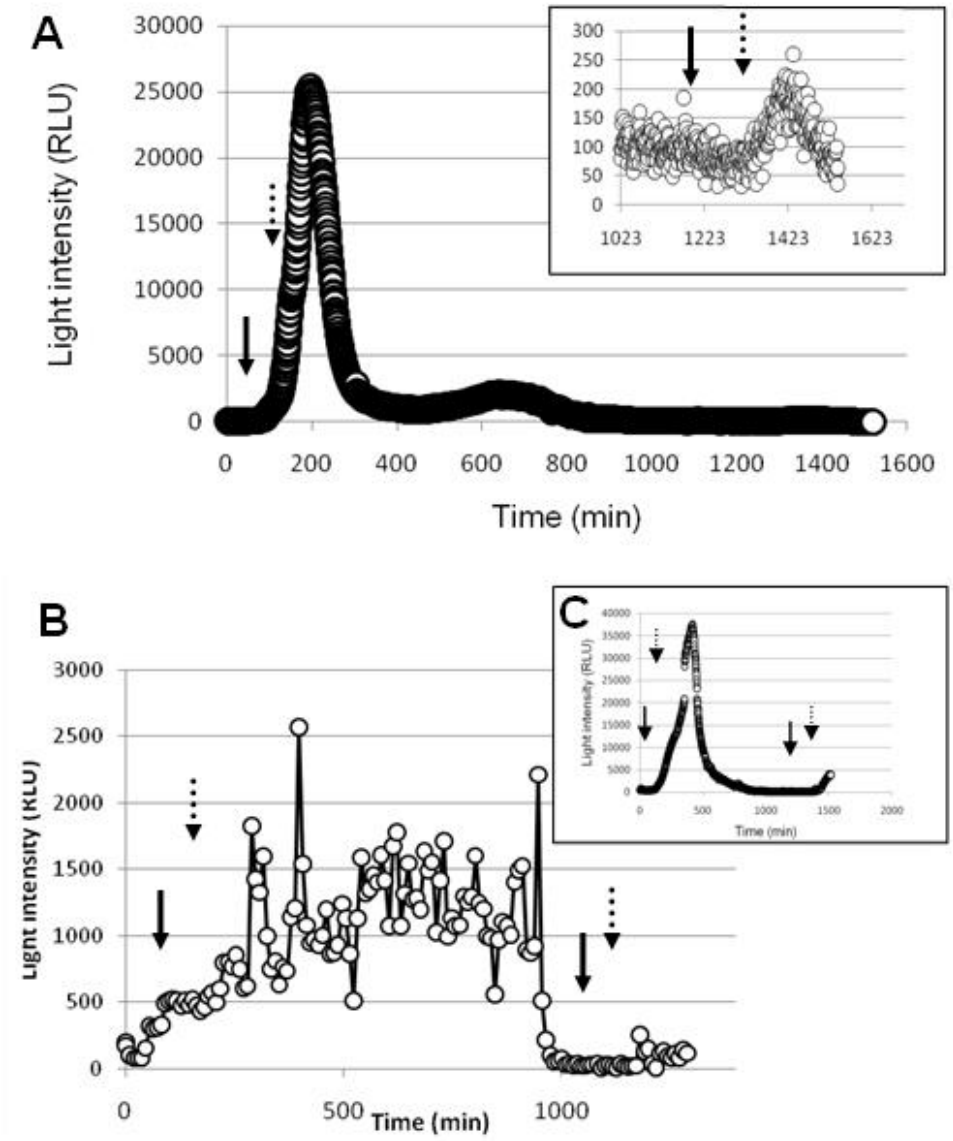

Figure 10: Analysis of river Meuse and tap water during a 24-h device operation. The solid arrows indicate the beginning and short dashed arrows the end of exposure of reporter strains to the positive controls. A. 24-h test with Meuse water, with strain TV1061 and 2% (v/v) ethanol as the positive control at t= 80-136 min and at t=1209-1273min (20-21 h). B. 24-h test with river Meuse water, with strain TV1061 and 2% (v/v) ethanol as the positive control at t= 36-53 min and at t=1000-1059min (16.6-17.6 h). C. 24-h test with tap water, with strain DPD2794 and 0.8 mg/L mitomycin C as the positive control at t = 52-120 min and at t= 1300-1360 min (21.6-22.6 h).

3.5 Storage behavior of reporter cells immobilized in calcium alginate on the fiber optic.

3.5.1 Comparison of different storage temperatures and two cryoprotectants

Viability of the immobilized reporter bacteria at various temperatures is shown in figures 11 to 13. In all these cases 20% (v/v) glycerol was used as a cryoprotectant. The sharpest decrease in the bacterial viability was observed at room temperature. In all measurements, it can be seen that the decrease in the storage temperature increased the time of viability of the fixed bacteria. At -80ºC the number of bacteria alive was almost the same as that at the onset of the experiment, even after almost 3 months.
Differences in the viability of reporter cells on the fiber optic using two well known cryoprotectants (20% (v/v) glycerol or 24% (w/v) sucrose) at various temperatures are represented in figure 14. In the case of the higher storage temperatures (RT and +4 ºC) sucrose gave a higher viability of bacteria than glycerol, while at the lower temperatures (-20 and -80 ºC), glycerol showed much better results. Thus, for long term storage, freezing at 80 ºC using glycerol as a cryoprotectant seems best.

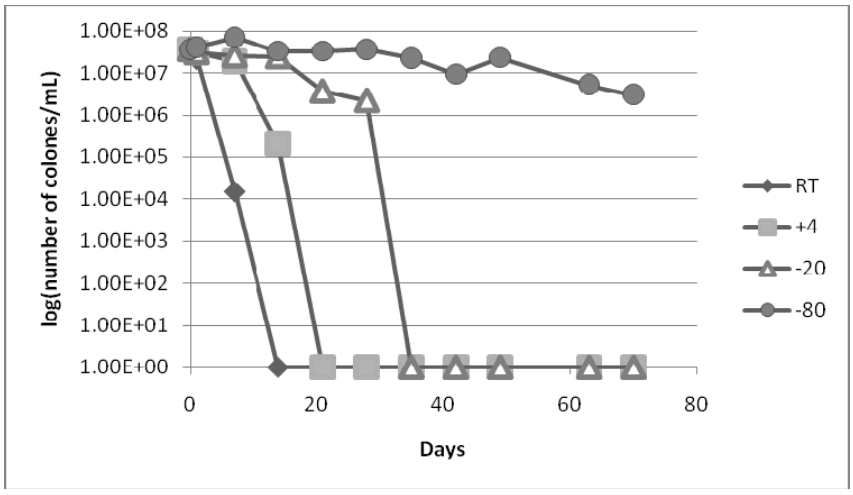

Figure 11: Comparison of the viability in time of the TV1061 strain stored at different temperatures (run 1). Diamonds represent storage at room temperature, squares at 4 ℃, triangles at -20 ℃, and circles at -80 ℃.

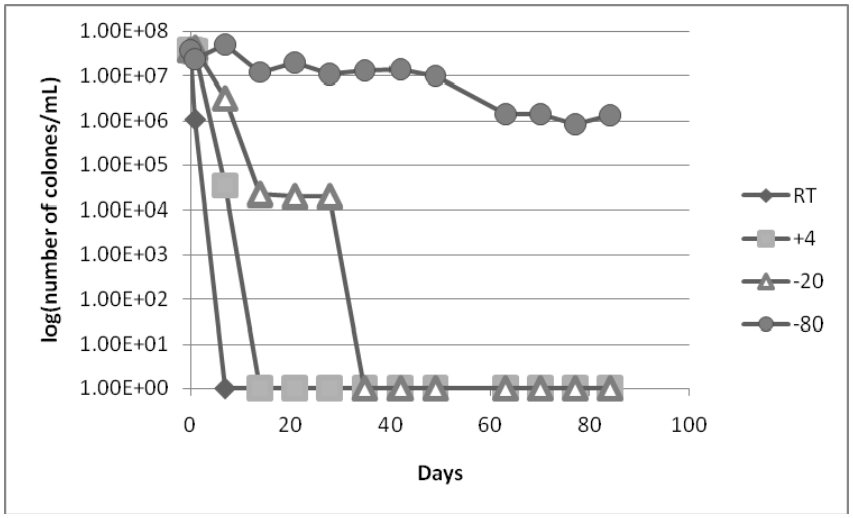

Figure 12: Comparison of the viability of the TV1061 strain stored at different temperatures (run 2). Diamonds represent storage at room temperature, squares at 4 ℃, triangles at -20 ℃, and circles at -80 ℃.

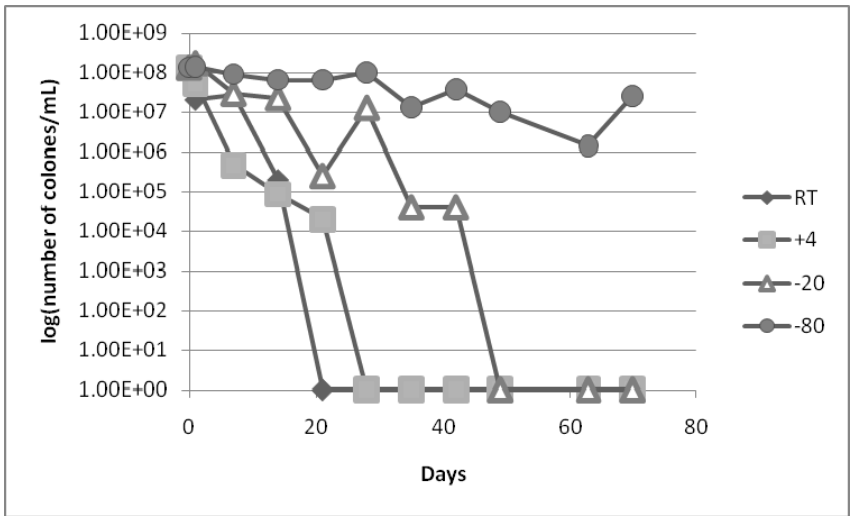

Figure 13: Comparison of the viability of the TV1061 strain stored at different temperatures (run 3). Diamonds represent storage at room temperature, squares at 4 ℃, triangles at -20 ℃, and circles at -80 ℃.

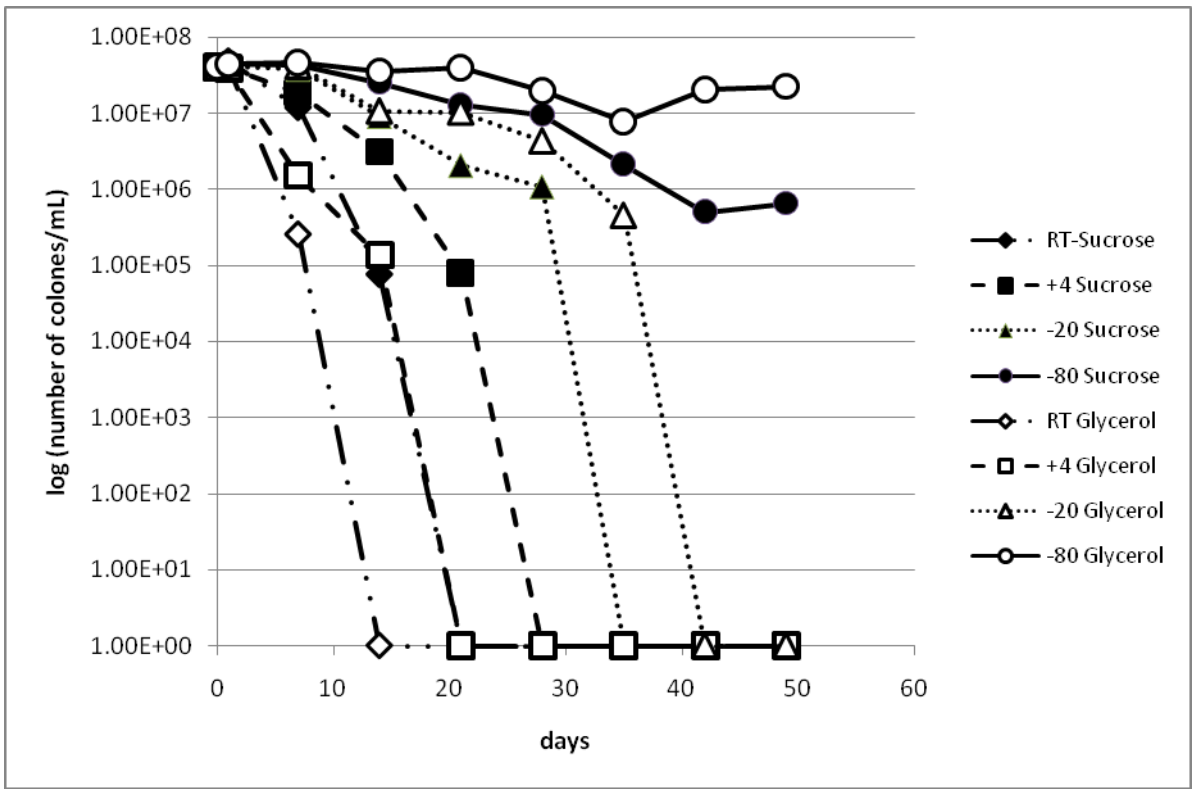

Figure 14: Comparison of TV1061 strain freezing with two different cryoprotectants, sucrose and glycerol. Diamonds represent storage at room temperature, squares at 4 ℃, triangles at -20 ℃, and circles at -80 ℃. Closed symbols represent storage in sucrose, closed symbols that in glycerol.

Preliminary experiments have indicated that storage of alginate beads in conventional *E. coli* media, LB broth medium for example, offers a good preservation condition, but causes a strong outgrowth of the immobilized cells from the matrix, even at low temperatures. The data obtained in these earlier experiments, where bacteria were stored at +4°C in LB medium in alginate beads, also showed that

immobilized cells retained both their luminescence (up to 70-75% from original luminescence) and viability relatively well for almost two months (data no shown). However, in these storage experiments there was a medium refreshment that prolonged the lifetime of the stored bacteria. In our setup in the present study, the bacteria were fixed on the fiber optic and stored for a couple of months without any refreshment of the stored medium. From the results we may observe a fast decrease in the bacterial viability at the higher temperatures, with the fastest decrease rate observed at RT. At +4^{o}C the metabolism rate of bacteria slows down, which can be observed in our results as a slower rate of decrease of the viability as compared to that of RT.
Only a few approaches in storing sensors in a frozen state are described in the literature for the preservation of immobilized cells in alginate at freezing conditions. In one approach, *Lactobacillus bulgaricus* cells were entrapped in beads of calcium alginate and evaluated for their ability to survive freezing processes. In this work, glycerol and mannitol were the cryoprotectives (but glucose was not) and each was added (6%) separately to the beads (co-entrapped with the cells [47]). In an other approach, Gram-negative bacterial cells *Pantoae* (*Enterobacter*) *agglomerance* strain IC 1270 were immobilized in calcium alginate spherical beads by co-entrapment of glycerol and colloidal chitin at different amounts, and then freeze-dehydrated (freeze-dried). Dried alginate beads with immobilized bacteria were stored at three different temperatures (ambient, 4^{o}C and -20^{o}C) and bacterial viability was examined over the course of 1 year. Further improvement in bacterial survival was achieved by adding 1% colloidal chitin to the alginate solution. At the ambient temperature, the bacteria survived poorly; however, this was slightly improved by the addition of chitin to the beads. Nevertheless, cell viability still decreased dramatically: no viable bacteria were detected after 3 and 4 weeks in beads without and with chitin, respectively. Storage at lower temperatures markedly improved bacterial survival. Immobilization in beads containing chitin preserved cell viability at 4 and -18 °C for 7 and 12 months, respectively, with no decrease in bacterial population. When the bacteria were immobilized in beads without chitin, no reduction in the number occurred for 3 and 8 months at 4 and -18 °C, respectively [48]. In other storage studies, the recombinant bioluminescent E.*coli* reporters for toxicity monitoring were freeze-dried in cell suspensions using four cryoprotectants (trehalose, sucrose, sorbitol or mannitol) at a concentration of 24% w/v. Trehalose and sucrose showed the best freeze-drying efficiency among the tested cryoprotectants [49].

3.5.2 Lyophilization

The second methodology and possibly preferred one for long-term fiber optic preservation, was the use the freeze-drying technique (lyophilization). This technique is very popular in the literature and used for many applications [50]. The lyophilization process was performed according to the protocol from the literature, where a liquid bacterial solution was freeze-dried. As we intend to store optic fibers containing bacteria, a modification of this protocol for a bacterial solution was required into one for optic fibers with a alginate gel [31]. However, lyophilization of the fiber optic according to the adapted protocol was not successful at all. Bacterial viability decreased so drastically that even directly after lyophilization, it was found to be only 1% (data not shown). For example, at 2% alginate the number of live bacteria decreased from 10^8 to 10^3.
Another problem was the loss of integrity of the alginate matrix after the lyophilization process. Various concentrations of the $CaCl_2$ solution and alginate concentrations were tested, without any significant effect on integrity maintenance. For example, from 10 prepared specimens (4% alginate+ equimolar concentration of $CaCl_2$ instead of NaCl in the LB) only 4 of the alginate matrices were left undamaged. Further increase in the percentage of alginate (higher than 4%) did not show improvement, either. The original alginate matrix is transparent. However, after the lyophilization process and when then alginate concentration was higher than 3%, the color of the matrix changed to white and the matrix lost its transparency.
Further research on lyophilization should try the use of softer lyophilization and rehydration conditions, which will be more suitable not only for matrix integrity but also for a better bacterial survival. For example, the freezing process of the fibers before lyophilization could be conducted not directly in liquid nitrogen, but at -80^{o}C

4 Conclusions

A new flow-through fiber-optic-based system for online monitoring of pollutants in water has been developed. An LB-medium addition of 7.5% to the tested water has been found to be optimal for long-term analyses with flowing water. The sensitivity of this flow-through system is roughly comparable to that of the static system where responses were found at 10–5000 µg/L p-chlorophenol and 32–2000 µg/L mitomycin C. To detect any exceedence of the target values for drinking water (10 ng/L for genotoxic compounds and 0.1 µg/L for other compounds), the sensitivity of the system must be increased. Optimal concentrations of positive controls were not found yet, this remains to be further studied in future research. It was found that 1.4% (v/v) bleach (i.e. 560 mg chlorine per L) was necessary to sufficiently disinfect the collected river water before disposal. The 24-h surface water measurements demonstrate the ability of the device to run for such a time period. A problem for long-term use is the apparent loss of functionality of the bacteria after 20 h in surface water and the oscillations seen in their response during this time. Thus, this system seems promising for use as water quality monitor in the future, but the mentioned issues first need to be studied and solved in future research.
Various temperatures and methods for storage of optic fibers with immobilized bacteria were studied. The most viable storage condition was found at -80°C with the addition of 20% of glycerol. Fibers with bacteria can be stored for at least 3 months in this condition. Lyophilization according to the adapted protocol for a liquid solution was not successful at all. As lyophilization may in theory still be a useful method (and more convenient than storage at -80 °C), a different protocol may be studied in future research.

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