Analyst

PAPER

View Article Online
View Journal | View Issue



Cite this: *Analyst*, 2014, **139**, 4696

# A dip-stick type biosensor using bioluminescent bacteria encapsulated in color-coded alginate microbeads for detection of water toxicity†

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The use of genetically engineered bioluminescent bacteria, in which bioluminescence is induced by different modes of toxic action, represents an alternative to acute toxicity tests using living aquatic organisms (plants, vertebrates, or invertebrates) in an aqueous environment. A number of these bacterial strains have been developed, but there have been no attempts to develop a hand-held type of biosensor for monitoring or identification of toxicity. We report a facile dip-stick type biosensor using genetically engineered bioluminescent bacteria as a new platform for classification and identification of toxicity in water environments. This dip-stick type biosensor is composed of eight different optically color-coded functional alginate beads that each encapsulates a different bioluminescent bacterial strain and its corresponding fluorescent microbead. These color-coded microbeads exhibit easy identification of encapsulated microbeads, since each microbead has a different color code depending on the bioluminescent bacterial strain contained and improved cell-stability compared to liquid culture. This dip-stick type biosensor can discriminate different modes of toxic actions (*i.e.* DNA damage, oxidative damage, cell-membrane damage, or protein damage) of sample water tested by simply dipping the stick into the water samples. It was found that each color-coded microbead emitted distinct bioluminescence, and each dip-stick type biosensor showed different bioluminescence patterns within 2 hours, depending on the toxic chemicals contained in LB medium, tap water, or river water samples. This dip-stick type biosensor can, therefore, be widely and practically used in checking toxicity of water in the environment primarily *in situ*, possibly indicating the status of biodiversity.

Received 12th February 2014
Accepted 16th June 2014

DOI: 10.1039/c4an00308j

www.rsc.org/analyst



## Introduction

Water in the environment is what human beings should always be able to use, touch and drink. If there were any toxic or hazardous compounds in the source of water it would be very harmful and dangerous to humans and biodiversity in environment. Thus, water in the environment and its sources should be clean and safe. However, people who live in countries with a shortage of water purification systems or in a region where all water supplies have been stopped have no choice but to use contaminated or polluted water. In addition, the increased usages of pharmaceutics, herbicides and pesticides, or man-made materials, resulting in toxic chemical discharge, are threatening the water in the environment and leading to the formation of polluted water.[1–3] Therefore, fast and accurate toxicity sensing of water in the environment including drinking water is urgent and needed in order to protect human health and environmental biodiversity. Many studies on the discharges of toxic materials and monitoring their effects on living organisms have been reported.[4–6] However, they still suffer from long detection times and expensive instruments. Therefore, a fast and simple method to pre-screen toxicity is required.

Bioluminescent bacteria have been constructed by DNA recombination of a plasmid containing various stress promoters with the *luxCDABE* gene.[7–9] Bioluminescent bacteria have been used for toxicity screening, due to their easy manipulation, fast response and low cost. Once a toxic chemical induces a stress promoter inside the bacteria, the promoter starts the transcription of *luxCDABE* to produce luciferase, which plays an important role in light emission. There are well-known stress promoters that respond to toxicants inducing DNA damage, oxidative damage and so on, and many different stress specific response strains could have been constructed.[10] According to the light emission from bacteria that have promoters induced by specific damages, the mode of toxic

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† Electronic supplementary information (ESI) available: Supplementary note, Fig. S1–S3 and Table S1. See DOI: 10.1039/c4an00308j

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action of compounds can be screened. To date, our research group has been working on using bioluminescent bacteria for the detection of various toxic compounds with various platforms such as gas sensors,[11] multichannel systems[12] and cell-chips with immobilization of bacteria.[13,14] Herein, we take advantage of the intrinsic stabilization of bioluminescent bacteria in alginate microbeads and prepare color-coded alginate microbeads[14] that can be visualized and used in discrimination of encapsulated bioluminescent bacteria, thereby generating a portable dip-stick type biosensor for easy monitoring and identification of toxicity in water samples for the first time compared to other biosensors developed by our group (Table S1†). To demonstrate the dip-stick type biosensor, we encapsulated eight different bioluminescent bacterial strains in alginate microbeads separately with different fluorescent beads to address color-coded visualization for the easy identification of each bead. We tested LB medium, tap-water, or river water samples containing toxic chemicals (single-dose or multiple-dose) to test the performance of the biosensor. The bioluminescence of bacterial strains used in this study was induced by different modes of toxic action such as DNA damage, oxidative damage, cell membrane damage, or protein damage.[15–21] Each color-coded alginate microbeads was set into each hole of the dip-stick, and bioluminescence was observed by the CCD camera.

# Materials and methods

### Bacterial strains and cell culture

In this study, eight different stress-specific recombinant bioluminescent bacterial strains are used. Strain names, plasmids, hosts and damaging stresses characteristics are listed above in Table 1. The bacterial strains contained plasmids from either *Vibrio fischeri* or *Photorhabdus luminescens* with specific *stress promoter::luxCDABE* genes that are constructed based either on pUCD615 [22] or pDEW201,[23] respectively. DPD2794,[15] BBTSbmC and EBAlkA [16] specifically express in response to DNA damage, EBSoxS,[17] DPD2511 [18] and EBHJ2 [19] express in response to oxidative damage, DPD2540 expresses in response to cell membrane damage and TV1061 [20] expresses in response to protein damage. Each strain is streaked on agar plates that contain 50 μg $ml^{-1}$ ampicillin (Sigma) and stay overnight. One colony was inoculated and grown in 1 ml of LB medium containing 50 μg $ml^{-1}$ ampicillin in a 15 ml Falcon tube in a shaking incubator at 37 °C and 250 rpm for 4 hours. A certain volume of strains in LB medium was collected and poured into 50 ml of fresh LB medium to give an OD value of 0.02, and cells are grown until they reach early stationary phase (OD = 0.8). After that, cells are centrifuged at 20 °C and 3000 rpm for 20 min, and the supernatant is discarded.

### Preparation of color-coded alginate microbeads containing bioluminescent bacteria

Centrifuged bioluminescent bacteria and 2.9 ml of 2% (w/v) sodium alginate solution in distilled water were mixed, and 0.1 ml of fluorescent beads ($1.0 \times 10^{10}$ beads per mL) (FluoSpheres® Polystyrene Microspheres, 1.0 μm, Invitrogen) were added to identify each cell strain using red, orange and green fluorescence. Fluorescent beads used for each strain are described in Table 1. We used an electrospray method to make microbeads. First, sodium alginate solution with bioluminescent bacteria and fluorescent microbeads were placed into 12 ml syringe, which was connected to a 5 cm silicon tube and 30 G needle. The solution in the syringe was extruded through the needle by pushing the syringe pump at 5 ml $h^{-1}$ and dropped into 50 ml sterilized 2% $CaCl_2$ solution on a stirrer by electrostatic and gravitational force. Electrostatic potential was generated by a high voltage DC unit with the positive electrode connected to aluminium foil on the stirrer and the negative electrode to the middle of needle. We generated 7 kV of electrostatic potential to synthesize 900–1000 μm-sized beads. After forming, microbeads went through a hardening process by stirring in 2% $CaCl_2$ solution for 30 min. Then, microbeads were washed with fresh LB medium 2 times and stored in 50 ml tubes at 4 °C to maintain humidity until used (Fig. 1(a)).

### Fabrication of optically coded functional microbead biosensor

Dip-stick type biosensors were fabricated with transparent glass based on computer-aided design and cut using laser engraving and a cutting machine (Fig. 2). The size of the dip-stick is 85.25 mm × 8 mm × 1.5 mm, and it has 10 holes that are 1.25 mm in diameter, and the top and bottom sections are reverse trapezoid in shape; therefore, the microbeads do not escape through the bottom and water samples are well merged

Table 1 Stress-specific recombinant bioluminescent bacteria and their corresponding fluorescent codes

| Strain names | Plasmid/host | Damaging stresses | Corresponding fluorescent codes (volume portion) | Reference |
|---|---|---|---|---|
| DPD2794 | pRecALux/RFM443 | DNA damage | Red | Vollmer *et al.*, 1997 |
| BBTSbmC | pJMsbmCLux/RFM443 | DNA damage | Red + green (2 : 1) | Ahn *et al.*, 2009 |
| EBAlkA | pCHalkALux/RFM443 | DNA damage (alkylation) | Orange | Ahn *et al.*, 2009 |
| EBSoxS | pBCsoxSLux/RFM443 | Oxidative damage (superoxide radical) | Green | Kim *et al.*, 2005 |
| DPD2511 | pKatGLux/RFM443 | Oxidative damage (hydroxyl radical) | Red + orange (2 : 1) | Belkin *et al.*, 1996 |
| EBHJ2 | pSodALux/RFM443 | Oxidative damage (response to both hydroxyl and superoxide radical) | No fluorescence | Lee *et al.*, 2003 |
| DPD2540 | pFabALux/RFM443 | Membrane damage | Orange + green (1 : 1) | Choi *et al.*, 2001 |
| TV1061 | pGrpELux/RFM443 | Protein damage | Red + orange (1 : 1) | Van Dyk *et al.*, 1995 |

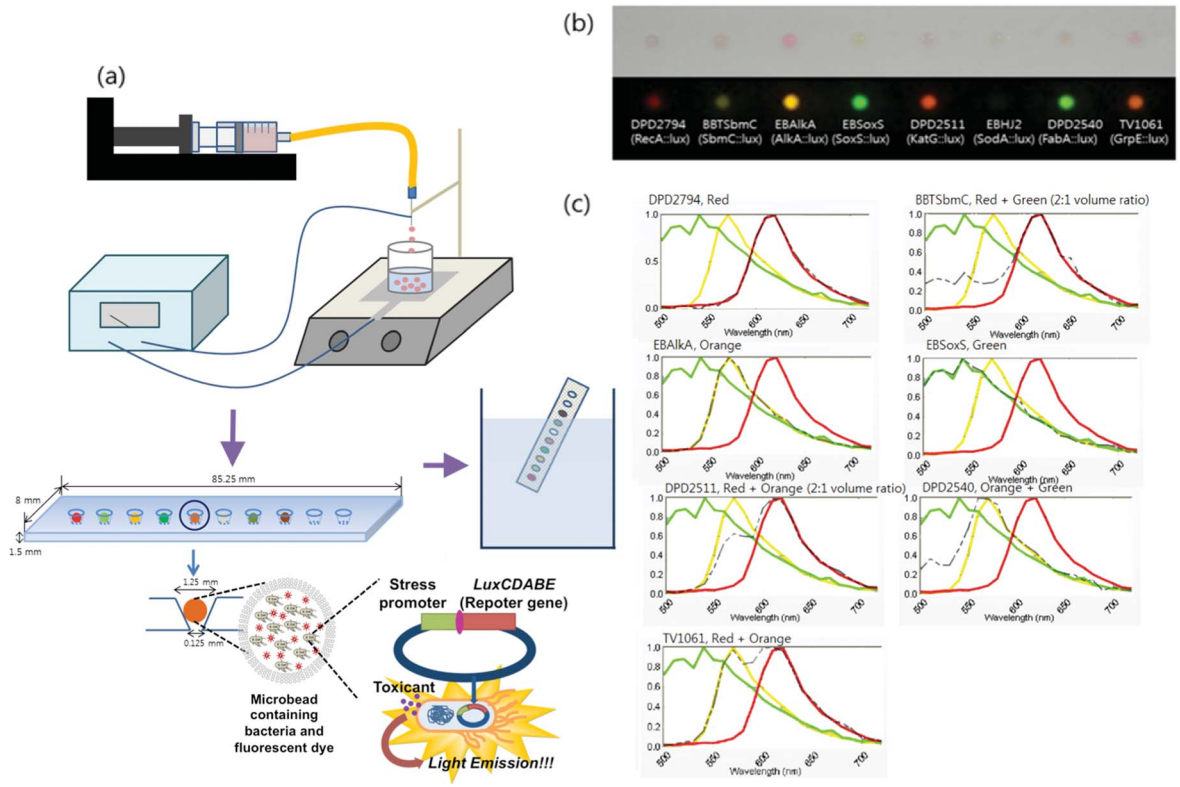


Fig. 1 (a) Schematic diagram of fabricating a microbead on the dip-stick type biosensor and an example of usage of the dip-stick type biosensor. (b) Real and fluorescent picture of the dip-stick type biosensor fabricated with eight different strains that are fluorescent coded. (c) Fluorescent spectral data of microbeads containing DPD2794 (Red), BBTSbmC (Red + Green (2 : 1 volume ratio)), EBAlkA (Orange), EBSoxS (Green), DPD2511 (Red + Orange (2 : 1 volume ratio)), EBHJ2 (no fluorescent), DPD2540 (Orange + Green) and TV1061 (Red + Orange) are shown as dashed lines. Three basic fluorescences (red, orange and green) are shown as red, yellow and green lines, respectively.

into the hole. To sterilize each stick, we wash them with deionized water and 70% ethanol on clean benches. Then, the sticks are dried under UV light for 30 min. Each sterilized stick was located in a Petri dish filled with 4 ml of fresh LB media. We used a 100 μl pipet with tip and glass capillary connected by paraffin film to pick and place individual microbeads into the hole of the stick. After fabrication, dip-stick type biosensors were stored at 4 °C until used. Using an *in vivo* multispectral imaging system (Maestro 2™, Cambridge Research & Instrumentation (CRI), Inc), the fluorescence of each color-coded microbead was taken. Three basic fluorescent dyes, *i.e.*, green, orange and red, were excited at 505, 540 and 580 nm, and the emission wavelengths were detected at 515, 560 and 605 nm, respectively. 10 nm bands from 500 to 720 nm were scanned for acquisition settings. Emission wavelength was analysed by Maestro software 2.8.0 (Cambridge Research & Instrumentation, Inc). The microbeads containing two different fluorescences are analysed based on a wavelength diagram of green, orange and red fluorescence.

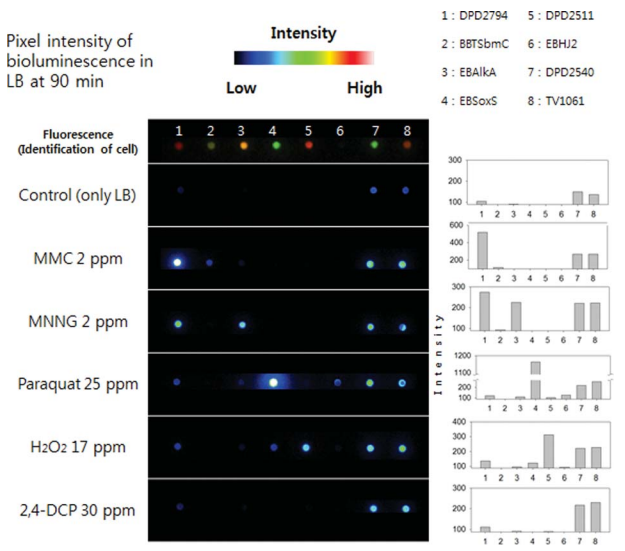


Fig. 2 General characterization of bioluminescent response to 5 model chemicals.

## Test chemicals and toxicity screening

To demonstrate bioluminescence emission in response to each type of damage, 5 model chemicals were used, *i.e.*, Mitomycin C (MMC), 1-methyl-1-nitroso-*N*-methylguanidine (MNNG), paraquat, hydrogen peroxide ($H_2O_2$) and 2,4-dicholorophenol (2,4-DCP). Stock chemical solutions in distilled water were diluted with fresh LB medium, tap water or river water sample

(Han River in Seoul, Korea, filtered by motor filtering) for toxicity tests. Before toxic chemical exposure, we extracted the biosensor fabricated with microbeads from 4 °C and incubated them at 37 °C for 30 min to activate the bacteria, which recovered bacterial sensing capabilities. After that, the biosensor was placed in a square-shaped case (30 mm × 40 mm × 10 mm) that is made of Teflon and able to have three separate biosensors, and then, biosensors were immersed in each chemical solution (1 ml). Biosensors were incubated in a water bath at 30 °C, and then placed in the dark box to take pictures of the bioluminescence using a cooled charge-coupled device camera (Cooled CCD camera) with constant focal plane and integration time (45 s). Bioluminescence was taken every 15 min up to 1 h, and then every 30 min up to 2 h after 1 h. The bioluminescence intensity was analysed by MetaVue 5.0r6. The data were transmitted to Microsoft office EXCEL 2007 software to analyse bioluminescence induction.

## Results and discussion

### Bioluminescent responses of the dip-stick type biosensor to toxic compound

A dip-stick type biosensor was fabricated with eight different bioluminescent bacterial microbeads that showed their own distinct stress responses. Each microbead has a different fluorescent color-code for identification and decrease the chance of confusing bioluminescent bacteria encapsulated in microbeads with others in the fabrication process. The bacterial strains were placed on the dip-stick chips (Fig. 1(b)) from left to right in this order: DPD2794 coded with red, BBTSbmC with red + green (2 : 1 volume ratio), EBAlkA with orange, EBSoxS with green, DPD2511 with red + orange (2 : 1 volume ratio), EBHJ2 with no fluorescence, DPD2540 with orange + green and TV1061 with red + orange, which are responsive to general DNA damage, DNA damage cascade, alkylation DNA damage, superoxide radical oxidative damage, hydroxyl radical oxidative damage, both superoxide and hydroxyl radical oxidative damage, cell surface damage and protein damage, respectively. The eight microbeads were distributed in a hole that has a reversed trapezoid shape; thus, the microbeads cannot escape from the bottom hole of the biosensor and water samples are well merged into the hole with the microbeads (Fig. 1(a)). Using a multispectral imaging system, fluorescent codes of all microbeads were taken and analysed (Fig. 1(b)). Based on three basic spectra of each fluorescent dye (red, orange and green) used, any color-coded microbeads can be identified by their own emission spectra (Fig. 1(c)), and their own bacteria also can be identified based on the corresponding fluorescence. Depending on the chemicals used, bacterial responses induced by different kinds of damage are distinguishable. First, we conducted a characterization of bioluminescent responses in the dip-stick type biosensor by spiking single chemical species at various concentrations.[24] In Fig. 2 and S1,† when treated with any individual chemical, high responses from DPD2540 and TV1061 were observed. This indicates that all chemicals treated cause cell membrane and protein damage. When MMC, an antineoplastic agent that causes DNA damage by production of DNA cross-linked product and cellular SOS response[25,26] was applied, DPD2794 showed a strong response and BBTSbmC showed a weak response. Moreover, it was hard to detect, but still EBAlkA emitted light slightly. MNNG, a direct-acting methylating agent,[27] caused a response from DPD2794 and EBAlkA, which indicates that MNNG causes general DNA damage and DNA alkylation. Paraquat generates superoxide, and bacterial response to superoxide-generating agents[28] generates resistance to paraquat toxicity. Then, paraquat induced a response from EBSoxS and EBHJ2. $H_2O_2$, generating hydroxyl radicals,[29] caused a response from EBSoxS and DPD2511. It also induced DPD2794 slightly, because ROS accumulated and damaged the DNA.[30] 2,4-DCP, which is a phenolic compound and changes lipid-to-protein ratios of cytoplasmic and outer membranes of *Escherichia coli*,[31] generated a response from DPD2540 and TV1061. Dose-dependency of a dip-stick type biosensor was shown in Fig. S2 and S3.† Each chemical addressed dose-dependent induction of bioluminescence up to a critical concentration. Bioluminescence over the critical concentrations of chemicals was decreased because chemical toxicity was too strong for the bacteria in the microbeads to survive.

### Multiple-dose test

LB medium containing two, three or five chemicals were prepared and applied to a dip-stick type biosensor (Fig. 3) to investigate the bioluminescent response patterns and compare to single species doses. We used 4 different combinations of the mixed chemicals ((a) MNNG (2 ppm) and 2,4-DCP (30 ppm), (b) MMC (2 ppm) and paraquat (25 ppm), (c) MMC (2 ppm), paraquat (25 ppm) and 2,4-DCP (30 ppm), and (d) MMC (2 ppm), MNNG (2 ppm), paraquat (25 ppm), $H_2O_2$ (17 ppm) and 2,4-DCP (30 ppm)) in LB. The induced bioluminescence in the combination of two and three chemicals showed the integrated patterns of individual chemical tests, although their intensities were decreased (Fig. 3(a)–(c)). The high loading of toxic chemicals (the five chemical mixture case) could not induce all the bioluminescence that appeared in the individual chemicals' tests. Most strains treated by all chemicals showed lower intensity compared to the single chemical exposed test, which indicates that all five chemicals were too toxic to induce a response of bacterial cells in microbeads (Fig. 3(d)). In practical use, unexpected multiple pollutants may not induce the bioluminescent intensity as expected because of the high toxic strength that gives cells a metabolic burden for producing bioluminescence. In that case, the test of diluted water samples may show the induction of bioluminescence intensity if the samples tested have high toxic strength.[32,33]

### Spiking test in LB medium, tap water and river water sample

Chemical-spiked LB medium, tap water, or river water samples were treated on a dip-stick type biosensor to see whether a dip-stick type biosensor can be applied to test the toxicity of tap water or river water (Fig. 4). The induced patterns of bioluminescence triggered by chemicals in tap water or river water were similar to those in LB medium, although the intensity of

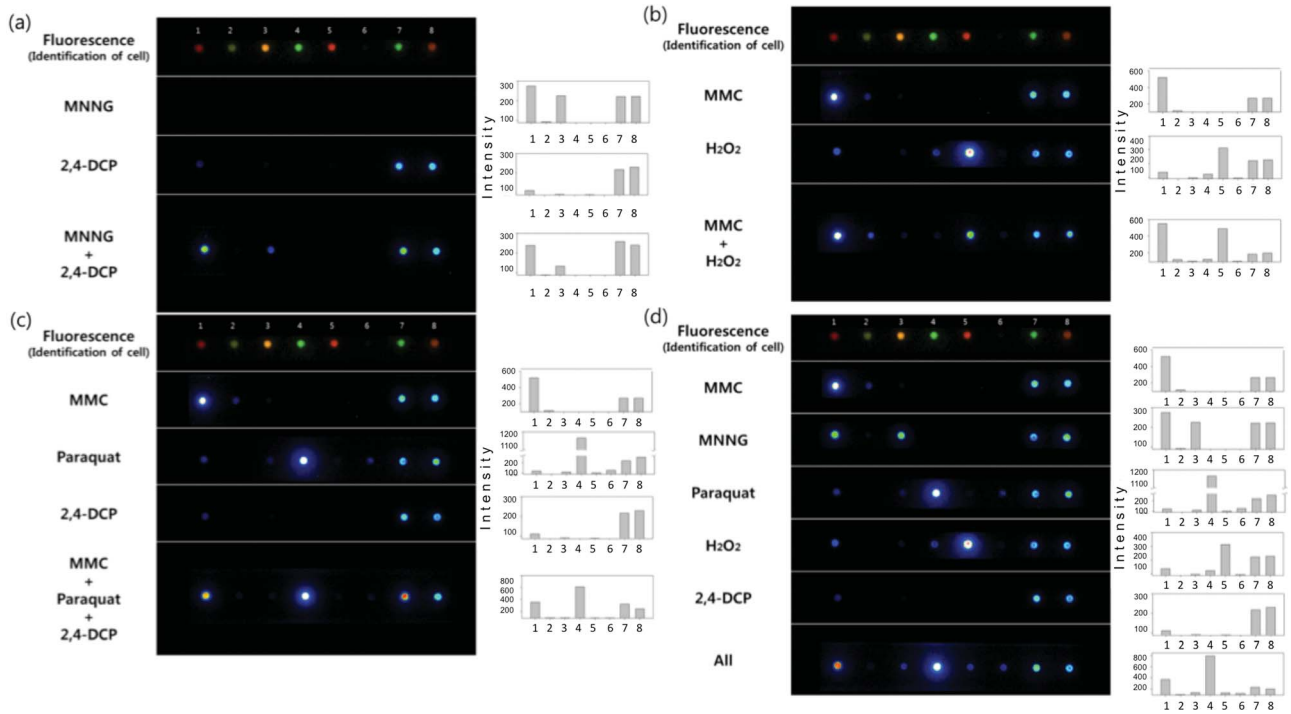


Fig. 3 Bioluminescent data of the multiple-dose test using mixed chemicals. (a) MMC (2 ppm) and paraquat (25 ppm) (b) MNNG (2 ppm) and 2,4-DCP (30 ppm) (c) MMC (2 ppm), paraquat (25 ppm) and 2,4-DCP (30 ppm) (d) All chemicals (MMC (2 ppm), MNNG (2 ppm), paraquat (25 ppm), $H_2O_2$ (17 ppm) and 2,4-DCP (30 ppm)).

bioluminescence in tap or river water samples was less than that in LB. The nutrients in LB may gradually affect bacterial cell activity inside the microbeads. These may make bacterial cells more robust during induction of stress triggered by the toxic spiked chemicals compared to the tests performed with tap or river water. Although the intensity of bioluminescence was not same, this dip-stick type biosensor is suitable for detecting toxicity of chemicals in tap water or river water in describing the patterns of the induced bioluminescence. In practical use, we considered that this dip-stick type biosensor could be used as a fast pre-screening tool for whether tested water samples have potential toxicity. The specific induction of bioluminescence and variation of quantitative intensities in each microbead to various toxic substances in water samples can give us useful

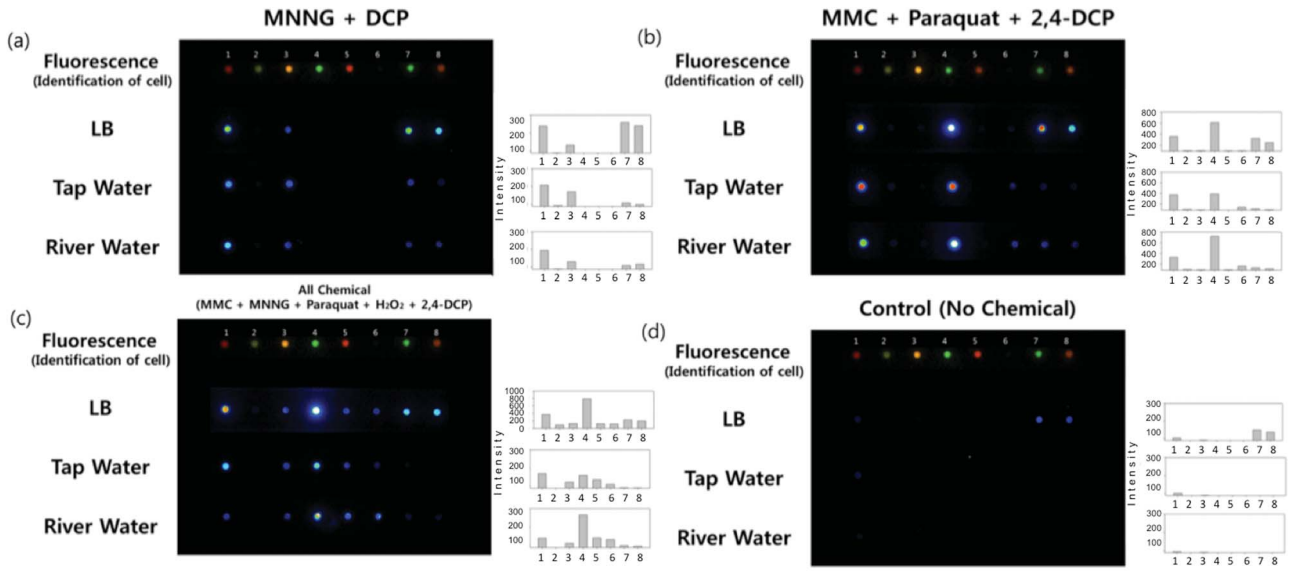


Fig. 4 Bioluminescent response induced by chemical spiked LB medium, tap water sample and river water sample. (a) No chemical spiking, (b) MNNG 2 ppm + 2,4-DCP 30 ppm, (c) MMC 2 ppm + Paraquat 25 ppm + 2,4-DCP 30 ppm, and (d) all chemical (MMC 2 ppm, MNNG 2 ppm, Paraquat 25 ppm, $H_2O_2$ 17 ppm and 2,4-DCP 30 ppm).

information about certain kinds of toxicity and toxic strength. This type of diagnosis for environmental water could not be achievable with instrumental analysis or other biological toxicity test systems.

## Conclusions

In this study, we successfully developed a dip-stick type biosensor using eight microbeads containing different bioluminescent bacterial strains, respectively. The size of dip-stick type biosensor is 85.25 mm × 8 mm × 1.5 mm. The holes' diameter is 1.25 mm with a section with reverse trapezoid shape from top to the bottom; thus, the microbeads would not be released out from the hole naturally. Each microbead has its own color code; thus, it is very easy to distinguish the bacterial strain that is present in a microbead. This dip-stick type biosensor is portable and can be easily used with an appropriate bioluminescent reader for detecting any toxicity of water in the environment. Using this dip-stick type biosensor, DNA damage, oxidative damage, cell surface damage and protein damage in response to toxic chemicals can be detected within 1 h by measuring bioluminescence using a CCD camera. This dip-stick type biosensor can, therefore, be widely and practically used in checking toxicity of water in the environment primarily *in situ*, possibly indicating the status of biodiversity.

## Acknowledgements

This study was supported by the National Research Foundation of Korea Grant funded by the Korean Government (MEST, NRF-2010-220-D00019, Global Research Network Program). The authors are grateful for their support.

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