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A novel continuous toxicity test system using a luminously modified freshwater bacterium

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Abstract

An automated continuous toxicity test system was developed using a recombinant bioluminescent freshwater bacterium. The groundwater-borne bacterium, *Janthinobacterium lividum* YH9-RC, was modified with *lux*AB and optimized for toxicity tests using different kinds of organic carbon compounds and heavy metals. *luxAB*-marked YH9-RC cells were much more sensitive (average 7.3–8.6 times) to chemicals used for toxicity detection than marine *Vibrio fischeri* cells used in the Microtox® assay. Toxicity tests for wastewater samples using the YH9-RC-based toxicity assay showed that EC_{50}-5 min values in an untreated raw wastewater sample (23.9 ± 12.8%) were the lowest, while those in an effluent sample (76.7 ± 14.9%) were the highest. Lyophilization conditions were optimized in 384-multiwell plates containing bioluminescent bacteria that were pre-incubated for 15 min in 0.16 M of trehalose prior to freeze–drying, increasing the recovery of bioluminescence and viability by 50%. Luminously modified cells exposed to continuous phenol or wastewater stream showed a rapid decrease in bioluminescence, which fell below detectable range within 1 min. An advanced toxicity test system, featuring automated real-time toxicity monitoring and alerting functions, was designed and finely tuned. This novel continuous toxicity test system can be used for real-time biomonitoring of water toxicity, and can potentially be used as a biological early warning system.

Keywords: Recombinant luminescent freshwater bacterium; *lux*AB; Microtox®; Continuous toxicity test; Biological early warning system

1. Introduction

Continuous and rapid detection of environmental toxicity caused by hazardous materials is of great value for conserving natural ecosystems and protecting human health. Many biological early warning systems (BEWS; Kramer and Botterweg, 1991; van der Schalie et al., 2001) have been developed to monitor physiological responses of whole organisms such as fish, protozoa, and algae to toxic chemicals in wastewater and soils (van Hoof et al., 1994; Borcherding and Jantz, 1997; Gerhardt et al., 1998; Pardos et al., 1999; van der Schalie et al., 2001). Most of these tests, however, require long-term observation and time-consuming propagation of test organisms. As contrasted with higher organism-based BEWS, biosensors and toxicity evaluation systems based on microorganisms have been widely used in acute toxicity tests because they are easy to use, non-invasive, costing little, rapid and reproducible, and have a statistical advantage due to the large number of bacteria used (see D'Souza, 2001).

Microbial toxicity test systems employing whole cell bioluminescence are generally categorized into two systems based on physiological response of luminescent bacteria to toxic chemicals, luminescence emission or reduction. The microorganisms that emit luminescence when they are exposed to toxic chemicals are genetically produced with a plasmid coding for luciferase genes (*lux*AB or *lux*CDABE; de Lorenzo et al., 1990; Flemming et al., 1994; Van Dyk et al., 1994) and a promoter for specific chemicals (e.g. *nah*G for naphthalene and analogues; Heitzer et al., 1994), or a

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stress promoter such as *grp*E, *dna*K, and *rec*A (Van Dyk et al., 1994; Belkin et al., 1997; Gu et al., 1999). In this system, the genetic control mechanism synthesizes luciferase through the induction of a promoter triggered by chemicals, leading to an increase in luminescence emission. However, because a specific promoter responds only to specific chemicals, a single bacterium harboring a specific promoter cannot be used to detect the broad range of toxic substances found in natural environments.

Systems that respond to toxicity through bioluminescence reduction employ the marine bacterium *Vibrio fischeri*. The Microtox® system, which uses *V. fischeri* as a toxicity test organism, has been commercialized and is widely used as a toxicity test system (Munkittrick et al., 1991; Gustavson et al., 1998; Salizzato et al., 1998; Pardos et al., 1999). Although the Microtox® assay has been shown to be rapid, reproducible, and sensitive to a wide range of inorganic and organic chemicals, the use of a marine bacterium has some shortcomings for applications in freshwater environments. It has been shown, for example, that salinity values over 2% NaCl in the Microtox® assay alter the mode of binding of chemicals to *V. fischeri* cells (Sinclair et al., 1999), which may distort toxicity results. The Microtox® assay was also shown to be less sensitive than an assay using aquatic invertebrates in toxicity tests for wastewater effluents (Calleja et al., 1994). A toxicity measurement system based on luminously modified terrestrial bacteria would, therefore, have a broader range of applications than the Microtox® system, incorporating freshwater, wastewater, soil, and groundwater environments. If the system offers continuous and real-time information on toxicity levels in a given environment, luminously modified terrestrial bacteria can be used as BEWS to monitor water quality.

The main purpose of this study, therefore, is to develop an advanced continuous toxicity test system by employing a *lux*-marked terrestrial bacterium. Initially, a freshwater-borne bacterium, which is very sensitive to organic substances and heavy metals, was isolated and luminously modified using *lux*AB. We have also developed a working system for BEWS that consists of three compartments: 384-multiwell plates containing freeze–dried luminescent bacteria; instrumentation for continuous measurements of bioluminescence; and application software (BactoTox®), which is capable of real-time monitoring of toxicity and telecommunications for alarming. The conditions for toxicity tests and freeze–drying were finely tuned, and the system was monitored using model toxic chemicals in a continuous manner.

2. Materials and methods

2.1. Construction of a bioluminescent strain

An oligotrophic bacterium, *Janthinobacterium lividum* YH9, was isolated from a groundwater sample used for natural mineral water on R2A (Reasoner and Geldreich, 1985) plates. Rifampicin-resistant YH9-R was generated by spontaneous mutation from the wild type. For the generation of *lux*AB-tagged YH9-R, *Escherichia coli* S17-1 (λ*pir*) was electroporated with pUT*lux*AB [mini-Tn5 harboring plasmid derived from *E. coli* CC118 (λ*pir*) pUT*lux*AB, a gift from Timmis (de Lorenzo et al., 1990)], and selected on Luria–Bertani (LB) agar supplemented with 12.5 μg ml^{-1} tetracycline. YH9-R *lux*AB (Rif^R Tc^R) was obtained by mating *E. coli* S17-1 (λ*pir*) pUT*lux*AB (Tc^R) with YH9-R (Rif^R) using the filter conjugation method (Herrero et al., 1990; Cho and Kim, 1999). Exconjugants, marked with *lux*AB by insertion of the hybrid transposon mini-Tn5 *lux*AB, were obtained by spreading the cell suspension on R2A medium supplemented with 50 and 12.5 μg ml^{-1} of rifampicin and tetracycline, respectively. A clone showing the highest relative light unit (RLU), designated as YH9-RC, was chosen among the exconjugants and used throughout the study.

2.2. Chemicals and wastewater samples

Chemical standards, including heavy metals and organic chemicals, were analytical grade and were used without further purification. All chemicals were purchased from the Aldrich. Wastewater samples were collected from a food industry plant located at Cheongju, Korea. Untreated raw wastewater, a wastewater sample from an aerobic treatment plant, and an effluent sample were collected. The basic environmental parameters of the wastewater samples including COD, ammonium-N, nitrite-N, nitrate-N, phenol, cyanide, cobalt, and lead concentrations were measured by procedures previously described (APHA, 1995).

2.3. Toxicity test

Toxicity tests were performed with two luminescent bacteria, *lux*AB-marked YH9-RC and *V. fischeri*. *V. fischeri* was used as a reference strain in order to compare the sensitivity of YH9-RC-based toxicity test with the conventional Microtox® assay (*V. fischeri* toxicity test). The *lux*AB-marked YH9-RC cells were grown in R2A broth on an orbital shaker at 200 rpm at 30 °C until early-exponential phase ($OD_{600} = 0.2$) and *V. fischeri* cells were grown in LB broth containing 2.5% NaCl at 25 °C until early-exponential phase. Each test sample was added to bacterial suspensions and incubated for 5, 15 (or 10), and 30 min prior to luminescence measurement. As the *lux*AB-marked YH9-RC cells lack the genes for synthesis of the luciferase substrate, *n*-decyl aldehyde was added to test tubes at a final concentration of 0.013%. In the case of the *V. fischeri*-based toxicity test system, all test solutions were adjusted to a final concentrations of 20‰ of salinity using NaCl. Bioluminescence was measured using a luminometer (TD-20/20, Turner designs, CA, USA). The toxicity test results were expressed as EC_{50}, defined as the effective concentration or

percentage of a sample that causes 50% reduction of bioluminescence. The values of average and standard deviation were obtained from at least triplicate measurements.

2.4. Generation of 384-multiwell plate containing lyophilized cells

The 384-multiwell plates as cartridges for supplying luminescent bacteria were made using lyophilized YH9-RC cells supplemented with trehalose as a cryo-protective agent. YH9-RC cells were grown in 50 ml of R2A broth until mid-exponential phase. To optimize freeze–drying conditions, two approaches were performed; one was that trehalose was supplemented to final culture broths immediately before freeze–drying. The other was that trehalose was added to growing culture broths, and cell cultures were subsequently incubated for 15 min. Trehalose concentrations added to culture broths were 0, 0.08, 0.16, and 0.32 M. In each case, 30 μl of cultures was loaded in each well of 384-multiwell plates and the plates were rapidly frozen in liquid nitrogen for 5 min. Frozen samples were dried on a freeze–drier (compact freeze–dryers FD1.0, Heto Lab. Equipment, Denmark) for 4 h. To determine the survival rate of cells and bioluminescence reduction during freeze–drying, lyophilized cells were rehydrated with 30 μl of R2A broth. Bioluminescence was measured by a luminometer and survival of lyophilized cells was determined by measuring the number of colony forming units (CFU) on R2A agar. Bioluminescence and number of CFU were measured in triplicate.

2.5. Instrumentation

A schematic diagram of the automated and continuous toxicity test system developed in this study is shown in Fig. 1, and a brief description of the system is given here. Full details, including robot arms for stage transport, electronic circuit diagrams, pumping apparatus, and the software BactoTox® are being processed for a patent. The system was built with a sample supplier, solution dispenser compartment, 384-multiwell cartridge storage tray, cartridge-loading compartment, PMT sensor, PMT sensor-transporting compartment, temperature controller, multi-controller compartment including RS232C serial port, and an IBM computer for data acquisition using the BactoTox® software. Luminously modified strain YH9-RC was lyophilized in 384-multiwell plates as described in Section 2.4, and plates were tightly sealed with a transparent plastic film and placed in the multiwell cartridge storage tray. The PMT sensor measured bioluminescence in each well, and data was transmitted to a PC running BactoTox® program via a RS232C serial port. The BactoTox® software, which has features for on-line monitoring of toxicity and alerting functions, was developed using Visual Basic software.

2.6. Real-time toxicity monitoring

Continuous toxicity tests were performed with the system developed in this study. Three different concentrations of phenol (5, 25, 100 mg l^{-1}) and an untreated raw wastewater sample obtained from a food industry plant (see Section 2.2) were used for toxicity tests. Toxicity monitoring was performed on 40 wells in a 384-multiwell plate at 5 min interval. After 30 μl of sample was pumped into a reaction well, freeze–dried cells were rehydrated. Thereafter, *n*-decyl aldehyde was added into a reaction well, and bioluminescence was subsequently measured automatically. In case of continuous toxicity test for phenol, 37 wells were used as control wells (no toxicity) and the remaining three wells were used as acute toxicity test wells. Initially, 5, 25, and 100 of mg l^{-1} of phenol were pumped into the 8th well (35 min), 16th well (75 min), and 32nd well (155 min), respectively. Similarly, 30 and 100% of the wastewater sample were pumped into the 12th well (55 min) and 24th well (115 min), respectively. All bioluminescence data were monitored through the BactoTox® software.

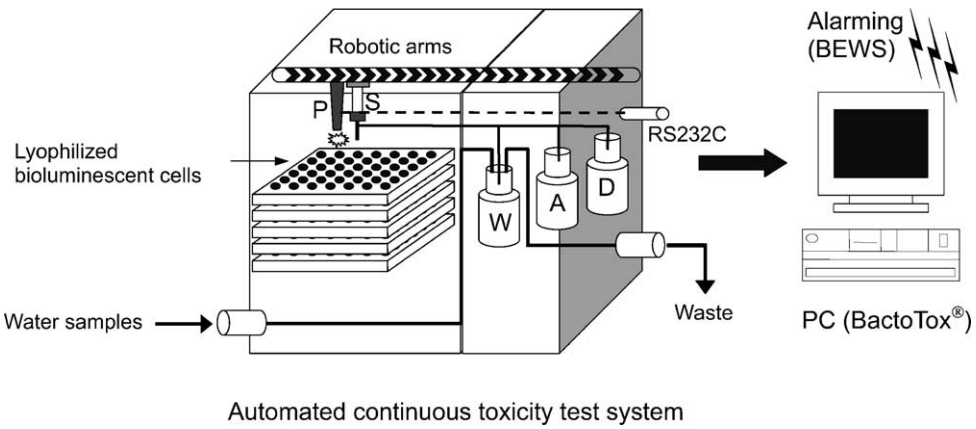

Fig. 1. Schematic diagram of an automated continuous toxicity test system using the luminously modified YH9-RC cells. P, photomultiplier (PMT) sensor; S, syringe; W, water sample; A, *n*-decyl aldehyde; D, distilled water.

Table 1
Toxicity values (EC_{50}) of various chemicals obtained by using *luxAB*-marked YH9-RC and *V. fischeri*

Chemical	EC_{50} (mg l^{-1})[a]					
	5 min		15 min		30 min	
	V. fischeri	YH9-RC	*V. fischeri*	YH9-RC	*V. fischeri*	YH9-RC
Phenol	138.8 ± 33.6[b]	26.5 ± 3.8	114.4 ± 33.0	12.0 ± 2.1	112.9 ± 52.1	12.7 ± 2.1
Benzene	74.3 ± 6.2	75.2 ± 5.9	104.7 ± 17.9	50.9 ± 5.8	105.6 ± 10.8	35.1 ± 4.6
Toluene	82.5 ± 9.8	5.6 ± 2.4	90.6 ± 8.9	5.7 ± 1.8	116.2 ± 12.7	6.3 ± 1.1
Cu	31.1 ± 13.9	10.1 ± 2.1	37.1 ± 7.8	9.5 ± 1.5	36.0 ± 14.6	10.5 ± 1.9
Al	16.1 ± 1.2	0.9 ± 0.3	15.4 ± 2.3	0.8 ± 0.4	16.5 ± 2.2	0.8 ± 0.3
As	25.2 ± 5.2	28.4 ± 2.7	16.5 ± 3.7	9.3 ± 1.6	18.8 ± 3.6	4.3 ± 1.2
Cd	22.2 ± 2.4	4.6 ± 1.3	21.7 ± 2.9	1.8 ± 0.4	21.8 ± 2.2	1.1 ± 0.3
Co	20.5 ± 3.5	1.1 ± 0.3	16.9 ± 3.2	1.8 ± 0.7	12.5 ± 1.1	1.9 ± 0.8
Cr(VI)	18.9 ± 2.3	5.9 ± 1.8	17.2 ± 2.4	7.5 ± 2.2	17.5 ± 1.9	6.3 ± 1.5
Fe	15.9 ± 1.8	6.7 ± 0.9	16.5 ± 0.9	9.2 ± 2.5	13.1 ± 1.4	11.0 ± 1.8
Hg	1.6 ± 0.6	0.3 ± 0.2	0.8 ± 0.5	0.2 ± 0.2	0.8 ± 0.2	0.2 ± 0.1
Zn	13.3 ± 1.5	1.3 ± 0.5	12.8 ± 1.3	1.3 ± 0.7	13.4 ± 1.9	1.3 ± 0.5

[a] Effective concentrations of a sample that causes 50% reduction of the luminescence emitted.
[b] Average ± S.D. (standard deviation); average values were obtained from at least triplicate measurements.

3. Results and discussion

3.1. Sensitivity of luminescent strain YH9-RC to chemicals

Toxicity test results using three organic chemicals and nine heavy metals are represented as EC_{50} values in Table 1. The luminously modified terrestrial luminescent bacterium, YH9-RC, showed higher sensitivity to chemicals tested than marine *V. fischeri*. Comparison of the toxicity assay based on *luxAB*-marked YH9-RC to the Microtox® (*V. fischeri* assay) showed that EC_{50} values for the YH9-RC-based toxicity test were significantly lower than those obtained using the Microtox® assay ($P < 0.01$, *t*-test). The ratio of EC_{50}-5 min, EC_{50}-15 min, EC_{50}-30 min between the two toxicity assays ranged from 0.9 to 18.6 (average; 7.3), 1.8 to 19.3 (average; 7.6), and 1.2 to 20.6 (average; 8.6), respectively. Among the organic chemicals tested, the EC_{50}-30 min value for toluene in the YH9-RC-based toxicity assay was 18.4 times lower than *V. fischeri* assay. Similarly, the EC_{50}-5 min values of aluminum, cobalt, and zinc were 17.9, 18.6, and 10.2 times lower than *V. fischeri* assay, respectively.

We used freshly cultured *V. fischeri* as a test bacterium for the Microtox® assay instead of lyophilized *V. fischeri*. In our results, the EC_{50} value for phenol in freshly cultured *V. fischeri* was not significantly different from lyophilized *V. fischeri* ($P > 0.6$, *t*-test; data not shown). Although we did not use the original Microtox® assay in this study, it was apparent that the YH9-RC-based assay was more sensitive to the chemicals studied than the Microtox® assay, indicating that YH9-RC can be used to test the toxicity of samples containing very low concentrations of toxic chemicals. Boyd et al. (1997) used a *luxAB* insert in *Pseudomonas fluorescens* to measure toxicity of groundwater samples. This study indicated that the sensitivity of the *P. fluorescens* assay was nearly the same as that of the Microtox® assay, when used to analyze contaminated groundwater samples.

J. lividum YH9 was isolated as a test bacterium from the groundwater, which was being used for natural mineral water. *J. lividum* is a very common species found in groundwater (Cho and Kim, 2000). Because the transport of organic or inorganic chemicals from surface soil to groundwater is very limited, there is little chance for bacteria in pristine groundwater to come into contact with toxic chemicals. This may be the reason why YH9-RC showed high sensitivity to the chemicals assayed.

3.2. Toxicity test in wastewater samples

The acute toxicity system developed in this study was applied to evaluate its ability to measure wastewater toxicity, including raw wastewater, aerobic treatment tank, and effluent samples. Resulting EC_{50} values (as percentage values) are represented in Table 2. The EC_{50} values in the untreated raw wastewater sample were the lowest, while those in the effluent were the highest. This study suggests that *luxAB*-marked bacterium YH9-RC can be used to bioassay the ecotoxicity of wastewater and effluent samples contaminated with COD compounds. Higher concentrations of COD in wastewater samples resulted in lower EC_{50} values (Table 2). A comparison of EC_{50} values and chemical analysis data indicated that there was no correlation between EC_{50} values, inorganic nitrogen compounds, and heavy metals. The concentrations of lead and cobalt in wastewater samples were not significantly different, and the concentrations of phenol were highest in the raw wastewater sample. The existence of relatively high concentrations of phenol and COD compounds in the raw wastewater sample may be responsible for the higher toxicity readings associated with these samples. However, the wastewater samples

Table 2
Toxicity values (EC_{50}) obtained using *luxAB*-marked YH9-RC, and chemical concentrations of wastewater samples collected from a food industry plant

Wastewater samples	EC_{50} (%)[a]			COD ($mg\,l^{-1}$)	Phenol ($mg\,l^{-1}$)	NH_4^+–N ($mg\,l^{-1}$)	NO_2^-–N ($mg\,l^{-1}$)	NO_3^-–N ($mg\,l^{-1}$)	CN^- ($mg\,l^{-1}$)	Pb ($mg\,l^{-1}$)	Co ($mg\,l^{-1}$)	Cd ($mg\,l^{-1}$)
	5 min	10 min	30 min									
Raw wastewater	23.9 ± 12.8[b]	23.3 ± 12.3	38.5 ± 18.1	29,900	3.99	26.3	30	17.0	0.062	0.36	0.58	0.009
Aeration tank	45.5 ± 9.8	31.8 ± 7.9	39.3 ± 12.3	1,333	0.02	191.0	ND[c]	64.0	ND	0.18	0.38	ND
Effluents	76.7 ± 14.9	57.9 ± 10.7	50.5 ± 10.8	294	0.06	166.5	ND	122.5	0.089	0.15	0.8	ND

[a] Effective dilution ratio of a sample that causes 50% reduction of the luminescence emitted by YH9-RC.
[b] Average ± standard deviation (S.D.); average values were obtained from at least triplicate measurements.
[c] ND, not detected.

contained a mixture of compounds; for this reason, synergetic, antagonistic, and additive modes of toxic action should be considered (Thomulka et al., 1993; Sixt et al., 1995). Further studies such as toxicity identification evaluation (TIE) procedures (USEPA, 1991) are necessary to identify which toxicants are responsible for the high toxicity observed in wastewater samples. Additional environmental samples and effluents are also required to evaluate the application of this acute toxicity test system to natural environments.

3.3. Optimization of freeze–drying conditions

Although freeze–drying has often been used for long-term preservation of microorganisms, we have found that freeze–drying of luminescent bacteria results in decreased luminescence and viability. To minimize reduction of luminescence and cell viability during freeze–drying, concentrations of the cryo-protective agent trehalose (Leslie et al., 1995) were optimized (Fig. 2). YH9-RC cells lyophilized without trehalose addition showed a 9.8–12.1% survival rate. The supplement of 0.16 M of trehalose into cell cultures immediately before freeze–drying restored 26.9% of bioluminescence and 27.7% of cell viability (Fig. 2A). An additional 15 min incubation step prior to freeze–drying showed a 48.7% increase in bioluminescence and a 54.1% increase in culturability (Fig. 2B). The EC_{50} value for cadmium measured using non-lyophilized YH9-RC cells in 10 replicates was $2.4 \pm 0.99\,mg\,l^{-1}$ and the corresponding value measured using lyophilized YH9-RC cells treated with trehalose was $2.5 \pm 1.62\,mg\,l^{-1}$ (data not shown). As the difference in EC_{50} values for cadmium between non-lyophilized cells and lyophilized cells was insignificant ($P > 0.5$, *t*-test), it was postulated that the capacity of YH9-RC cells to measure EC_{50} values was not altered during the freeze–drying process.

3.4. Real-time toxicity monitoring under laboratory conditions

The developed continuous toxicity test system was operated using a 384-multiwell plate containing luminescent YH9-RC cells, with different concentrations of toxicants (phenol and wastewater) added in pulses to specific monitoring wells. The response of bioluminescence to the addition of phenol (5, 25, $100\,mg\,l^{-1}$), raw wastewater from a food factory (30 and 100%), and distilled water as a control are shown in Fig. 3. The average RLU and percentage coefficient of variation (%CV) obtained from the control wells (distilled water) in a plate where phenol was subjected to the specific wells were 3493.3 and 1.99%, respectively. In wells where wastewater samples were added in a pulse, the average RLU and %CV from the control wells were 3208.4 and 2.19%, respectively. Therefore, the variations of RLU in the control wells could be regarded as negligible. When 5, 25, and $100\,mg\,l^{-1}$ of phenol flowed into the wells, 3103, 1894, and 25 RLU values were observed, respectively. Similarly,

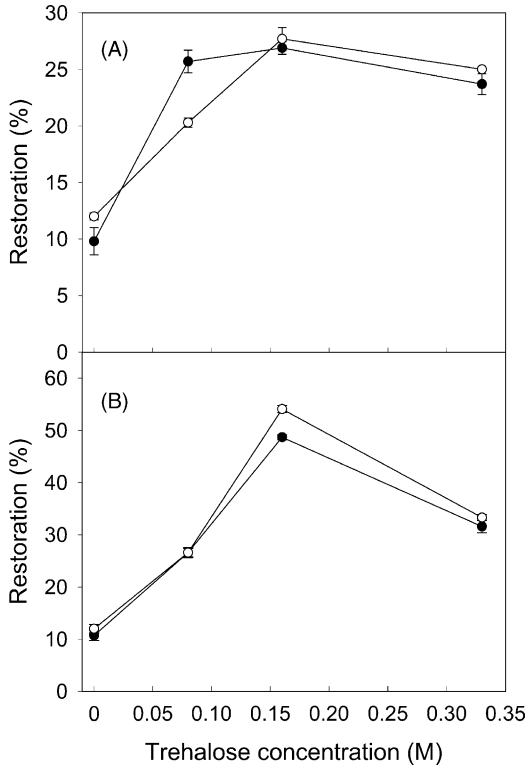

Fig. 2. Recovery of YH9-RC cells expressed as bioluminescence (RLU (●)) and heterotrophic plate counts (HPC (○)) after freeze–drying in trehalose. (A) Trehalose was added into cell cultures immediately before freeze–drying and (B) trehalose was added into culture broths and cell cultures were subsequently incubated for 15 min. Note the differences in scale on the *y*-axis between (A) and (B). Error bars; standard deviation.

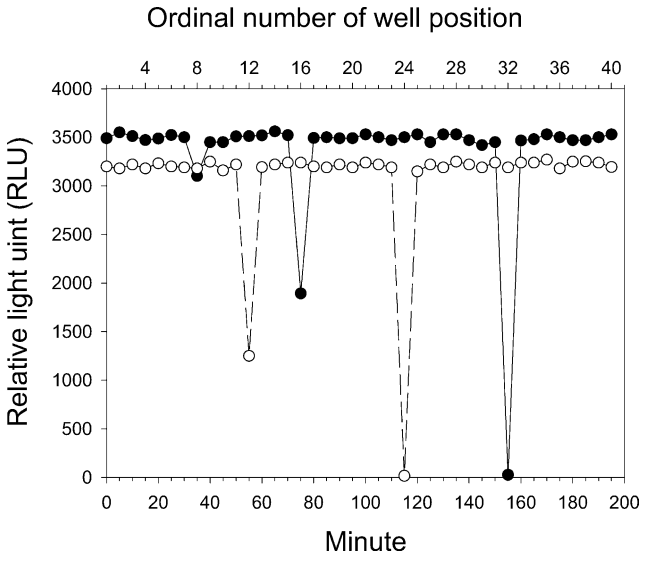

Fig. 3. Real-time bioluminescence signals responding to different concentrations of phenol (●) and a wastewater sample (○), subjected to the toxicity test system in a pulse. Phenol stream (5, 25, and 100 mg l^{-1}) was pumped into the 8th well (35 min), 16th well (75 min), and 32th well (155 min), respectively. Wastewater sample stream (30 and 100%) was pumped into the 12th well (55 min) and 24th well (115 min), respectively.

when 30 and 100% of wastewater samples flowed into the wells, 1250 and 15 RLU values were measured, respectively.

Chun et al. (1996) designed a flow-through cell with immobilized *Photobacterium phosphoreum* and continuously monitored the toxicity of 4-nitrophenol and salicylate. In their study, bioluminescence of immobilized *P. phosphoreum* decreased with a short time lag when the cells were exposed to toxicants, and bioluminescence was recovered rapidly when the feed was switched from the toxicant to a maintenance medium. In addition, the recovered bioluminescence decreased rapidly when the cells were continuously maintained in the medium. In contrast, our study showed neither a lag period nor a decrease in bioluminescence. Since our study used lyophilized cells in a physiologically equal state, there is little possibility of showing a time lag when the cells encounter toxicants or a decrease in bioluminescence when the cells encounter non-toxic samples.

4. Conclusions

The main function of the toxicity test system developed in this study is to monitor the toxicity of samples automatically and continuously without an operator. The system can be equipped with twelve 384-multiwell plates, which can be maintained for a month without operator intervention if toxicity tests are performed at 10 min intervals. Although bioluminescence decreased up to 20% of the initial value after storing lyophilized cells for 1 month, the bioluminescence after 1 month was high enough to measure EC_{50} values of various chemicals and wastewater. When water samples are pumped from rivers, streams, or effluents, the solution dispenser compartment starts to dispense water samples and *n*-decyl aldehyde into a well containing luminescent bacteria. After incubating for 30 s at 25 °C, a PMT sensor starts to measure the luminescence emitted from each well; thereafter, the ADC compartment converts analog values derived from the PMT sensor to digital signals, and an RS232C serial port transmits the digital values to the BactoTox® software. The major functions of the BactoTox® software are to transform bioluminescence values into the predefined data structure for on-line monitoring, to raise the alarm in the event of high toxicity, and to communicate with the main server through a network.

The results reported in this study indicate that the *luxAB*-marked *J. lividum* YH9-RC bioassay employing a freshwater bacterium was more sensitive, in terms of toxicity detection, than the Microtox® bioassay using a marine bacterium. A mechanically and electronically developed toxicity test system based on optimized trehalose-assisted freeze–drying conditions and the construction of 384-multiwell plates could measure the toxicity of various chemicals and wastewater samples in a continuous manner. This work finally suggests that the simple, fast, cost-effective, automatic, and continuous toxicity test system we developed is suited for real-time biomonitoring of water toxicity, and thus can be used as a biological early warning system in the future.

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References

APHA, 1995. Standard methods for the examination of water and wastewater, 19th ed. American Public Health Association, New York, USA.

Belkin, S., Smulski, D.R., Dadon, S., Vollmer, A.C., Van Dyk, T.K., LaRossa, R.A., 1997. A panel of stress-responsive luminous bacteria for toxicity detection. Water Res. 31, 3009–3016.

Borcherding, J., Jantz, B., 1997. Valve movement response of the mussel Dreissena polymorpha—the influence of pH and turbidity on the acute toxicity of pentachlorophenol under laboratory and field conditions. Ecotoxicology 6, 153–165.

Boyd, E.M., Killham, K., Wright, J., Rumford, S., Hetheridge, M., Cumming, R., Meharg, A., 1997. Toxicity assessment of xenobiotic contaminated groundwater using lux modified *Pseudomonas fluorescens*. Chemosphere 35, 1967–1985.

Calleja, M.C., Persoone, G., Geladi, P., 1994. Comparative acute toxicity of the first 50 multicentre evaluation of in vitro cytotoxicity chemicals to aquatic non-vertebrates. Arch. Environ. Contam. Toxicol. 26, 69–78.

Cho, J.-C., Kim, S.-J., 1999. Green fluorescent protein-based direct viable count to verify a viable but non-culturable state of Salmonella typhi in environmental samples. J. Microbiol. Methods 36, 227–235.

Cho, J.-C., Kim, S.-J., 2000. Increase in bacterial community diversity in subsurface aquifers receiving livestock wastewater input. Appl. Environ. Microbiol. 66, 956–965.

Chun, U.H., Simonov, N., Chen, Y., Britz, M.L., 1996. Continuous pollution monitoring using Photobacterium phosphoreum. Resour. Conserv. Recy. 18, 25–40.

de Lorenzo, V., Herrero, M., Jakubzik, U., Timmis, K.N., 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. 172, 6568–6572.

D'Souza, S.F., 2001. Microbial biosensors. Biosens. Bioelectron. 16, 337–353.

Flemming, C.A., Lee, H., Trevors, J.T., 1994. Bioluminescent most-probable-number method to enumerate lux-marked Pseudomonas aeruginosa UG2Lr in soil. Appl. Environ. Microbiol. 60, 3458–3461.

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

Gu, M.B., Gil, G.C., Kim, J.H., 1999. A two-stage minibioreactor system for continuous toxicity monitoring. Biosens. Bioelectron. 14, 355–361.

Gustavson, K.E., Svenson, A., Harkin, J.M., 1998. Comparison of toxicities and mechanism of action of N-alkanols in the submitochondrial particle and the *Vibrio fischeri* bioluminescence (Microtox) bioassay. Environ. Toxicol. Chem. 17, 1917–1921.

Heitzer, A., Malachowsky, K., Thonnard, J., Bienkowski, P., White, D., Sayler, G., 1994. Optical biosensor for the environmental on-line monitoring of naphthalene and salicylate bioavailability with an immobilised bioluminescent catabolic reporter bacterium. Appl. Environ. Microbiol. 60, 1487–1494.

Herrero, M., de Lorenzo, V., Timmis, K.N., 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. 172, 6557–6567.

Kramer, K.J.M., Botterweg, J., 1991. Aquatic biological early warning systems: an overview. In: Jeffrey, D.J., Madden, B. (Eds.), Bioindicators and Environmental Management. Academic Press, London, pp. 95–126.

Leslie, S.B., Israeli, E., Lighyhare, B., Crowe, J.H., Crowe, L.M., 1995. Trehalose and sucrose protect both membrane and proteins in intact bacteria drying. Appl. Environ. Microbiol. 61, 3592–3597.

Munkittrick, K.R., Power, E.A., Sergy, G.A., 1991. The relative sensitivity of Microtox, Daphnid, Rainbow trout, fat-head Minnow acute lethality tests. Environ. Toxicol. Water Qual. Int. J. 6, 35–62.

Pardos, M., Benninghoff, C., Guéguen, C., Thomas, R., Dobrowolski, J., Dominik, J., 1999. Acute toxicity assessment of Polish (waste)water with a microplate-based Hydra attenuata assay: a comparison with the Microtox® test. Sci. Total Environ. 243–244, 141–148.

Reasoner, D.J., Geldreich, E.E., 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. 49, 1–7.

Salizzato, M., Bertato, V., Pavoni, B., Ghirardini, A.V., Ghetti, P.F., 1998. Sensitivity limits and EC50 values of the Vibrio fischeri test for organic micropollutants in natural and spiked extracts from sediments. Environ. Toxicol. Chem. 17, 655–661.

Sinclair, G.M., Paton, G.I., Meharg, A.A., Killham, K., 1999. Lux-biosensor assessment of pH effects on microbial sorption and toxicity of chlorophenols. FEMS Microbiol. Lett. 174, 274–278.

Sixt, S., Altschuh, J., Bruggemann, R., 1995. Quantitative structure-toxicity relationships for 80 chlorinated compounds using quantum chemical descriptors. Chemosphere 30, 2397–2414.

Thomulka, K.W., McGee, D.J., Lange, J.H., 1993. Use of bioluminescent bacterium Photobacterium phosphoreum to detect potentially biohazardous materials in water. Bull. Environ. Contam. Toxicol. 51, 538–544.

USEPA, 1991. Methods for aquatic toxicity identification evaluations: phase I toxicity characterization procedures, EPA 600/6-91-003. National Effluent Toxicity Assessment Center, Duluth, Minnesota, USA.

van der Schalie, W.H., Shedd, T.R., Knechtges, P.L., Widder, M.W., 2001. Using higher organisms in biological early warning systems for real-time toxicity detection. Biosens. Bioelectron. 16, 457–465.

Van Dyk, T.K., Majarian, W.R., Konstantinov, K.B., Young, R.M., Dhurjati, P.S., LaRossa, R.A., 1994. Rapid and sensitive pollutant detection by induction of heat shock gene-bioluminescence gene fusions. Appl. Environ. Microbiol. 60, 1414–1420.

van Hoof, F., Sluyts, H., Paulussen, J., Berckmans, D., Bloemen, H., 1994. Evaluation of a bio-monitor based on the phototactic behavior of Daphnia magna using infrared detection and digital image processing. Water Sci. Technol. 30, 79–86.