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# New applications of genetically modified *Pseudomonas aeruginosa* for toxicity detection in water

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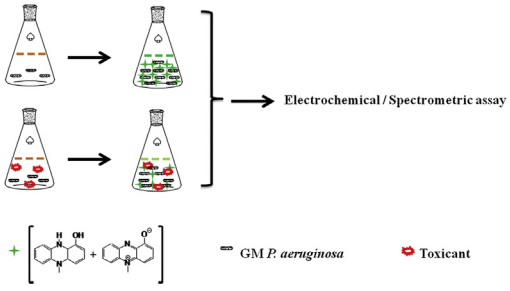
## HIGHLIGHTS

- A novel and external mediator-free water toxicity detection was firstly developed.
- A genetically modified *Pseudomonas aeruginosa* was used as the model microorganism.
- The pyocyanin (PYO) produced by GM *P. aeruginosa* was used as the indicator.
- Toxicity detection was assessed with electrochemical and spectroscopic assays.
- The toxicity of sample solution with 3, 5-DCP could be estimated visually by naked eyes.

## GRAPHICAL ABSTRACT

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## ABSTRACT

A novel mediator-free method based on genetically modified bacteria was developed for detecting water toxicity, where genetically modified *Pseudomonas aeruginosa* (GM *P. aeruginosa*) was selected as the biosensor strain and pyocyanin (PYO) produced by this strain was used as the indicator. The toxicity response of GM *P. aeruginosa* to 3, 5-dichlorophenol (3, 5-DCP) was measured electrochemically and spectroscopically, and the half maximal inhibitory concentration ($IC_{50}$) of 3, 5-DCP was determined to be 15.1 mg/L. Strikingly, the toxicity of sample solution with 3, 5-DCP could also be estimated visually by naked eyes at a concentration as low as 10 mg/L. The present study provided a convenient, sensitive and cost-effective method for water toxicity detection, and extended biosensing application of the genetically modified bacterium.



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## 1. Introduction

Various types of pollutants such as heavy metal ions and pesticides with high toxicity existed in water threaten the safety of humans seriously. Various detection techniques for water toxicity have been developed in recent years (Pasco et al., 2001; Hsieh et al., 2004; Kim et al., 2007). The algae, fish, daphnia or microorganism were used as the indicator for water toxicity detection by biological method (Beasley et al., 2015; Kim et al., 2015; Li et al., 2015; Shuhaimi-Othman et al., 2015). Among them, the microbial toxicity detection attracted much attention due to its fast response, long-term stability, high sensitivity and low cost (Catterall et al., 2010). Many kinds of water toxicity biosensors based on luminescent bacteria have been developed in the past decades (Ghirardini et al., 2009; Lopes et al., 2012). However, complicated maintenance procedures were required to obtain the reliable results. Recently, microbial toxicity detection by electrochemical method has attracted considerable attention, due to its durable with high concentration of cells from the turbid medium. Meanwhile, the toxicity detection method based on genetically modified microorganism shows great promise. For example, plasmids with toxicant responsive promoter (*fabA*, *dnaK* or *grpE* etc.) and reporter gene (*lacZ* (β-galactosidase) or *gfp* (fluorescent properties luciferase) etc.) were introduced into microorganisms and employed as water toxicity biosensor. Once exposed to toxicants, the responsive promoter would activate the expression of report gene. The activity of the corresponding protein or enzyme expressed from the reporter gene, correlated with the concentration of the toxicants could be detected by conventional physic-chemical assays (Tessema et al., 2006). Nevertheless, expensive instruments or substrates for enzymes were usually required, which limited the practical application owing to high testing cost.

To overcome these drawbacks, electrochemical biosensors attracted much attention. The direct electrochemical interaction between the bacteria and electrode without mediator is quite favorable for the development of electrochemical biosensors, which would simplify the testing procedure and instrument requirement (Si et al., 2016). It is well known that the soluble, redox active molecules produced by bacteria can be used as electron mediators (Hernandez et al., 2004; Sun et al., 2017). For instance, *Pseudomonas aeruginosa* (*P. aeruginosa*) releases some phenazine compounds, including phenazine-*l*-carboxamide (PCA), pyocyanin (PYO) and 1-hydroxy-phenazine (1-HP) (Chang and Blackwood, 1969; Watson et al., 1986). PYO exhibits reversible redox activity with an oxidation potential of −0.034 V (pH = 7), while the concentration of PYO produced by wild *P. aeruginosa* is too low to detect. Wang et al. successfully constructed a *P. aeruginosa* strain that produces higher concentrations of phenazine under anaerobic condition by over expression of the PqsE effector in a PQS negative mutant (Wang et al., 2013). Yong et al. greatly improved the PYO production with genetically modified *Pseudomonas aeruginosa* (GM *P. aeruginosa*), and a pigment-based biosensor that could sensitively detect *N*-butyryl homoserine lactone (BHL) was developed (Yong and Zhong, 2009).

In this work, GM *P. aeruginosa* with improved PYO production efficiency is used as the biosensor and PYO produced by the GM *P. aeruginosa* is used as the indicator for water toxicity detection. To the best of our knowledge, it is the first report that GM *P. aeruginosa* is used to assess toxicity of environmental pollutants. Both electrochemical and spectroscopic measurements are carried out to detect the water toxicity by the biosensor. After the oxidation of reduced state PYO in ambient condition, it changes to green color, and can be visually observed by naked eyes. The performance of the biosensor developed here is also comparable with other reported biosensors such as Microtox (Rathinam and Mohanan, 1998), Cellsense (Wang et al., 2008), Micredox (Pasco et al., 2001), Baroxymeter (Tzoris et al., 2005) and so on. The present proposed method is promising for sensitive and cost-effective assessment of water toxicity.

## 2. Experimental details

### 2.1. Chemicals and reagents

Peptone was purchased from Beijing Aoboxing Bio-tech Co., Ltd., glycerol was purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd., $MgCl_2$ and $K_2SO_4$ were purchased from Beijing Chemical Works, tetracycline was purchased from Sigma-Aldrich, ampicillin was purchased from Amresco and 3, 5-DCP was purchased from Sigma-Aldrich. Antibiotics stock solution was prepared by dissolving 500 mg ampicillin and 1 g tetracycline in 50 mL deionized water, then filter-sterilized with 0.22 μm membrane. The pseudomonas broth (PB, 20 g/L peptone, 1.4 g/L $MgCl_2$, 10 g/L $K_2SO_4$ and glycerol (v:v = 1:50)) was adjusted to pH 7.0 with 1 M NaOH and sterilized at 121 °C for 20 min. The fresh toxicant solutions of 3, 5-DCP with desired concentration were prepared by diluting the high-concentration stock solution (1000 mg/L) with deionized water. Unless otherwise stated, all chemicals were used as received without further purification and all solutions were prepared using deionized water (18.25 MΩ/cm) purified with a Milli-Q Gradient System (Millipore).

### 2.2. Instruments

Electrochemical measurements including Cyclic Voltammetry and Chronoamperometry were performed with an electrochemical analyzer (CHI 832B, Shanghai Chen Hua Instrument Co., Ltd.). A conventional three-electrode system was used, which consisted of a self-made Ag/AgCl (saturated KCl) electrode as the reference electrode, a platinum plate as the counter electrode and a self-made Pt ultramicroelectrode array (UMEA) composed of 25 pieces of 10.0-μm-radius platinum disks as the working electrode (Yu et al., 2013), respectively. For toxicity test, the current-time curves were used to quantitatively measure the reduced mediator, and the working potential was set at −0.1 V. All potentials given here were versus the Ag/AgCl reference electrode, and all tests were performed at room temperature. The surface of UMEA was successively polished with silicon carbide abrasive paper from 180, 600, 1200, 2000, 3000 to 5000, and thoroughly cleaned before use. The UV–vis absorption spectra were obtained with a Varian 50 Conc UV–visible Spectrophotometer (Mulgrave, Australia).

### 2.3. Bacteria and culture conditions

GM *P. aeruginosa* was constructed by overexpression of *rhlIR* gene clsuter as described elsewhere (Yong et al., 2011) by using *P. aeruginosa* CGMCC 1.860 (Sun et al., 2006) as the parent strain. Here, bacteria were routinely grown by shaking (220 rpm) in PB broth (Ishida et al., 2007) at 30 °C for 10 h or so. Antibiotics including 200 μg/mL ampicillin and 100 μg/mL tetracycline were used for plasmid maintenance in GM *P. aeruginosa*. Culture supernatants for electrochemical and spectroscopic toxicity tests were harvested by centrifugation at 10,000 rpm for 10 min at room temperature.

### 2.4. Principle of the toxicity detection

The basic principle of the toxicity detection is based on the changes in the respiration chain activity of GM *P. aeruginosa* induced by toxicants. Schematic illustration of the mediator-free toxicity detection was shown in Scheme 1. It is obvious that less reduced state pyocyanin ($PYO_{red}$) was produced by cells in the toxic sample than the control sample after incubation, because the respiratory and metabolic activity of microorganisms were inhibited in the presence of toxicants, and less $PYO_{red}$ could be transformed to oxidized state pyocyanin ($PYO_{oxi}$). Therefore, the measured oxidation current of $PYO_{red}$ and UV–vis absorbance value of PYO decreased, and green color of the test solution became lighter. So, two test assays, both electrochemical and spectroscopic assays could be adopted in the toxicity detection.

For the electrochemical and spectroscopic assays, the toxicities could be expressed by the inhibition % shown in equation (1) and equation (2), respectively:

$$\text{Inhibition}\ (\%) = 100 \times (i_{con} - i_{tox})/i_{con} \quad (1)$$

$$\text{Inhibition}\ (\%) = 100 \times (Abs_{m-con} - Abs_{m-tox})/Abs_{m-con} \quad (2)$$

The inhibition ratio was calculated according to equation (1) when electrochemical assay was used, $i_{tox}$ and $i_{con}$ represented the limiting currents obtained in the presence and absence of toxicants, respectively. The inhibition ratio for spectroscopic assay was calculated according to equation (2), $Abs_{m\text{-}tox}$ and $Abs_{m\text{-}con}$ represented the UV–vis absorbance of the solution in the presence and absence of toxicants, respectively. Meanwhile, the toxicity results, the lowest concentration (LC) could be visually estimated by naked eyes with colorimetry assay.

### 2.5. Toxicity tests

The samples including final concentrations of (a) 0 mg/L (control), (b) 2.5 mg/L, (c) 5 mg/L, (d)10 mg/L and (e) 20 mg/L 3, 5-DCP were prepared. GM *P. aeruginosa* cell suspensions (18 mL PB broth containing appropriate antibiotics and cells ($OD_{600} \approx 0.05$, final concentration)) were mixed with 2 mL samples of different concentration 3, 5-DCP. Then, the cell suspension with 3, 5-DCP was cultivated at 30 °C by shaking (220 rpm) for 10 h, next, the PYO production and color changes of these suspensions were monitored. To avoid possible interference from cells, the culture supernatants were collected by centrifugation before electrochemical or spectrometric assay.

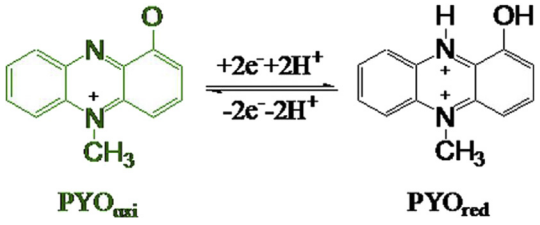
## 3. Results and discussion

### 3.1. Electrochemical detection of water toxicity with GM *P. aeruginosa*

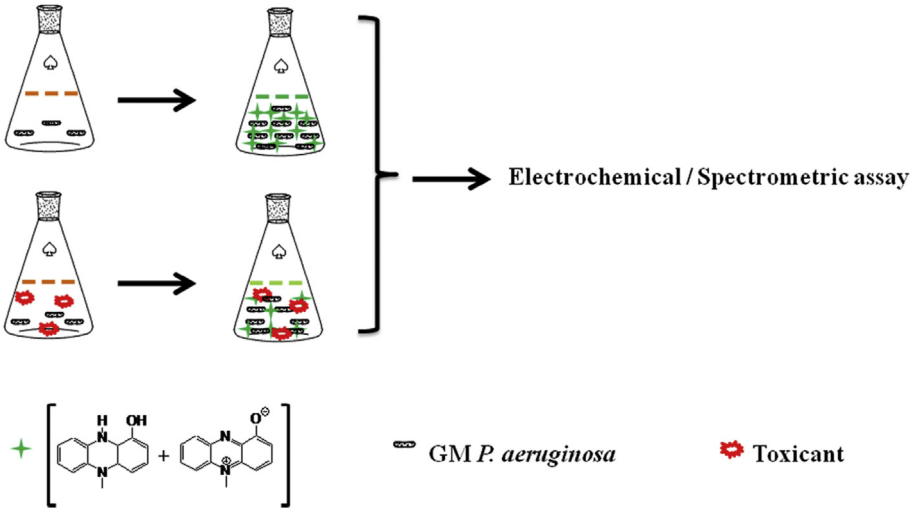
#### 3.1.1. Validation of feasibility

Due to its various advantages, UMEA became an important tool in electroanalytical chemistry. The current measured by UMEA is able to reach to the steady state in a short time because nonlinear diffusion controlled kinetics played the dominant role. Furthermore, higher current can be collected by UMEA than a single microelectrode (Yu et al., 2013). As shown in Scheme 2, $PYO_{red}$ produced could be oxidized easily to $PYO_{oxi}$ by dissolved oxygen (Webster and Goluch, 2012), thus the cultured PB broth may contain both colorless $PYO_{red}$ and green $PYO_{oxi}$ after incubation. So, UMEA was used to detect $PYO_{red}$ quickly in order to avoid the oxidation of $PYO_{red}$.

By simple overexpression of *rhlIR* gene cluster, the yield of PYO from GM *P. aeruginosa* was greatly improved compared to its parent strain as evidenced by HPLC and MS analysis (Fig. S1). Thus, it is expected to use this PYO overproducing strain for toxicity detection. Fig. 1(A) and (B) showed the color changes of GM *P. aeruginosa* inoculated in PB broth before and after incubation, respectively, which suggested GM *P. aeruginosa* produced significant amount of PYO (colorless $PYO_{red}$ produced could be partially oxidized to green $PYO_{oxi}$ in the air after culture). Fig. 1(C) showed the Cyclic




**Scheme 2.** Schematic illustration of transformation between $PYO_{red}$ and $PYO_{oxi}$.




**Scheme 1.** Schematic illustration of mediator-free toxicity detection.

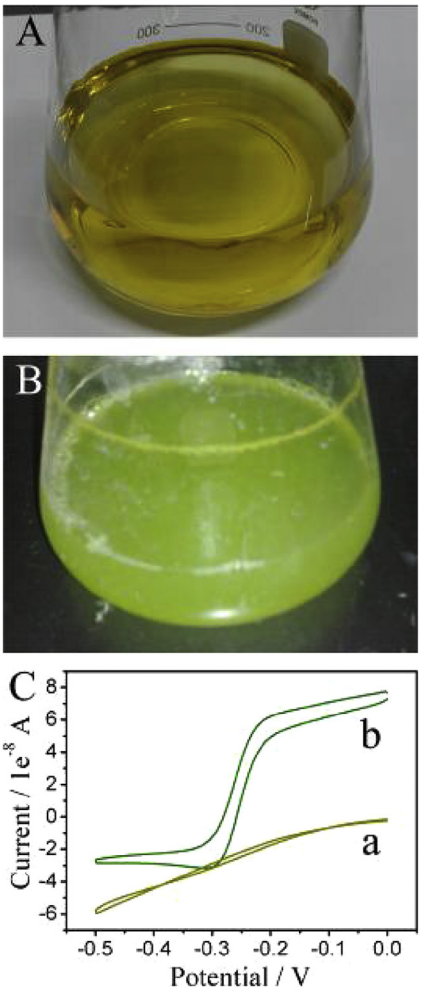



**Fig. 1.** (A) PB broth solution containing GM *P. aeruginosa* before incubation; (B) PB broth solution containing GM *P. aeruginosa* after incubation for 15 h; (C) CVs of GM *P. aeruginosa* solution before (a) and after 15 h incubation (b) at a scan rate of 50 mV/s in −0.5–0 V.

Voltammetry (CV) curves of the GM *P. aeruginosa* solution between −0.5 and 0 V before and after incubation for 15 h obtained with UMEA at a scan rate of 50 mV/s. The electrochemical redox reaction was a diffusion control process at 10–100 mV/s scan rate (Yong et al., 2011). It was clearly shown in Fig. 1(C) that the pair of redox peaks is attributed to electrochemical activity of PYO produced by GM *P. aeruginosa*. The results of HPLC/MS and electrochemical tests indicated that high concentration of PYO produced by GM *P. aeruginosa* possesses good electrochemical activity, suggesting it is feasible to detect water toxicity with electrochemical assay using GM *P. aeruginosa*.

### 3.1.2. Toxicity assay

3, 5-DCP is widely used as the model toxicant for water toxicity monitoring (Pasco et al., 2001; Liu et al., 2011). Therefore, the toxicity response of GM *P. aeruginosa* to 3, 5-DCP was studied. The CV curve of GM *P. aeruginosa* culture after incubation for 10 h was recorded as shown in Fig. 1(C). It was obvious that $PYO_{red}$ can be oxidized to $PYO_{oxi}$ in the voltage range of −0.22–0 V. For toxicity tests, the amperometric i-t curves were collected at a constant potential of −0.1 V, at which the electrochemical oxidation process of $PYO_{red}$ is diffusion control and the steady-state currents can be obtained in less than 10 s. The amperometric i-t curves can be used to quantitatively measure the reduced mediator of $PYO_{red}$. The feasibility of the proposed toxicity biosensor was further examined in Fig. 2, the currents obtained decreased along with increasing concentration of 3, 5-DCP. The inhibition ratios calculated according to equation (1) for 2.5, 5.0, 10.0 and 20.0 mg/L 3, 5-DCP were 12.47%, 14.10%, 22.34% and 47.62%, respectively.

### 3.2. Spectrometric detection of water toxicity with GM *P. aeruginosa*

The PYO, a yellow-green pigment produced by GM *P. aeruginosa*, can be spectroscopically determined at 660 nm by UV–vis spectrum. So, it is expected to analyze GM *P. aeruginosa* response to the toxicity of 3, 5-DCP with spectroscopic assay. Fig. 3(A) showed the inhibitory curves of GM *P. aeruginosa* grown with different concentrations of 3, 5-DCP. It was clearly observed that the UV–vis absorbance decreased along with increasing concentrations of 3, 5-DCP, indicating less PYO produced by GM *P. aeruginosa* due to the metabolic and respiratory activities of microorganisms were inhibited in the presence of 3, 5-DCP. Inhibition ratios calculated according to equation (2) for 2.5, 5.0, 10.0 and 20.0 mg/L 3, 5-DCP were 5.54%, 11.92%, 27.53% and 72.47%, respectively. The corresponding half maximal inhibitory concentration ($IC_{50}$) of 3, 5-DCP was calculated to be 15.1 mg/L according to the plots in Fig. 3(B).

As shown in Fig. 4, the color of samples became sequentially lighter along with the samples of “b”, “c”, “d” and “e”, and the color of sample “a” without 3, 5-DCP was the darkest. The color differences are remarkable between the control sample “a” and the sample “d” and “e”. The color of sample “d” was lighter than that of the sample “e” observed by naked eyes. Therefore, we further examined GM *P. aeruginosa* response to 3, 5-DCP toxicity with colorimetric assay, where PYO was used as the colorimetric indicator and GM *P. aeruginosa* was used as the model bacterial. The toxicant 3, 5-DCP with the LC value of 10.0 mg/L could be easily distinguished from the color lightness by naked eyes, and a more accurate LC estimation would be obtained by optimizing toxicant concentration in further research. The colorimetric biosensor developed in this work doesn't need special equipment, which can dramatically simplify operations and reduce the test cost. With regard to the sensitivity and test cost, the colorimetric biosensor shows promising potentials in environmental determination.

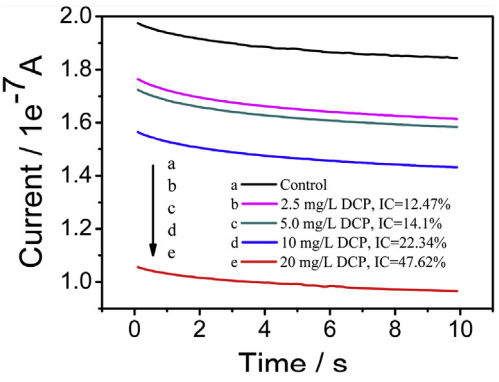



**Fig. 2.** The amperometric i-t curves recorded electrochemical responses of GM *P. aeruginosa* supernatant cultured with different concentration of 3, 5-DCP ((a) 0.0 mg/L, (b) 2.5 mg/L, (c) 5.0 mg/L, (d) 10.0 mg/L and (e) 20.0 mg/L) after incubation for 10 h. Working electrode was poised at a constant of potential −0.1 V.

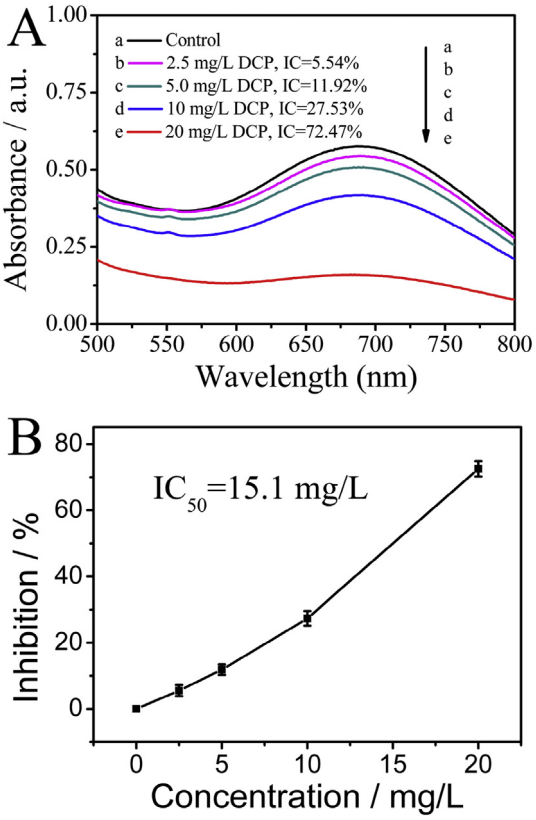



**Fig. 3.** (A) UV–vis spectra of GM *P. aeruginosa* supernatant cultured with different concentration of 3, 5-DCP ((a) 0.0 mg/L, (b) 2.5 mg/L, (c) 5.0 mg/L, (d) 10.0 mg/L and (e) 20.0 mg/L) after incubation for 10 h; (B) inhibitory curves of bacterial respiration for different concentrations of 3, 5-DCP.

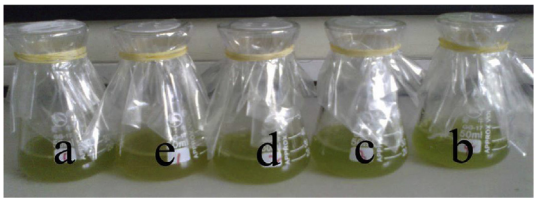



**Fig. 4.** Color changes of GM *P. aeruginosa* suspension cultured with different concentration of 3, 5-DCP ((a) 0.0 mg/L, (b) 2.5 mg/L, (c) 5.0 mg/L, (d) 10.0 mg/L and (e) 20.0 mg/L) after incubation for 10 h.

### 3.3. Comparison with other water toxicity analysis methods

The developed biosensor with two different detection assays was compared with several other toxicity detection sensors on essential parameters such as LC, concentration for 50% of maximal effect ($EC_{50}$) or $IC_{50}$, experimental requirements and duration of test (Table 1). LC and $IC_{50}$ for 3, 5-DCP were compared with $EC_{50}$ and $IC_{50}$ obtained from other toxicity sensors. The LC value of 10.0 mg/L for 3, 5-DCP is comparable with the $EC_{50}$ obtained by other biosensors based on respiration inhibition (Micredox, Cellsense, Baroxymeter and Respirometer), and it is a little higher than the $EC_{50}$ from the biosensors based on fluorescent fading (Toxalert, Microtox and Lumistox). However, considering the equipment requirement and time consumption, the assay developed here was more cost-effective for water toxicity detection. In addition, our sensing method may be further optimized by adjusting the culture conditions and integration of other genetic engineering strategies. For example, oxygen concentration and pH played sophisticated roles on the bioelectrochemical activity of exoelectrogens (Liang et al., 2013; Liu et al., 2014), especially oxygen is an important inducer for PYO synthesis (Yong and Zhong, 2009). Thus, optimization of culture conditions (oxygen, pH, etc.) in the future study could be expected to improve PYO production and the biosensor's sensitivity. In addition, engineering PQS quorum sensing system, which greatly enhanced PYO secretion (Wang et al., 2013), could be another promising strategy for biosensor improvement.

## 4. Conclusions

A novel, sensitive and cost-effective water toxicity biosensor based on GM *P. aeruginosa* was reported in this work. The toxicity of 3, 5-DCP was successfully determined by the biosensor with electrochemical (mediator-free) and spectroscopic assays (absorbance assay and colorimetric assay). The calculated $IC_{50}$ was 15.1 mg/L with spectrometric assay, and the toxicity can be visually estimated by naked eyes at a toxicant concentration as low as 10.0 mg/L. In summary, our method offers novel, cheap and sensitive strategies to detect low concentration of toxicants, and shows potential application in determining the water toxicity. This method is also a unique example for toxicity biosensor based on GM *P. aeruginosa*, which could inspire other new ideas for the development of biosensors with genetically engineered bacteria.

**Table 1**
Comparison of LC, $IC_{50}$ or $EC_{50}$ values of 3, 5-DCP obtained with different methods.

| Method and equipment | Microorganism | $IC_{50}/EC_{50}$ | Matrix | Experimental equipment | Duration of test (min) | Reference |
|---|---|---|---|---|---|---|
| Spectroscopic assay | GM *P. aeruginosa* | 15.1 | Distilled water | Yes | † | This work |
| | | 10.0* | | No | † | |
| Colorimetric biosensors | *E. coli* | 12.5* | Groundwater | No | 30 | (Zhai et al., 2013) |
| | | 12.5* | South lake water | No | 30 | |
| Toxalert | *Vibrio fischeri* | 4.42 | | Yes | 5 | (Jennings et al., 2001) |
| Microtox | | 4.09 | | Yes | 5 | |
| Lumistox | | 4.8 | | Yes | 5 | |
| Micredox | *Bacillus subtilis* | 7.5 | Distilled water | Yes | 60 | (Tizzard et al., 2004) |
| | *Pseudomonas putida* | 8.5 | | Yes | 60 | |
| | *E. coli* | 7.0 | | Yes | 60 | |
| CellSense | Activated sludge | 9.8 | Distilled water | Yes | 15–30 | (dos Santos et al., 2002) |
| | *Vibrio fischeri* | 37.5 | | Yes | 15–30 | |
| Baroxymeter | | 10 | | Yes | | # |
| Respirometer | Activated sludge | 12.97 | Synthetic sewage | Yes | 180 | (Gutierrez et al., 2002) |
| | | 5.19 | | Yes | 60 | (Ricco et al., 2004) |

*: LC.
†: incubation for 10 h or so, need no additional reaction time.
#: http://www.baroxymeter.com/index.php?page=dose-responseand-ec50.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2017.05.154.

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