Research Square

Preprints are preliminary reports that have not undergone peer review. They should not be considered conclusive, used to inform clinical practice, or referenced by the media as validated information.

Luminescent Bacterial Biosensors with Different Promoters

Guey-Horng Wang[1], Teh-Hua Tsai[2], Chun-Chi Kui[3], Chiu-Yu Cheng[3], Tzu-Ling Huang[3], Ying-Chien Chung[3,*]

[1] Research Center of Natural Cosmeceuticals Engineering, Xiamen Medical College, Xiamen 361008, China; wanggh@livemail.tw

[2] Department of Chemical Engineering and Biotechnology, National Taipei University of Technology, Taipei, Taiwan; thtsai@ntut.edu.tw

[3] Department of Biological Science and Technology, China University of Science and Technology, Taipei 11581, Taiwan; cckui2020@gmail.com (C.-C.K.); cycheng@cc.cust.edu.tw (C.-Y.C.); tlhuang2020bb@gmail.com (T.-L.H)

* Correspondence: ycchung@cc.cust.edu.tw; Tel.: +886-2782-1862; Fax: +886-2786-5456

Abstract:

In this study, we constructed recombinant luminescent *Escherichia coli* with T7, T3, and SP6 promoters inserted between *tol* and *lux* genes as toluene biosensors and evaluated their sensitivity, selectivity, and specificity for measuring bioavailable toluene in in groundwater and river water. The luminescence intensity of each biosensor depended on temperature, incubation time, ionic strength, and concentrations of toluene and coexisting organic compounds. Toluene induced the highest luminescence intensity in recombinant *lux*-expressing *E. coli* with the T7 promoter [T7-*lux*-*E. coli*, limit of detection (LOD) = 0.05 μM], followed by that in *E. coli* with the T3 promoter (T3-*lux*-*E. coli*, LOD = 0.2 μM) and SP6 promoter (SP6-*lux*-*E. coli*, LOD = 0.5 μM). Luminescence activities may have been synergistically or antagonistically affected by coexisting organic compounds other than toluene; nevertheless, low concentrations of benzoate and toluene analogs had no such effect. In reproducibility experiments, the biosensors had low relative standard deviation (4.3%–5.8%). SP6-*lux*-*E. coli* demonstrated high adaptability to environmental interference. T7-*lux*-*E. coli* biosensor—with low LOD, wide measurement range (0.05–500 μM), and acceptable deviation (−14.3% to 9.1%)—is an efficient toluene biosensor. This is the first study evaluating recombinant *lux E. coli* with different promoters for their potential application in toluene measurement in actual water bodies.

Keywords: Biosensor, Groundwater, Promoter, Toluene

Introduction

The large-scale consumption of petroleum-derived fuels has led to groundwater and soil contamination through their leakage from fuel tanks and pipelines. Because of its moderate solubility in water and toxicity, toluene is a petrochemical contaminant of particular concern [1]. Even at low concentrations, toluene can be carcinogenic, can exhibit mutagenic properties, and can damage the kidney, liver, and central nervous system [2]. In Taiwan, environmental agencies have set acceptable limits for toluene in drinking water and groundwater at considerably low levels (7.6–10.9 μM) [3, 4]. In addition, toluene measurement is paramount for the monitoring and clean-up of contaminated groundwater and surface water. Thus, the need for sensitive toluene detection is high, but its design is challenging. In particular, toluene is found in various water bodies, including rivers, as well as coastal water and groundwater; even drinking water contains toluene at trace concentrations (μg/L).

Conventional analytical techniques, such as gas chromatography (GC) and high-performance liquid chromatography, are sensitive and reliable for toluene detection but are time-consuming, expensive, and laboratory-bound, and they require large equipment and specialized training [5, 6]. By contrast, biological methods can be useful alternatives for organics detection because they are low cost, easy to use, portable, small, and highly specific and can detect bioavailability [7–9]. Of the biological methods, biosensors are suitable for application as environmental sensors, even for on-field measurements.

Over the last 20 years, biosensors have been developed and are widely used as simple and practical approaches for the sensitive and specific detection of various compounds, including organic compounds (pesticides and chlorophenol), heavy metals (mercury, zinc, and cadmium), and some inorganic compounds [9–11]. Whole-cell biosensors rely on gene expression analysis: transcriptional fusions between a promoter and a reporter gene are created, and the extent of reporter gene expression is used to indicate the pollutant

concentration [12]. Several engineered biosensors have specifically discriminated between alkyl-substituted benzene derivatives in water samples [13].

A biosensor of this type can be genetically engineered by placing a reporter gene, such as *lacZ*, *gfp*, *luc*, or *lux*, under the control of a transcriptional activator [11, 14]. Under appropriate conditions (e.g., in the presence of specific pollutant), the biosensor can produce a detectable signal (color or luminescence) that is directly correlated to the pollutant concentration [12, 15]. This can aid in directly correlating the toluene concentration with the reporter enzyme activity. Various biosensors for benzene, toluene, ethylbenzene, and xylene detection have been developed on the basis of the *tol* plasmid of *Pseudomonas putida* mt-2 [16, 17]. In particular, bioluminescence is highly applicable as a reporter for pollutant detection because its instrumentation is sensitive for detecting light production [18]. However, *Escherichia coli* cells harboring this plasmid often express various response levels when constructed with different reporter genes or promoters that can lead to various linear measurement ranges and limits of detection (LODs) [19]. For instance, among induction-based biosensors, *luc*-, *lux*-, and aequorin-based biosensors have the LODs of 11, 7.5, and 1 μM, respectively [20–22]. Of the reporter genes, *lux* has acceptable sensitivity for signal production [18]. Measurement of toluene at very low concentration levels is a main goal of current environmental research; therefore, for the practical application of these biosensors, efforts toward overcoming the aforementioned limitations are warranted [23]. Rational selection of a suitable promoter or reporter gene is essential for increasing the sensitivity, signal intensity, and response speed of whole-cell biosensors.

SP6, T3, and T7 promoters, which are widely used for in vitro transcription, have similar but distinct promoter specificities [24]. They are classified as strong or weak promoters according to their RNA polymerase affinities. T7 is a strong promoter that maintains gene expression tuned to the highest level, thus potentially producing high signal intensity [25].

By contrast, weaker promoter (T3 and SP6) may correspond or adapt environmental variation, which produces different signal characteristics [26]. Thus, the linear measurement ranges and LODs of whole-cell biosensors would be expanded or improved if recombinant luminescent bacteria with suitable promoters are constructed.

In this study, we applied this strategy to construct recombinant *E. coli* strains carrying the *tol* plasmid from *P. putida* and including various promoters (T7, T3, or SP6) controlling *lux* expression. By optimizing the promoter and regulating the *lux* expression level in *E. coli*, the recombinant luminescent biosensors could detect bioavailable toluene under different environmental conditions.

Results and Discussion

Comparison of time-dependent induction of our three recombinant luminescent *E. coli* strains with toluene

Figure 1 illustrates the construction of the three recombinant plasmids. According to the preliminary experiment, the logarithmic growth phases of the three recombinant *E. coli* strains occurred from 6 to 15 h of incubation, and the relationship between OD of bacterial growth and RLU emitted from the three recombinant *E. coli* strains was linear from 8 to 14 h of incubation. Accordingly, the inoculation time of the three recombinant *E. coli* strains for the subsequent experiment was set as 12 h after incubation. Figure 2 presents a comparison of the time-dependent induction of luminescence emitted from T3-*lux*-*E. coli*, SP6-*lux*-*E. coli*, and T7-*lux*-*E. coli* caused by different toluene concentrations. As shown in Figure 2, the induction of luminescence caused by different toluene concentrations occurred time-dependently, regardless of the promoter type. The luminescence intensity continuously increased, leveled off, and then began to considerably decrease during incubation, all potentially due to the biochemical nature of the reporter gene *lux* [27].

The results demonstrated that luminescence was stable and the highest at 2–2.5 h after

incubation for T3-*lux-E. coli* and T7-*lux-E. coli* or 1–1.5 h after incubation for SP6-*lux-E. coli*. The time was equal to or shorter than that previously reported for the *lux*-based bioluminescent bioreporter *P. putida* TVA8 (2 h) and luminescence bacterial biosensors without the T7 promoter (3 h) for toluene measurement [6, 21]. Therefore, on average, 20-min consecutive measurements were recorded when T3-*lux-E. coli* and T7-*lux-E. coli* were cultured for 2 h and when SP6-*lux-E. coli* was cultured for 1 h. The maximum average luminescence induced by 200 μM toluene for T3-*lux-E. coli*, SP6-*lux-E. coli*, and T7-*lux-E. coli* was 1020 ± 17.6, 510 ± 14.1, and 2120 ± 63.8 RLU, respectively. Moreover, at the same toluene concentration, the signal intensity of luminescence decreased as follows: T7-*lux-E. coli* > T3-*lux-E. coli* > SP6-*lux-E. coli*. However, SP6-*lux-E. coli* had the shortest stable period for luminescence induction. de Las Heras A1 and de Lorenzo V. (2012) used a similar strategy to produce a considerable increase in bioluminescence emission by fusing the T7 promoter to control expression of the *lux* operon [28].

Effects of culture conditions on luminescence activity

The effects of incubation temperature and ionic strength on the induction of luminescence biosensors for toluene were evaluated according to practical considerations. Figure 3A illustrates the effects of incubation temperature on the luminescence activity induced by 100 μM toluene for T7-*lux-E. coli*. The experimental results demonstrated the optimal temperature range of luminescence activity for T7-*lux-E. coli* to be 30–37 °C, with nonsignificant differences ($p > 0.05$). Similar results were observed for T3-*lux-E. coli* and SP6-*lux-E. coli*. Moreover, luminescence activities of the three recombinant *E. coli* strains at 20 and 40 °C were 12.1%–15.3% and 24.4%–26.8% lower than those at 37 °C, respectively. The effect of high temperature on the luminescence activity of the recombinant *E. coli* strain was more noticeable. It should be attributed to the physiological characteristics of the *E. coli* [29]. Thus, subsequent experiments were performed at 37 °C

for all three recombinant *E. coli* strains.

Figure 3B shows the effects of ionic strength on the luminescence activities of the recombinant *E. coli* with the T3, SP6, or T7 promoter that were induced by 100 μM toluene. The results demonstrated almost no effect of different ionic strengths on the luminescence activity for SP6-*lux-E. coli*, but the ionic strength had relatively high effects on that of T7-*lux-E. coli*. When the ionic strength was 0.55 M, the luminescence activity of T7-*lux-E. coli* decreased by 12.5% ± 0.6%. This inconsistency among the recombinant *E. coli* with different promoters was presumed to be related to promoter structure and composition, which determine the strength of various types of promoter–target DNA bonds [30]. In general, the ranges of ionic strengths of groundwater, river water, seawater, and polluted water are 0.01–0.02, 10^{-3}–10^{-2}, 0.45–0.55, and $>10^{-2}$ M, respectively. Thus, SP6-lux-*E. coli* is suitable for application in various water environments (groundwater, river water, and seawater), whereas T7-*lux-E. coli* is suitable for use in relatively low ionic strength environments, except seawater.

Effects of coexisting carbon sources, intermediates, and toluene analogs on luminescence activity

Figure 4A illustrates the effects of coexisting carbon sources at 100 μM on the luminescence activity of T7-*lux-E. coli*. The tested chemicals are considered potential inhibitors or activators (indirect or direct inducers) of *xylS* and *xylR* and may deviate significantly or be additive effect in relation to theoretically expected effects, calculated on the basis of individual chemicals [12, 31, 32]. The current results demonstrated that the coexistence of sodium lactate or glycerin with toluene induced higher luminescence activity than did toluene alone. Sodium lactate and glycerin synergistically increased luminescence by 21% ± 8.6% or 14% ± 1.8%, respectively. If sodium lactate and glycerin were diluted to 70 or 85 μM, this synergistic increase disappeared. By contrast, the coexistence of sodium

acetate with toluene induced lower luminescence activity than did toluene alone; luminescence decreased by 32% ± 1.5%. However, for other chemicals, the coexistence had negligible effect on the detection of toluene by T7-*lux*-*E. coli*.

Figure 4B illustrates the effects of the benzoate concentration on the luminescence activity of T7-*lux*-*E. coli*. Benzoate is the most important metabolite produced during toluene biodegradation [33], which may affect *XylS* expression [31]; thus, we evaluated the effect of the benzoate concentration on the luminescence activity of T7-*lux*-*E. coli*. The results demonstrated that a high benzoate concentration could induce higher luminescence activity than did toluene alone, as detected using T7-*lux*-*E. coli*. However, 50–150 μM benzoate did not affect luminescence activity, whereas 250 and 300 μM benzoate improved luminescence activity by 26% ± 3.5% and 34% ± 2.8%, respectively. In other words, the effect of low concentrations of benzoate on luminescence activity was limited when toluene was detected by T7-*lux*-*E. coli*.

Figure 4C illustrates the effects of toluene analogs and their concentrations on the luminescence activity of T7-*lux*-*E. coli*. The results demonstrated that the various concentrations of *o*-xylene and *p*-xylene had negligible effects on toluene detection by the recombinant *E. coli* biosensor; moreover, even when 250 μM *o*-xylene was used, only a 4.3% increase in luminescence activity was observed. However, 250 μM *m*-xylene and 250 μM benzene induced T7-*lux*-*E. coli* to produce relatively high luminescence activity (12.5% and 14.6%, respectively). By contrast, the effect of the toluene analog concentration of ≤200 μM on toluene detection was limited (<8%). The effect of the synergistic mode was far lower than that observed in the *P. putida* mt-2 KG1206 biosensor [12].

Taken together, these results illustrate that our recombinant luminescent biosensor possesses high selectivity and specificity when detecting a group of analytes with similar chemical structures. Because the included chemicals mainly affect the regulatory genes *xylS* or *xylR*, but not the T3, SP6, or T7 promoter, their effects on the magnitude of luminescence

activity among all three recombinant *E. coli* biosensors were similar [12]. Figure 4 exemplifies the case of T7-*lux-E. coli*.

Relationship of toluene concentration with luminescence activity

Under optimal operating conditions, we determined the relationships between the toluene concentration and the luminescence activity of the three recombinant *E. coli* strains. Two sets of linear relationships were observed between the toluene concentration and luminescence activity at different concentration ranges. Figure 5A presents a set of regression equations for the toluene concentration and the luminescence activity of T7-*lux-E. coli*, T3-*lux-E. coli*, and SP6-*lux-E. coli* when the toluene concentration was 10–500 μM: $y = 6.140x + 724.9$, $y = 3.233x + 302.2$, and $y = 1.560x + 154.9$, respectively. Figure 5B presents another set regression equations for T7-*lux-E. coli*, T3-*lux-E. coli*, and SP6-*lux-E. coli* when the toluene concentration was ≤10 μM: $y = 40.515x + 46.9$, $y = 11.666x + 24.5$, and $y = 7.868x + 17.6$, respectively. The coefficients of determination for these equations was high (>0.99), indicating their reliability. The concentration-dependent differences in these linear relationships may have been due to differences in promoter characteristics [34]. Moreover, for T7-*lux-E. coli*, T3-*lux-E. coli*, and SP6-*lux-E. coli*, the LODs for toluene were 0.05, 0.2, and 0.5 μM, respectively. Therefore, T7-*lux-E. coli* was the most sensitive. Willardson et al. (1998) constructed a bacterial biosensor with the reporter gene *luc*, Casavant et al. (2003) constructed a site-specific recombination-based biosensor with *tbu*A1UBVA2C promoter, Li et al. (2008) constructed a *lux*-based bacterial biosensor, Zeinoddini et al. (2010) constructed a aequorin-based *E. coli* biosensor, Zhong et al. (2011) constructed a monooxygenase biosensor, and Ray et al. (2018) constructed a protein-based biosensor; their LODs for toluene were 10, 0.2, 7.5, 1, 3, and 3.3 μM, respectively [13, 20–22, 35, 36]. Compared with the aforementioned biosystems, T7-*lux-E. coli* has lower LOD (0.05 μM), indicating acceptable sensitivity. Moreover, the LOD of our

luminescence-based *E. coli* biosensor may be significantly improved if promoters positioned before the reporter gene *lux* are included.

Hence, on the basis of the aforementioned reliable equations or the calibration curve for 0.05–10 or 10–500 μM toluene, the toluene concentration in the water samples can be rapidly determined. In addition, the broad detection ranges of T7-*lux-E. coli* indicate that it is a practical toluene measurement tool.

Reproducibility

To evaluate the reproducibility of the biosensors for detecting toluene, T7-*lux-E. coli*, T3-*lux-E. coli*, and SP6-*lux-E. coli* were tested under identical conditions by using TMM containing 10 μM toluene. Relative standard deviation (RSD) for T7-*lux-E. coli*, T3-*lux-E. coli*, and SP6-*lux-E. coli* was 4.3%, 5.1%, and 5.8%, respectively (n = 10). Batch-to-batch variation was also tested by comparing the luminescence activity from the five sets, which was tested using TMM containing 10 μM toluene, and the RSD for T7-*lux-E. coli*, T3-*lux-E. coli*, and SP6-*lux-E. coli* was 6.2%, 6.5%, and 9.4%, respectively. These results are comparable to the reproducibility reported for two induction-based toluene biosensors: RSD = 9.5% for n = 3 and RSD = 7.4% for n = 8 [36, 37]. Thus, our recombinant luminescent *E. coli* biosensors demonstrated operational stability. Similar results were obtained when these biosensors were applied for measuring 10 μM toluene after a 3-month cryogenic storage period.

Toluene measurement in groundwater and river water by using our three recombinant luminescent *E. coli* biosensors

Most luminescent biosensors have been applied for measuring toluene availability in artificial wastewater, but few have been applied in actual wastewater. Table 1 summarizes the measured toluene concentrations in seven groundwater samples and three river water

samples using our three recombinant luminescent *E. coli* biosensors and the standard GC–MS method. The results demonstrated that the toluene concentration determined using our biosensors and through GC–MS demonstrated excellent correlation ($r^2 > 0.997$); moreover, the deviation between the toluene concentrations measured through GC–MS and those measured using T7-*lux-E. coli*, T3-*lux-E. coli*, and SP6-*lux-E. coli* was −14.3% to 9.1%, −60.0% to 70.7%, and −75.0% to 46.3%, respectively. Considering the measurement ranges and accuracy, T7-*lux-E. coli* provided the most accurate and reliable toluene measurement in these aqueous matrices. The deviation in the toluene concentration measured by the T3-*lux-E. coli* biosensor appeared to be high. Nevertheless, for toluene concentrations more than its LOD (i.e., 0.2 μM), this deviation range for T3-*lux-E. coli* narrowed to −10.7% to 26.7%. Similarly, the deviation range for SP6-*lux-E. coli* narrowed to −3.6% to 4.2% for toluene concentrations more than its LOD (0.5 μM). Therefore, under appropriate toluene concentration ranges, SP6-*lux-E. coli* could be the best biosensor in terms of accuracy, and its genetic assembly is relatively less susceptible to environmental interference [26]. The measurement deviation of T7-*lux-E. coli* and SP6-*lux-E. coli* were comparable to that (−16.7% to 7.5%) of electrochemical inhibition bacterial sensor array for toluene detection [38]. Taken together, these results indicate that the developed recombinant luminescent bacterial biosensors can determine toluene concentration in different water bodies.

Conclusions

In this study, recombinant luminescent *E. coli* biosensors containing different promoters (T3, T7, and SP6) positioned before the reporter gene *lux* were developed for the accurate measurement of toluene concentrations in groundwater and river water. Of these biosensors, T7-*lux-E. coli* was the most sensitive to toluene, with optimal LOD and widest measurement range for toluene concentrations. Moreover, SP6-*lux-E. coli* had the shortest reaction time and highest adaptability to environmental interference but the poorest LOD.

T7-*lux-E. coli* exhibited competitive advantages over previously reported biosystems, particularly for optimal LOD and wide measurement range. According to the results of reproducibility experiments and the test on actual water samples, our *lux*-based biosensors exhibited the high operational stability (i.e., low RSD) and acceptable measurement deviation. In conclusion, our biosensors, particularly T7-*lux-E. coli*, are sensitive, reliable, specific, and stable systems for preliminary in-field detection of toluene in water samples.

Materials and Methods

Bacterial strains, gene cloning, and biosensor plasmid construction

To clone the *tol* gene, partial *tol* in *P. putida* (ATCC 33015) was amplified using the primer set (forward 5′-GTTAACTGCATCCAGCCC-3′, reverse 5′-CCGGGCGATGCCAACCC-3′) through polymerase chain reaction (PCR). To clone T3-*lux*, SP6-*lux*, or T7-*lux*, *lux* in *Vibrio vulnificus* was amplified with the primer set for the corresponding genes (T7-*lux*, forward 5'- TAATACGACTCACTATAGGTCGACTTTATCGAGCCTGA-3´ and reverse 5'-CAGCTGTTTTTGCTCCT-3'; T3-*lux*, forward 5'-ATTAACCCTCACTAAAGGTCGACTTTATCGAGCCTGA-3' and reverse 5'-CAGCTGTTTTTGCTCCT-3'; SP6-*lux*, forward 5'-ATTTAGGTGACACTATAGGTCGACTTTATCGAGCCTGA-3' and reverse 5'-CAGCTGTTTTTGCTCCT-3') through PCR. All the resultant DNA fragments were inserted into the pGEM-T easy vector plasmid (Promega, Madison, WI, USA). The recombinant plasmids were named pTOL, pT3-*lux*, pSP6-*lux,* and pT7-*lux*. In brief, the plasmids were then transferred to the expression host *E. coli* DH5α and plated on Luria–Bertani (LB) agar plates. Then, isolated pTOL, pT3-*lux*, pSP6-*lux*, and pT7-*lux* plasmids were cut at cleavage sites using *Bst*EII/*Hin*dIII and *Hin*dIII/*San*I. Next, pTOL-T3-*lux*, pTOL-SP6-*lux*, and pTOL-T7-*lux* were constructed by ligating pTOL to pT3-*lux*, pSP6-*lux*, and pT7-*lux* fragments by using T4 DNA ligase (New England BioLabs, Beverly, MA,

USA), respectively. The resulting plasmids were inserted into the pGEM-T easy vector plasmid. Next, the plasmids were transformed into *E. coli* DH5α to create the corresponding whole-cell biosensors. All the restriction enzymes were purchased from New England BioLabs. Vector DNA was prepared using the QIAEX II gel extraction kit (Qiagen, Hilden, Germany).

Bacterial growth

E. coli with pTOL-T3-*lux* (T3-*lux-E. coli*), pTOL-SP6-*lux* (SP6-*lux-E. coli*), and pTOL-T7-*lux* (T7-*lux-E. coli*) (all initial concentration = 2×10^5 cfu/mL) were cultivated in LB broth containing 50 mg/L kanamycin at 37 °C at 200 rpm on an orbital shaker. Overnight cultures were then diluted 100-fold into toluene-mineral medium (TMM) containing 0.43 g/L K_2HPO_4, 0.23 g/L KH_2PO_4, 1 g/L NH_4NO_3, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2$, 0.05 mg/L $Fe_2(SO4)_3$, 0.25 mg/L $NaMoO_4 \cdot 2H_2O$, 50 mg/L kanamycin, and a specific concentration of toluene (in this case: 10 mg/L). The cultures were incubated at 37 °C at 200 rpm on an orbital shaker. The optical density (OD) measurements of the bacterial growth and the luminescence intensity released from recombinant *E. coli* were conducted at specific intervals. The OD of the cultures was measured at 600 nm on a UV-vis spectrophotometer (Shimadzu, Kyoto, Japan). The luminescence intensity [in relative light units (RLU)] was measured by adding 200 μL of the culture to a 96-well microplate and then placing it under a microplate luminometer (Titertek-Berthold, Pforzheim, Germany). All chemicals used in the experiment were of analytical grade (purity > 99%). Toluene was purchased from Sigma-Aldrich Corporation (St. Louis, MI, USA).

Determination of optimum conditions

After 12 h of cultivation in TMM, 1 mL of culture containing 5×10^7 cfu/mL T3-*lux-E. coli*, SP6-*lux-E. coli*, or T7-*lux-E. coli* was inoculated into 200 mL of TMM [with different final

concentrations (50–500 μM) of toluene] and incubated at 37 °C on an orbital shaker (200 rpm) for 5 h. The luminescence intensity was continuously measured until the luminescence intensity approached zero. The effects of temperature and ionic strength on bioluminescence emissions of the three recombinant *E. coli* strains were evaluated separately, and 100 μM toluene was used as an inducer in TMM. During incubation, temperature (15–40 °C) was controlled using thermostat, and ion strength (0.04–0.55 M) was adjusted using aqueous $NaOCl_4$. After 2-h incubation for T3-*lux-E. coli* and T7-*lux-E. coli* and 1-h incubation for SP6-*lux-E. coli*, 200 μL of the cultures were sampled, and the luminescence intensity (in RLU) of these biosensors was measured immediately. On average, 20-min consecutive measurements were recorded (i.e., one measurement every 0.5 s).

Various carbon sources (i.e., sodium acetate, sodium lactate, glucose, sucrose, fructose, glycerin, succinate, citrate, and pyruvate) were added to TMM to evaluate their effects on bioluminescence emissions of the three recombinant *E. coli* strains. In medium, final concentrations of coexisting carbon sources and toluene were 100 μM. After 2-h incubation for T3-*lux-E. coli* and T7-*lux-E. coli* and 1-h incubation for SP6-*lux-E. coli*, the luminescence intensity of each biosensor was measured immediately. Toluene analogs (i.e., benzene, *o*-xylene, *p*-xylene, and *m*-xylene) and intermediates of toluene degradation (benzoate) were added to TMM to evaluate the effects on the bioluminescence emissions of the three recombinant *E. coli* strains. Based on their solubility, *o*-xylene, *p*-xylene, and *m*-xylene were predissolved in 95% ethanol and added to TMM. The final concentrations of the toluene analogs, benzoate, and toluene in medium were 50–250, 50–300, and 100 μM, respectively. The cells were incubated for 2 h (T3-*lux-E. coli* and T7-*lux-E. coli*) or 1 h (SP6-*lux-E. coli*) at 37 °C; the luminescence intensity (in RLU) of these biosensors was then measured, as described above. Measurements were obtained from at least three independent experiments, each performed at least in triplicate.

Establishment of calibration curve and measurement of real water sample

To establish the relationships between the toluene concentration and the luminescence intensity of the three recombinant *E. coli* biosensors, we mixed 100 µL of toluene (0.01–500 µM), 50 µL of 4× TMM (without toluene), and 50 µL of recombinant luminescent *E. coli* cells (final concentration after mixing: 5×10^7 cfu/mL). We then operated at the optimal incubation time and conditions determined in previous experiments. Standard curves (known as calibration curves) were plotted from the linear regression of average luminescence intensity at each toluene concentration. To ensure that the established curves and methods were valid, we prepared similar solutions as mentioned above, but used groundwater (from Lin-Yuan Industrial Park, Kaohsiung City, Taiwan) and river water (from Tamsui River, New Taipei City, Taiwan) instead of pure toluene. The toluene concentration in the prepared solution was separately measured using the established GC–mass spectrometry (MS) method [39] as well as using our three recombinant *E. coli* biosensors. Considering practical application, the retention of illuminant activity of recombinant *E. coli* after its cryogenic storage is essential for biosensor usage; thus, similar experiments were conducted when the biosensors were cryogenically stored for 3 months. Data were obtained from at least three independent experiments, with each performed at least in triplicate.

Acknowledgments

The authors thank J.T. Kuo and Y.J. Liao for help with partially analytical measurements.

Author Contributions

All authors collaborated to carry out the work presented here. Conceptualization,

Guey-Horng Wang and Ying-Chien Chung; Formal analysis, Chun-Chi Kui and Chiu-Yu Cheng; Funding acquisition, Ying-Chien Chung; Investigation, Teh-hua Tsai, Chun-Chi Kui and Tzu-Ling Huang; Writing – original draft, Ying-Chien Chung; Writing – review & editing, Guey-Horng Wang, Teh-hua Tsai and Ying-Chien Chung All authors read and approved the manuscript.

Funding

This research was supported by NSC 99-2815-C-157-005-E and MOST 107-2313-B-157-001-MY2.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests..

References

1. Barberes GA, dos Reis RP, Spigolon ALD, Fonseca PE, de Mello CB, Barata MT. Groundwater natural contamination by toluene in Beja and Faro Districts, Portugal. Geosciences 2018;8:9.
2. Wang X, Bartha R. Effects of bioremediation on toxicity, mutagenesis, and microbiota in hydrocarbon-contaminated soils. In *Remediation of Hazardous Waste Contaminated Soils*, Wise, D.L.; Trantolo, D.J., Eds; Marcel Dekker; New York, USA, 1998, pp. 175–97.
3. EPA in Taiwan. Groundwater Pollution Control Standards. 2013 https://law.moj.gov.tw/ENG/LawClass/LawAll.aspx?pcode=O0110006.
4. EPA in Taiwan. Drinking Water Quality Standards. 2017. https://law.moj.gov.tw/ENG/LawClass/LawAll.aspx?pcode=O0040019.
5. Namieśnik J. Trends in environmental analytics and monitoring. Crit Rev Anal Chem. 2000;30:221–69.
6. Kuncová G, Ishizaki T, Solovyev A, Trögl J, Ripp S. The repetitive detection of toluene with bioluminescence bioreporter *Pseudomonas putida* TVA8 encapsulated in silica hydrogel on an optical fiber. Materials 2016;9:467.
7. Rodriguez-Mozaz S, de Alda MJL, Barceló D. Biosensors as useful tools for environmental analysis and monitoring. Anal Bioanal Chem. 2006;386:1025–41.
8. Xu TT, Close DM, Sayler GS, Ripp S. Genetically modified whole-cell bioreporters for environmental assessment. Ecol Indic. 2013;28:125–41.
9. Hashemi GN, Mirzaei H, Sahebkar A, Poursadeghiyan M, Masoudifar A. Malekshahi ZV, Negahdari B. Biosensors for the detection of environmental and urban pollutions. J Cell Biochem. 2018;119:207–12.
10. Cheng CY, Kuo JT, Lin YC, Liao YR, Chung YC. Comparisons of *Vibrio fischeri*, *Photobacterium phosphoreum*, and recombinant luminescent using *Escherichia coli* as

BOD measurement. J Environ Sci Health A 2010;45:233–8.

11. Gui Q, Lawson T, Shan S, Yan L, Liu Y. The application of whole cell-based biosensors for use in environmental analysis and in medical diagnostics. Sensors 2017;17:1623.

12. Kong IC. Effects of binary mixtures of inducers (toluene analogs) and of metals on bioluminescence induction of a recombinant bioreporter strain. Sensors 2014;14:18993–19006.

13. Ray S, Panjikar S, Anand R. Design of protein-based biosensors for selective detection of benzene groups of pollutants. ACS Sens. 2018;3:1632–38.

14. Burlage RS. Emerging technologies: Bioreporters, biosensors, and microprobes, In *Manual of Environmental Microbiology*, 2nd ed.; Hurst, C.J. Ed.; ASM: Washington, DC, USA, 2002, pp. 147–57.

15. Zhou Y, Fang Y, Ramasamy RP. Non-covalent functionalization of carbon nanotubes for electrochemical biosensor development. Sensors 2019;18:pii: E392.

16. Kim MN, Park HH, Lim WK, Shin HJ. Construction and comparison of *Escherichia coli* whole-cell biosensors capable of detecting aromatic compounds. J Microbiol Methods. 2005;60:235–45.

17. Burlage RS, Tillmann J. Biosensors of bacterial cells. J Microbiol Methods. 2017;138:2–11.

18. Axelrod T, Eltzov E, Marks RS. Bioluminescent bioreporter pad biosensor for monitoring water toxicity. Talanta 2016;149:290–7.

19. Nakamura, H. Current status of water environment and their microbial biosensor techniques-Part II: Recent trends in microbial biosensor development. Anal Bioanal Chem. 2018;410:3967–89.

20. Willardson BM, Wilkins JF, Rand TA, Schupp JM, Hill KK, Keim P, Jackson PJ. Development and testing of a bacterial biosensor for toluene-based environmental contaminants. Appl Environ Microbiol. 1998;64:1006–12.

21. Li YF, Li FY, Ho CL, Liao VHC. Construction and comparison of fluorescence and bioluminescence bacterial biosensors for the detection of bioavailable toluene and related compounds. Environ Pollut. 2008;152:123–9.

22. Zeinoddini M, Khajeh K, Behzadian F, Hosseinkhani S, Saeedinia AR, Barjesteh H. Design and characterization of an aequorin-based bacterial biosensor for detection of toluene and related compounds. Photochem Photobiol. 2010;86:1071–5.

23. Akbar M, Narayanan S, Restaino M, Agah M. A purge and trap integrated microGC platform for chemical identification in aqueous samples. Analyst. 2014;139:3384–92.

24. Eun HM. RNA polymerases, In *Enzymology Primer for Recombinant DNA Technology*, Elsevier Science Publishing Co Inc: San Diego, USA, 1996; pp 491–566.

25. Martin CT, Esposito EA, Theis, K, Gong P. Structure and function in promoter escape by T7 RNA polymerase. Prog Nucleic Acid Res Mol Biol. 2005;80:323–47.

26. Zhu LP, Li ZF, Sun X, Li SG, Li YZ. Characteristics and activity analysis of epothilone operon promoters from *Sorangium cellulosum* strains in *Escherichia coli*. Appl Microbiol Biotechnol. 2013;97:6857–66.

27. Dollard MA, Billard P. Whole-cell bacterial sensors for the monitoring of phosphate bioavailability. J Microbiol Methods 2003;55:221–9.

28. de Las Heras A, de Lorenzo V. Engineering whole-cell biosensors with no antibiotic markers for monitoring aromatic compounds in the environment. Methods Mol Biol. 2012;834:261–81.

29. Akkermans S, Logist F, Van Impe JF. An interaction model for the combined effect of temperature, pH and water activity on the growth rate of *E. coli* K12. Food Res Int. 2018;106:1123–31.

30. Blazeck J, Alper HS. Promoter engineering: recent advances in controlling transcription at the most fundamental level. Biotechnol J. 2013;8:46–58.

31. Kong IC, Suh H, Yang Z, Burlage RS. A bioluminescent reporter strain utilizing the

lower pathway promoter (Pm) of the *xyl* operon of *Pseudomonas*: optimization of a bioassay for *m*-toluate. Adv Environ Res. 2004;8:647–54.

32. Ikuma K, Gunsch C. Effect of carbon source addition on toluene biodegradation by an *Escherichia coli* DH5α transconjugant harboring the TOL plasmid. Biotechnol Bioeng. 2010;107:269–77.

33. Martínez-Lavanchy, PM, Müller C, Nijenhuis I, Kappelmeyer U, Buffing M, McPherson K, Heipieper HJ. High stability and fast recovery of expression of the TOL plasmid-carried toluene catabolism genes of *Pseudomonas putida* mt-2 under conditions of oxygen limitation and oscillation. Appl Environ Microbiol. 2010;76:6715–23.

34. Espinosa-Urgel M, Serrano L, Ramos JL, Ferna´ndez-Escamilla AM. Engineering biological approaches for detection of toxic compounds: a new microbial biosensor based on the *Pseudomonas putida* TtgR Repressor. Mo. Biotechnol. 2015;57:558–64.

35. Casavant NC, Thompson D, Beattie GA, Phillips GJ, Halverson LJ. Use of a site-specific recombination-based biosensor for detecting bioavailable toluene and related compounds on roots. Environ Microbiol. 2003;5:238–49.

36. Zhong Z, Fritzsche M, Pieper SB, Wood TK, Lear KL, Dandy DS, Reardon KF. Fiber optic monooxygenase biosensor for toluene concentration measurement in aqueous samples. Biosens Bioelectron. 2011;26:2407–12.

37. Stiner L, Halverson LJ. Development and characterization of a green fluorescent protein-based bacterial biosensor for bioavailable toluene and related compounds. Appl Environ Microbiol. 2002;68:1962–71.

38. Abu-Ali H, Nabok A, Smith TJ. Electrochemical inhibition bacterial sensor array for detection of water pollutants: artificial neural network (ANN) approach. Anal Bioanal Chem. 2019;411:7659–68.

39. USEPA Method 624.1: Purgeables by GC/MS., 2016.

Figure caption

Fig. 1 Construction of pTOL-T3-*lux*, pTOL-SP6-*lux* and pTOL-T7-*lux*.

Fig. 2 Comparison of time-dependent induction of luminescence from **(A)** T3-*lux-E. coli*, **(B)** SP6-*lux-E. coli*, and (**C**) T7-*lux-E. coli*; initial cell concentration: 5×10^7 cfu/mL, culture media: TMM with different toluene concentration, operational conditions: 37 °C and 200 rpm.

Fig. 3 (**A**) Effects of incubation temperature on luminescence activity of T7-*lux-E. coli* induced by 100 μM toluene for 2 h. (**B**) Effects of ionic strength on the luminescence activities of T3-*lux-E. coli*, SP6-*lux-E. coli* and T7-*lux-E. coli* induced by 100 μM toluene for 2 h (T3-*lux-E. coli* and T7-*lux-E. coli*) or 1 h (SP6-*lux-E. coli*).

Fig. 4 Effects of (A) coexisting carbon sources (100 μM), (B) benzoate, and (**C**) toluene analogs and their concentrations on luminescence activity of T7-*lux-E. coli* induced by 100 μM toluene for 2 h.

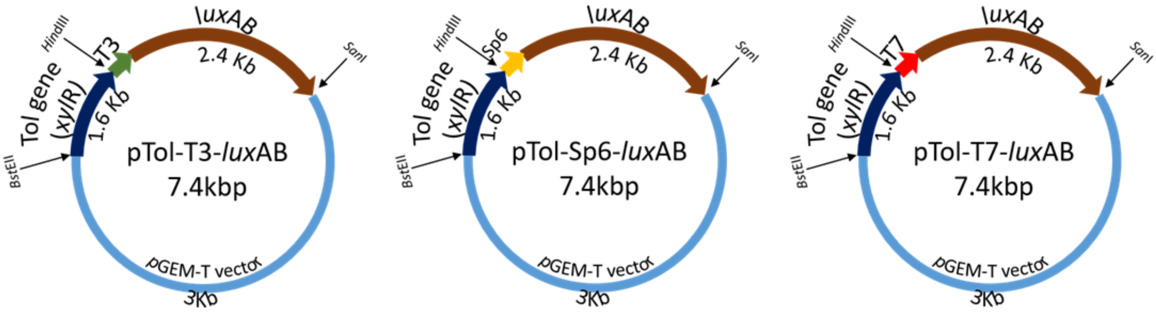
Fig. 5 Relationship between toluene concentration [(**A**) 0.01–500 and (**B**) 0.05–10 μM] and luminescence activity of recombinant *E. coli* with different promoters (initial cell concentration: 5×10^7 cfu/mL, culture media: TMM, operational condition: 37 °C and 200 rpm, incubation time: 2 h for T3/T7-*lux-E. coli* and 1 h for SP6-*lux-E. coli*).

Table 1 Toluene measurement from groundwater and river water by using the GC–MS method and biosensors

	Groundwater							River water		
GC–MS	0.15*	0.56	1.20	9.5	5.6	15.6	0.082	0.12	20.6	31.5
T7-biosensor	0.16 (6.7%)**	0.61 (8.9%)	1.31 (9.1%)	8.6 (-9.5%)	4.8 (-14.3%)	15.2 (-2.6%)	0.078 (-4.9%)	0.13 (8.3%)	18.5 (-10.2%)	30.6 (-2.9%)
T3-biosensor	0.06 (-60%)	0.50 (-10.7%)	1.52 (26.7%)	10.1 (6.3%)	6.1 (8.9%)	16.5 (5.8%)	0.14 (70.7%)	0.05 (-58.3%)	21.8 (5.8%)	32.6 (3.5%)
SP6-biosensor	0.09 (-40%)	0.54 (-3.6%)	1.25 (4.2%)	9.8 (3.2%)	5.8 (3.6%)	15.9 (1.9%)	0.12 (46.3%)	0.03 (-75%)	20.9 (1.5%)	32.1 (1.9%)

*Unit: µM

**Deviation compared with GC–MS-measured value



HindIII
T3
luxAB
2.4 Kb
SanI
Tol gene
(xylR)
1.6 Kb
BstEII
pTol-T3-luxAB
7.4kbp
pGEM-T vector
3Kb
HindIII
Sp6
luxAB
2.4 Kb
SanI
Tol gene
(xylR)
1.6 Kb
BstEII
pTol-Sp6-luxAB
7.4kbp
pGEM-T vector
3Kb
HindIII
T7
luxAB
2.4 Kb
SanI
Tol gene
(xylR)
1.6 Kb
BstEII
pTol-T7-luxAB
7.4kbp
pGEM-T vector
3Kb

Fig. 1

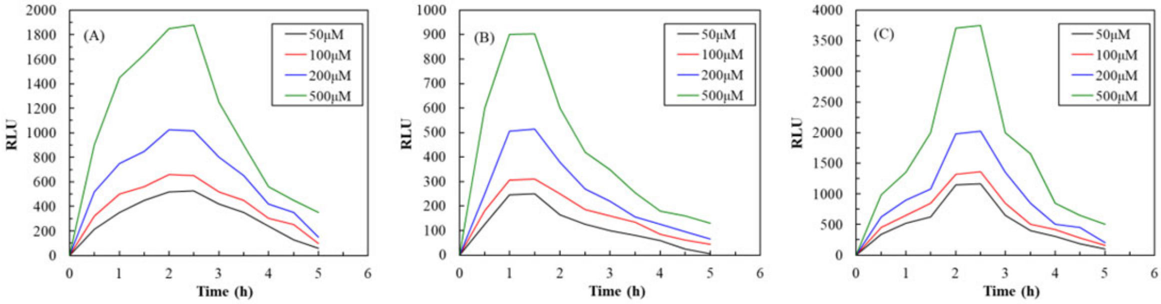
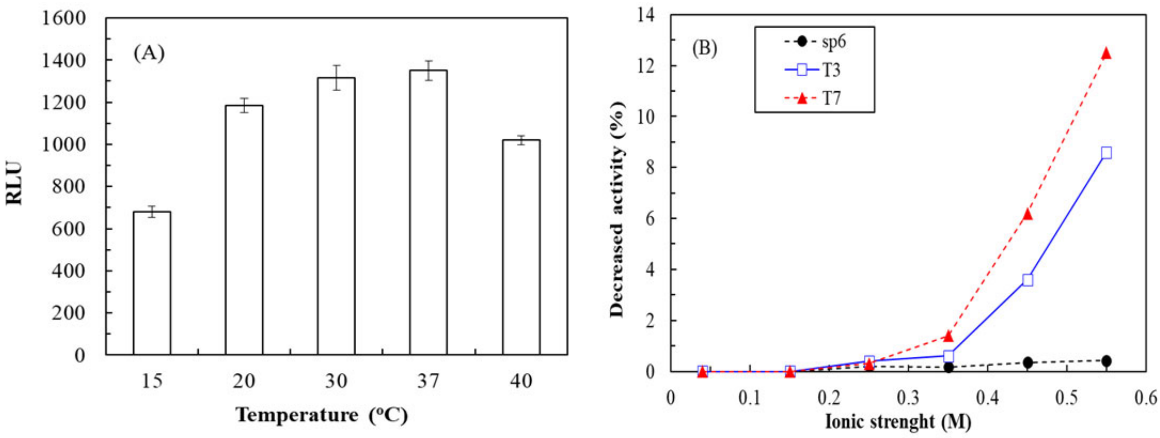
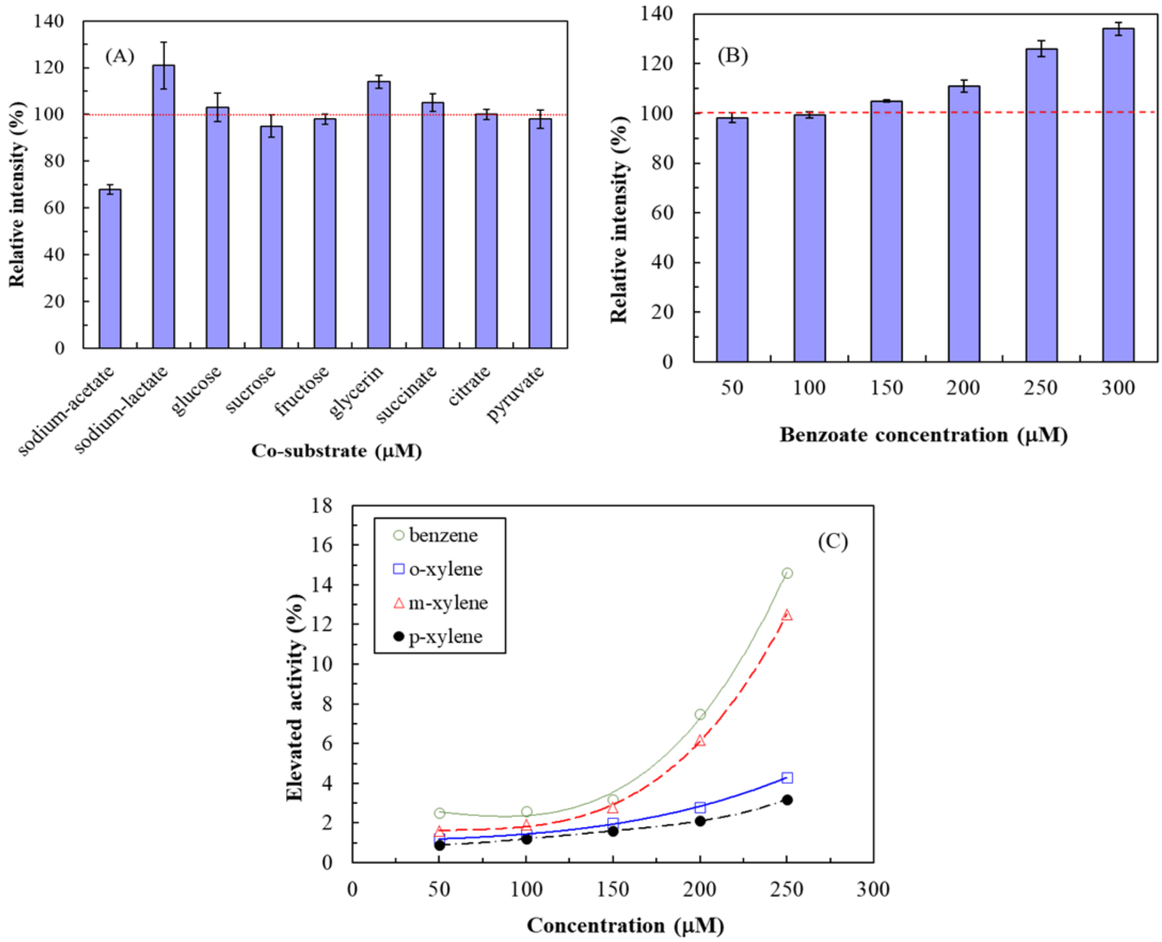


Fig.2



(A)
RLU
1600
1400
1200
1000
800
600
400
200
0
15
20
30
37
40
Temperature (°C)
(B)
sp6
T3
T7
Decreased activity (%)
14
12
10
8
6
4
2
0
0
0.1
0.2
0.3
0.4
0.5
0.6
Ionic strenght (M)

Fig. 3



(A)
Relative intensity (%)
140
120
100
80
60
40
20
0
sodium-acetate
sodium-lactate
glucose
sucrose
fructose
glycerin
succinate
citrate
pyruvate
Co-substrate (μM)
(B)
Relative intensity (%)
50
100
150
200
250
300
Benzoate concentration (μM)
(C)
benzene
o-xylene
m-xylene
p-xylene
Elevated activity (%)
18
16
14
12
10
8
6
4
2
0
Concentration (μM)

Fig. 4

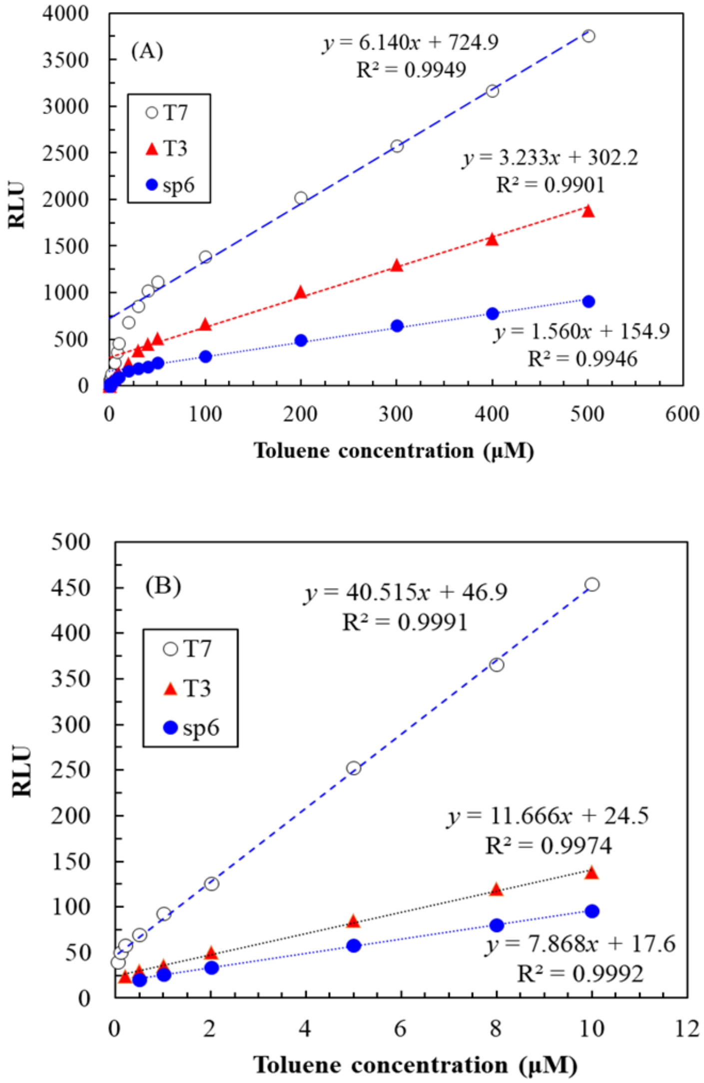

(A)
T7
T3
sp6
y = 6.140x + 724.9
R² = 0.9949
y = 3.233x + 302.2
R² = 0.9901
y = 1.560x + 154.9
R² = 0.9946
RLU
Toluene concentration (μM)
(B)
T7
T3
sp6
y = 40.515x + 46.9
R² = 0.9991
y = 11.666x + 24.5
R² = 0.9974
y = 7.868x + 17.6
R² = 0.9992
RLU
Toluene concentration (μM)

Fig. 5