

**Application of bacterial whole cell biosensor to rapid detection of  
cytotoxicity in heavy metal contaminated seawater**

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## Abstract

A toxicity biosensor *Acinetobacter baylyi* Tox2, carrying bioluminescence gene cluster *luxCDABE* on the plasmid pWH1274, was developed and applied to detect cytotoxicity of heavy metal contaminated seawater. The constitutively expressed bioluminescence in *A. baylyi* Tox2 reduces its intensity proportional to the concentrations of toxic compounds. *A. baylyi* Tox2 exhibits tolerance to salinity, hence applicable to seawater samples. *A. baylyi* Tox2 and *Mugilogobius chulae* were exposed to different concentrations of heavy metals ( $\text{Hg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Cd}^{2+}$ ) in artificial seawater and Pearson correlation analysis showed a significant correlation ( $p < 0.01$ ) between *A. baylyi* Tox2 toxicity detection and the fish (*M. chulae*) exposure test. It suggests that the performance of *A. baylyi* Tox2 is comparable to the conventional fish toxicity test using *Mugilogobius chulae* in terms of cytotoxicity detection of metal contaminated seawater. Furthermore, *A. baylyi* Tox2 was applied to evaluate cytotoxicity of seawater samples collected from the vicinity of six sewage discharge outlets in Shandong, Yellow Sea, China. The results indicate that there was a significant correlation between the luminescence inhibition ratio (IR) of *A. baylyi* Tox2 and heavy metal concentrations detected by ICP-MS in the samples. Two seawater samples, which contained a high concentration of total heavy metals exhibited stronger cytotoxicity than samples containing low concentrations of heavy metals. In conclusion, *A. baylyi* Tox2 can be used as an alternative tool to the aquatic animals for the evaluation of the cytotoxicity of heavy metal contamination in the marine environment.

**Keywords:** cytotoxicity; heavy metal; marine; luminescent bacteria test assay;

44    *Mugilogobius chulae* ; *Acinetobacter baylyi*;

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## 1. Introduction

In recent years, increasing pollutants discharged into the sea have led to serious marine contamination (Henry et al., 2016; Rusinol et al., 2014). Heavy metals are amongst the most typical marine pollutants that threaten marine animals' habitats (Kelley et al., 2016) and human health (Mance, 2012). In many cases, heavy metals such as  $\text{Hg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Cd}^{2+}$ , enter water bodies through industrial effluents (Wei and Yang, 2010). There are several direct or potential hazards associated with heavy metals, which not only affect the growth of fish and other aquatic organisms, but also threaten the health and survival of human beings through bioaccumulation (Vinodhini and Narayanan, 2008; Zhang et al., 2007). It is therefore important to assess the impact of heavy metals and a novel toxicity test method is urgently needed. Various methods based on exposure experiments using marine species including fishes, shrimps, copepods, and amphipods have been developed to detect the toxicity of marine pollutants (Campbell et al., 2014; Simpson and Spadaro, 2016). However, these methods are usually time-consuming, costly, and laborious, and can't meet the need for a rapid emergency response especially in the case of accidental marine pollution. Hence, a more rapid, reliable, high throughput, and sensitive method for the evaluation of general toxicity of contaminants in marine environment is currently imperative.

*Acinetobacter baylyi* ADP1 is a non-pathogenic soil bacterium, and its genome has been fully sequenced (Barbe et al., 2004). Remarkably, this bacterium is not only able to utilize a variety of compounds, but also can tolerate a high concentration of salt (Sand et al., 2011). Therefore, the biosensor derived from *A. baylyi* ADP1 is more robust than

that from *E. coli* in terms of viability, maintenance, and storage (Song et al., 2009). Although genotoxicity biosensor based on *A. baylyi* ADP1 has previously been developed to detect contaminated soil and groundwater (Song et al., 2009, Song et al., 2014, Jiang et al., 2015), cytotoxicity biosensor based on *A. baylyi* ADP1 has not been reported yet. In this study, we developed a cytotoxicity biosensor *A. baylyi* Tox2, which constitutively expressed bioluminescence and reduced its intensity in response to toxic compounds.

Aquatic animals such as fish toxicity test have been used as a standard toxicity method (Azmat et al., 2012; Klüver et al., 2015). *Mugilogobius chulae* is a species of small marine fish ( $35.56 \pm 5.97$  mm) found in warm waters (Li et al., 2012). They are widely distributed along the coast of Asia, living in shallow waters such as coastal seas, estuaries, and inland tidal rivers (Rainboth, 1996). Several advantages associated with *M. chulae*, including its wide distribution, salinity and temperature tolerance, and high reproductive capacity, make it a good model animal for water quality bio-monitoring. *M. chulae* has widely been used as a standard toxicity test species (Zhang and Zhu, 2014), and the 96 h-mortality ratio (MR) can be calculated to determine the cytotoxicity of contaminated seawater (Li et al., 2013). Currently, researchers at Guangdong Laboratory Animals Monitoring Institute in China have established the technique for indoor artificial breeding of this species (Cai et al., 2015), and have carried out a series of application tests (Wang et al., 2012b). Hence, the *M. chulae* toxicity test was chosen as a robust standard to evaluate the performance of *A. baylyi* Tox2 biosensor for the detection of cytotoxicity.

In this paper, we established a strong correlation between the biosensor *A. baylyi* Tox2 and the marine fish *M. chulae* in terms of heavy metal cytotoxicity. We also applied *A. baylyi* Tox2 biosensor to rapidly detect cytotoxicity of contaminated seawaters from Yellow sea.

## 2. Materials and Methods

### 2.1. Construction of *A. baylyi* Tox2 biosensor

*A. baylyi* Tox2 is *A. baylyi* ADP1 containing the pWH1274\_lux plasmid. A gene cassette *luxCDABE* was obtained from the plasmid pSB417 (Michael K. Winson et al., 1998) by PCR using the forward primer: 5'-gcggatccATGACTAAAAAATTTTCATTCATATTAACGG-3' and the reverse primer: 5'-gcggatccTCAACTATCAAACGCTTCGGTTAAGCTTAAAGCAC-3' that included *Bam*HI sites (underlined) flanking the *luxCDABE* gene cassette. The plasmid pWH1274 (Genbank accession JN381160.1; construction shown in Fig. S2) (Wang et al., 2012a) and the *luxCDABE* PCR product were both cut by *Bam*HI, ligated together (New England Biolabs, UK) and transferred into competent cells of *E. coli* JM109 (Promega, UK). The resulting plasmid pWH1274\_lux contained promoterless *luxCDABE* under the control of a constitutively promoter. The plasmid pWH1274\_lux was then transferred into *A. baylyi* ADP1 by gene transformation (Wang et al., 2012a) to make *A. baylyi* Tox2.

### 2.2. Chemicals

All chemicals are analytical-grade reagents (Sigma Aldrich, unless otherwise stated). Concentrated nitric acid (GR) was purchased from Merck, Germany. Environmental calibration standard solution containing multi-elements (1000 µg/mL of Ca, Fe, K, Mg, and Na, 10 µg/mL of Ag, Al, As, Ba, Be, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Sb, Se, Th, Tl, U, V, and Zn) and internal standard solution containing Li, Sc, Ge, Y, In, Tb, and Bi (10 mg L<sup>-1</sup>) were both obtained from SPEX CertiPrep Co., America. Liquid argon was 99.999% pure.

### *2.3. Preparation of test solutions containing heavy metals*

Artificial seawater was prepared from commercially available sea-salt (Instant Ocean, America); 35.95 g of sea salt was dissolved in 1 L of sterilized distilled water according to the manufacturer's instruction.

Stock solutions were prepared separately by dissolving 0.1 g of Hg<sup>2+</sup>, Zn<sup>2+</sup>, Cu<sup>2+</sup>, and Cd<sup>2+</sup> (HgCl<sub>2</sub>, ZnSO<sub>4</sub>•7H<sub>2</sub>O, CuSO<sub>4</sub>•5H<sub>2</sub>O, and CdCl<sub>2</sub>•2.5H<sub>2</sub>O) in 100 mL of distilled water. The following range of concentrations of test solutions were prepared by diluting with artificial seawater: Hg<sup>2+</sup> (0.20 mg/L, 0.17 mg/L, 0.14 mg/L, 0.11 mg/L, 0.08 mg/L, 0.05 mg/L, and 0.02 mg/L); Zn<sup>2+</sup> (200.00 mg/L, 150.00 mg/L, 100.00 mg/L, 50.00 mg/L, and 1.00 mg/L); Cu<sup>2+</sup> (1.25 mg/L, 1.00 mg/L, 0.75 mg/L, 0.50 mg/L, 0.25 mg/L, and 0.10 mg/L), and Cd<sup>2+</sup> (100.00 mg/L, 50.00 mg/L, 10.00 mg/L, 5.00 mg/L, 1.00 mg/L, 0.50 mg/L, 0.10 mg/L, and 0.05 mg/L).

### *2.4. Sampling of coastal seawaters containing heavy metals*

Seawater samples containing heavy metals were collected from the vicinity of

sewage discharge outlets in Weifang and Rizhao, China, where the seawater were impacted by sewage discharged from metal production plants or received chronic pollutants input. The sampling procedures were carried out according to standard protocol (The specification for marine monitoring- Part 3: Sample collection, storage and transportation. GB/T 17378.3-2007. <http://www.spsp.gov.cn/page/P428/392.shtml>). The geographical locations and detailed information of sampling sites are shown in Fig.1 and Table S7.

#### 2.5. Bioassays of artificial seawaters containing heavy metals by *A. baylyi* Tox2 and *M. chulae*

A single colony of *A. baylyi* Tox2 was inoculated into 50 mL of LB liquid medium containing 100 µg/mL of ampicillin and grown to stationary phase at 30°C under 150 rpm shaking for 16 h (OD=1.0). The bacterial culture was then transferred to 50 mL of LB medium with an inoculum size of 2% (v/v) and grown under the same conditions as previous for 8 h until the luminous intensity of the bacterial culture reached the maximum value. The culture was centrifuged at 6, 000 rpm for 10 min, and re-suspended in 3% NaCl prior to cytotoxicity testing. 190 µL of the samples (with sterilized artificial seawater as a negative control) and 10 µL of freshly prepared biosensor cells were mixed well in a white 96-well plate, then the luminous intensity was measured with a luminescence detector (SpectraMax i3 Multi-mode detection platform, Molecular Devices, America) at 30 °C. Each treatment was carried out in triplicates, and the luminescence inhibition ratio (IR) after 15 min exposure was calculated as following:



$$154 \quad IR = \frac{L_{NC} - L}{L_{NC}} \times 100\%$$

155 IR: luminescence inhibition ratio (%);  $L_{NC}$ : luminous intensity of the negative controls  
 156 (artificial seawater) (RLU); L: luminous intensity of the samples (RLU).

157 60-day-old individuals of *M. chulae* of similar size ( $14.55 \pm 2.04$  mm) and vitality  
 158 were kept in 200 mL of artificial seawater containing different toxicants. Each treatment  
 159 was performed in triplicates; the dead individuals in the negative control treatment  
 160 should not exceed 10%. The 96 h-mortality ratio (MR) was calculated as following:

$$161 \quad MR = \frac{N_{NC} - N}{N_{NC}} \times 100\%$$

162 MR: mortality ratio of *M. chulae* (%);  $N_{NC}$ : number of live individuals in the negative  
 163 control treatment (artificial seawater); N: number of live individuals in each sample.

## 164 2.6. Chemical analysis and bioassays of the contaminated seawater samples by *A.* 165 *baylyi* Tox2

166 The collected seawater samples were filtered through a  $0.22 \mu\text{m}$  pore-size membrane  
 167 filter, acidified by nitric acid before the measurement of heavy metals by ICP-MS  
 168 (Agilent ICP-MS 7500a, America). A tuning solution was used to tune the ICP-MS to  
 169 obtain the best analysis requirements for double charge, oxide, sensitivity, and  
 170 resolution. The radio frequency power was 1,350 W. The flow rates were  $15.0 \text{ L} \cdot \text{min}^{-1}$   
 171 for the plasma gas,  $1.18 \text{ L} \cdot \text{min}^{-1}$  for the carrier gas, and  $1.0 \text{ L} \cdot \text{min}^{-1}$  for the auxiliary  
 172 gas. The sampling depth was 6.5 mm, the spray chamber temperature was  $2.0^\circ\text{C}$ , the  
 173 sample uptake rate was  $1.00 \text{ mL} \cdot \text{min}^{-1}$ , the acquisition mode was the quantity, the  
 174 integration time was 0.5 s, the dwell time was 30 ms, and the number of replicates was  
 175 3.

Environmental calibration standard solutions with concentrations of 0.0, 10.0, 20.0, and 100.0  $\mu\text{g}\cdot\text{L}^{-1}$  were measured with internal standard solution containing 50.0  $\mu\text{g}\cdot\text{L}^{-1}$  of Li, Sc, Ge, Y, In, Tb, and Bi for calibration. A standard working curve was then established using the ratios of the signal value (CPS) of measured elements/the signal value (CPS) of internal standard elements and the concentrations of measured elements.

The luminous intensity of luminescent bacteria can be significantly affected by the salinity of water (Menz et al., 2013; Tan et al., 2016). To keep consistence, salinity of the field collected seawaters was adjusted to 30 by adding solid sodium chloride to the samples to eliminate its effect on bio-luminescence of *A. baylyi* Tox2 (Table S8). The toxicity test method was performed as mentioned above.

### 3. Results

#### 3.1. Cytotoxicity of four heavy metals determined by *A. baylyi* Tox2

The IR values of the luminescent bacterium *A. baylyi* Tox2 increased with increasing concentrations of  $\text{Hg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Cd}^{2+}$  and exhibited a significant correlation with the following concentrations of  $\text{Hg}^{2+}$  (0.02 mg/L  $\sim$  0.20 mg/L),  $\text{Zn}^{2+}$  (1 mg/L  $\sim$  250 mg/L),  $\text{Cu}^{2+}$  (0.1 mg/L  $\sim$  1.25 mg/L), and  $\text{Cd}^{2+}$  (0.05 mg/L  $\sim$  100 mg/L) (Fig. 2 and Table 1). 0.20 mg/L of  $\text{Hg}^{2+}$  exerted the maximum inhibitory effect on *A. baylyi* Tox2, whilst 250 mg/L of  $\text{Zn}^{2+}$ , 1.25 mg/L of  $\text{Cu}^{2+}$ , and 100 mg/L  $\text{Cd}^{2+}$  produced 92.3%, 79.9%, and 95.7% inhibition respectively. The sensitivity of the *A. baylyi* Tox2 biosensor was higher than previously reported animal tests, with levels as low as 0.02

mg/L of  $\text{Hg}^{2+}$ , 1 mg/L of  $\text{Zn}^{2+}$ , 0.2 mg/L of  $\text{Cu}^{2+}$ , and 0.05 mg/L of  $\text{Cd}^{2+}$  causing inhibition of *A. baylyi* Tox2 bioluminescence (Fig. 2).

### 3.2. Cytotoxicity of four heavy metals determined by *M. chulae*

In the fish exposure experiment carried out here, 0.05 mg/L of  $\text{Hg}^{2+}$  started having a lethal effect on *M. chulae* at 96 h, with 0.20 mg/L of  $\text{Hg}^{2+}$  causing a 96 h-MR of 78.8%. A  $\text{Zn}^{2+}$  concentration of 50 mg/L began to have a lethal effect on *M. chulae* at 96 h, whilst 250 mg/L of  $\text{Zn}^{2+}$  caused 100% MR after 96 h. No fish mortality was observed at less than 0.5 mg/L of  $\text{Cu}^{2+}$  after 96 h with 1.25 mg/L of  $\text{Cu}^{2+}$  only having a limited lethal effect on *M. chulae* (23.3%). 5.0 mg/L of  $\text{Cd}^{2+}$  started having a lethal effect on *M. chulae* at 96 h, with 50 mg/L of  $\text{Cd}^{2+}$  resulting in a 96 h-MR of 100% (Fig. 3).

### 3.3. Correlation of the toxicity results determined by *A. baylyi* Tox2 and *M. chulae*

As shown in Table 1, there was a significant correlation between the luminescent bacteria test assay (LBTA) method using *A. baylyi* Tox2 and the fish exposure method using *M. chulae* in a cytotoxicity test for heavy metals in seawater (the P values were all  $<0.01$ , except that of  $\text{Cu}^{2+}$ ). Therefore, given the advantages of the LBTA method with *A. baylyi* Tox2 in terms of its accuracy and correlation with the fish exposure method, its higher sensitivity, increased speed of detection, lower cost and high sample throughput it can be used to determine the cytotoxicity of heavy metal contaminated seawaters.

### 3.4. Biosensor *A. baylyi* Tox2 used for cytotoxicity of the field collected seawaters

The seawaters were collected from the vicinity of six sewage discharge outlets in

Shandong, China (Fig. 1). C5B072 caused the greatest inhibition of luminescence to *A. baylyi* Tox2, with the intensity dropping to 0 shortly after it was exposed to this sample. C5H075 also caused luminescence inhibition of *A. baylyi* Tox2 (15min-IR=29.5%), with the luminous intensity decreasing sharply to 0 at 1 h. However, the other four samples did not cause any significant inhibition (Fig. 4).

Fig. 4 shows that the toxicity detected by *A. baylyi* Tox2 is in good agreement with the chemical analysis results. C5B072 exhibited the highest cytotoxicity, where the 15min-IR of *A. baylyi* Tox2 was 100%, and chemical analysis revealed that the concentration of total heavy metals in C5B072 was the highest (about 1.69 mg/L) amongst the six seawater samples with Mn (0.95 mg/L) and Cu (0.65 mg/L) predominant (95.1%). C5H075 also exhibited high cytotoxicity, where the 15min-IR of *A. baylyi* Tox2 was 29.5%, and similarly the concentration of total heavy metals in C5H075 was also high with Mn predominant (85.3%) at a concentration of 0.90 mg/L. The concentration of Mn in C5H075 was comparable to that in C5B072, whilst the concentration of Cu in C5H075 (0.09 mg/L) was much lower than that in C5B072 (0.65 mg/L). The corresponding IR of C5H075 was also much lower than that of C5B072, suggesting that Cu might be a key factor exerting cytotoxicity on *A. baylyi* Tox2. The concentration of total heavy metals in C5H077 (0.74 mg/L, including 0.46 mg/L of Mn, 0.14 mg/L of Zn, and 0.11 mg/L of Cu) was much lower than those in C5B072 and C5H075. Therefore, C5H077 exhibited slight cytotoxicity, with the IR of *A. baylyi* Tox2 only being 3.0%, indicating heavy metal mixtures at such concentrations cause limited cytotoxicity to *A. baylyi* Tox2. In addition, C5B073, C5B076, and C5H074 had lower

concentrations of total heavy metals, with Cu being the predominant species in the heavy metal mixtures (0.26 mg/L, 0.08 mg/L, and 0.41 mg/L, respectively). However, the luminescence intensity of *A. baylyi* Tox2 was increased in these three samples.

#### 4. Discussion

Cytotoxicity indicates a general deteriorative effect on enzymes which leads to the inhibition of metabolic activity in cells. In normal condition, the *luxCDABE* in *A. baylyi* Tox2 is constitutively expressed at a high level, showing a strong bioluminescence. When the cells of Tox2 are exposed to toxic compounds, the machinery of gene transcription and protein translation in cells will be generally inhibited due to the cytotoxicity. This leads to less active luciferase (encoded by *luxAB*) and less expression of acyl-CoA reductase (*luxC*), acyl-ACP thioesterase (*luxD*), and acyl-protein synthetase (*luxE*) that produce an aldehyde as a substrate to luciferase for bioluminescence (Meighen, 1991). Hence, the stronger toxicity, the less intensity of bioluminescence.

##### 4.1. Feasibility of LBTA using *A. baylyi* Tox2 to detect the cytotoxicity of heavy metals in seawater samples

*Acinetobacter* sp. is a broadly distributed environmental microorganism, which is suitable for the pollution detection (Song et al., 2009) and *Acinetobacter* bioreporter has been previously applied to the detection of heavy metals in wastewater (Abd El Haleem et al., 2006). In this study, we compared *A. baylyi* Tox2 and fish (*M. chulae*)

exposure experiment to detect cytotoxicity caused by heavy metals in artificial seawater. Detection of cytotoxicity by the LBTA method is mainly based on the interference or inhibition of cell metabolic activity, whilst detection of cytotoxicity by the fish exposure experiment is mainly based on the effects of pollutants on the survival of the fish. Hence, there are some differences between the two methods due to their different modes of action (MoA). Nevertheless, Pearson correlation analysis suggests that both methods (IR of *A. baylyi* Tox2 and 96h-MR of *M. chulae*) exhibited high correlations (Fig. 3 and Table 1).  $\text{Hg}^{2+}$  and  $\text{Cd}^{2+}$  inhibit most organisms and *A. baylyi* Tox2  $\text{EC}_{50}$  values of  $\text{Hg}^{2+}$  (0.12 mg/L) and  $\text{Cd}^{2+}$  (43.67 mg/L) were similar to *M. chulae*  $\text{LC}_{50}$  values of  $\text{Hg}^{2+}$  (0.14 mg/L) and  $\text{Cd}^{2+}$  (39.83 mg/L).

Although zinc and copper are essential trace elements, high concentrations of zinc and copper can cause damage to aquatic organisms (Ebrahimpour et al., 2010). The  $\text{LC}_{50}$  value of  $\text{Zn}^{2+}$  detected by *M. chulae* (107.73 mg/L) was just 1.5 times that of the  $\text{EC}_{50}$  value detected by *A. baylyi* Tox2 (71.81 mg/L), but almost two order of magnitudes higher than that of the  $\text{EC}_{50}$  value detected by *Vibrio fischeri* (3.5 mg/L) (Codina et al., 2000; Fulladosa et al., 2005). Moreover, the  $\text{LC}_{50}$  value of  $\text{Zn}^{2+}$  ( $\text{LC}_{50}$  value was 102.90 mg/L in hard water) derived from another type of fish *Capoeta fusca* (Ebrahimpour et al., 2010) was also comparable to the  $\text{EC}_{50}$  value detected by *A. baylyi* Tox2. This indicated that the cytotoxicity of  $\text{Zn}^{2+}$  determined by the LBTA method using *A. baylyi* Tox2 showed much better agreement with the results of fish exposure experiments than that of LBTA method using *V. fischeri*. So, LBTA based on this new biosensor could be a potential alternative to Microtox test using *V. fischeri* especially

for application in cytotoxicity detection of seawater.

The cytotoxicity of  $\text{Cu}^{2+}$  is very strong, because 0.0167 mg/L of  $\text{Cu}^{2+}$  could inhibit 50% of the larvae of *Chironomus tentans* at first-instar in soft water (Gauss et al., 1985). Besides, 2.0  $\mu\text{g/L}$  dissolved Cu has an cytotoxicity effect on aquatic life, according to US-EPA Draft Aquatic Life Ambient Estuarine/Marine Water Quality Criteria for Copper-2016. In this study, the  $\text{EC}_{50}$  value of  $\text{Cu}^{2+}$  detected by the LBTA method using *A. baylyi* Tox2 was 0.68 mg/L, whilst the  $\text{LC}_{50}$  value of  $\text{Cu}^{2+}$  detected by *M. chulae* could not be obtained because of its low MR. Since the *M. chulae* in this test showed a relatively high tolerance to  $\text{Cu}^{2+}$ , it may not be a good model animal for copper toxicity in seawaters. Therefore, the sensitivity of *A. baylyi* Tox2 was much higher than that of *M. chulae* in the detection of cytotoxicity of  $\text{Cu}^{2+}$ .

In addition, according to the 15min- $\text{EC}_{50}$  values of the four measured heavy metals to *A. baylyi* Tox2, the cytotoxicity was in the order of  $\text{Hg}^{2+} > \text{Cu}^{2+} > \text{Cd}^{2+} > \text{Zn}^{2+}$ . The  $\text{EC}_{50}$  values of  $\text{Hg}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Cd}^{2+}$  obtained by LBTA using *A. baylyi* Tox2 was also comparable to the results detected by LBTA using *V. fischeri* (their 15min- $\text{EC}_{50}$  values were 0.05 mg/L, 3.8 mg/L, and 19 mg/L respectively) (Bitton et al., 1994).

#### 4.2. Correlation between LBTA using *A. baylyi* Tox2 and chemical analysis of the field collected seawaters

In the field study, there was a significant correlation between the results of LBTA using *A. baylyi* Tox2 and the results of chemical analysis of the contaminated seawaters from the vicinity of coastal sewage discharge outlets according to Pearson correlation

analysis (at 0.01 level, 2-tailed).

C5B072 and C5H075 exhibited remarkable cytotoxicity, and chemical analysis showed that the total concentration of heavy metals in these two samples were higher than the others. Mn and Cu might contribute most to the overall toxicity of the samples, considering 1) they were the predominant species of heavy metal in C5B072 and C5H075 (combined proportion 95.1% and 93.9%, respectively). 2) Both Cu and Mn could cause a generalized oxidative stress to fish (Vieira et al., 2012). Nevertheless, the significant inhibition of the field collected seawaters to *A. baylyi* Tox2 might also due to the combined toxicity (Beyer et al., 2014) of all the heavy metals in the samples. In contrast, IR of *A. baylyi* Tox2 exposed to the other three samples (C5B073, C5B076, and C5H074) were less than 0, and that might be ascribed to antagonistic mixture effects (Balistrieri and Mebane, 2014; Tipping and Lofts, 2013).

#### *4.3. Prospect of application of LBTA using A. baylyi Tox2 in marine environment*

To date, most luminescent bacteria have mainly been applied to detect the cytotoxicity of freshwater or sediment samples (Isidori et al., 2005; Jones et al., 2011). However, there are relatively few reports on the application of toxicity biosensors to marine environments. Microtox test using *V. fischeri* showed no relationship with PAH exposure concentrations in Gulf of Mexico seawater, in contrast, the LBTA using Tox2 agreed well with heavy metal concentrations in field collected seawaters (Fig. 4)(Echols et al., 2015). Nevertheless, stimulatory (negative) effects on light production might be a common problem in LBTA method (C5B073, C5B076, and C5H074 in Fig. 4)(Echols et al., 2015).



Although chemical analysis methods have advantages such as accuracy and quantification, it is impossible to evaluate the bioavailability and the biological effect of contaminated seawater from chemical data alone, and it is also time-consuming and cost-ineffective. The LBTA method using *A. baylyi* Tox2 is easy to operate, has a quick response time, is sensitive to various toxicants, comparable to the fish toxicity detection method, has a high-throughput, and is low in cost. Therefore, the LBTA method using Tox2 is promising in the evaluation of the cytotoxicity of contaminated seawater in the marine environment.

## 5. Conclusion

In this study, the genetically engineered luminescent bacterium *A. baylyi* Tox2 was used to detect the cytotoxicity of heavy metal amended artificial seawaters, and the results was verified by a fish exposure experiment using *M. chulae*. Pearson correlation analysis indicated that there was a significant correlation between these two toxicity test methods. Furthermore, the LBTA method using *A. baylyi* Tox2 could be directly related to the chemical analysis results in the cytotoxicity detection of field collected seawaters from the vicinity of sewage discharge outlets in Shandong, China. Therefore, *A. baylyi* Tox2 can be used as a rapid sensitive biosensor to evaluate the cytotoxicity of field collected seawater.

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## **Appendix A. Supplementary data**

Supplementary data related to this article can be found.

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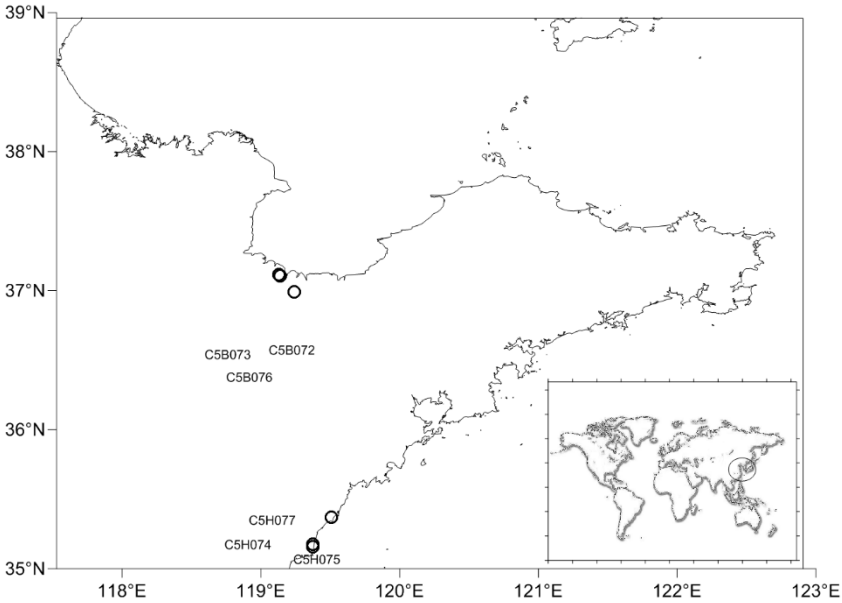
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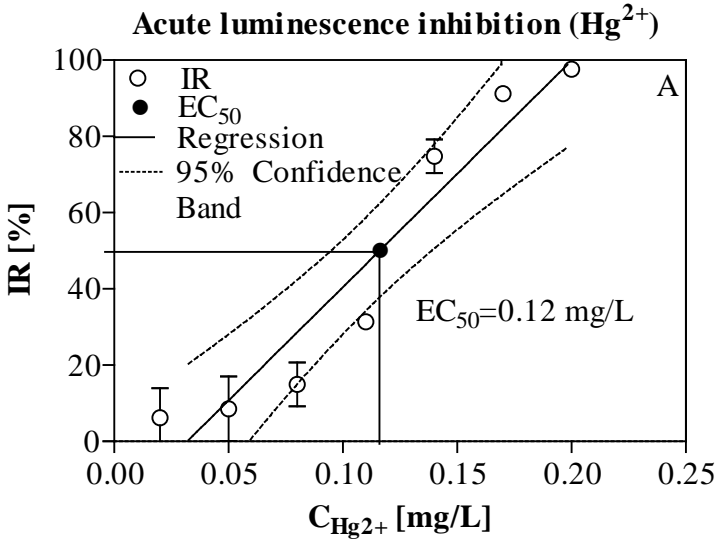
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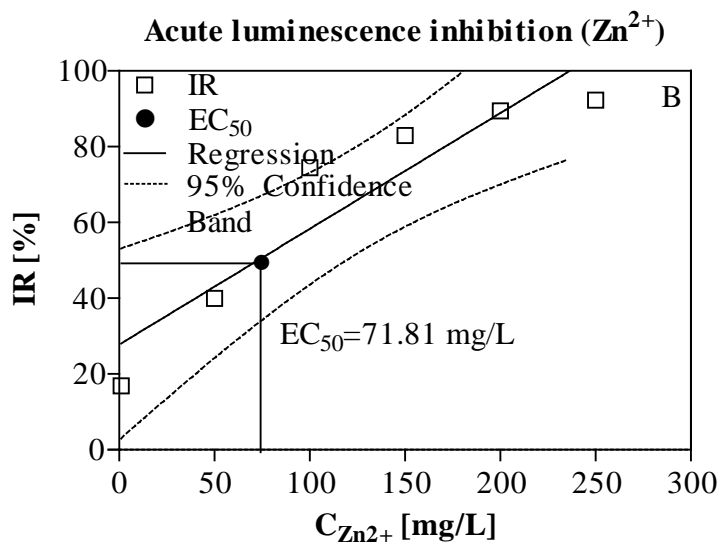
Figures



**Fig. 1.** Geographical locations of the sampling sites for field collected seawaters.

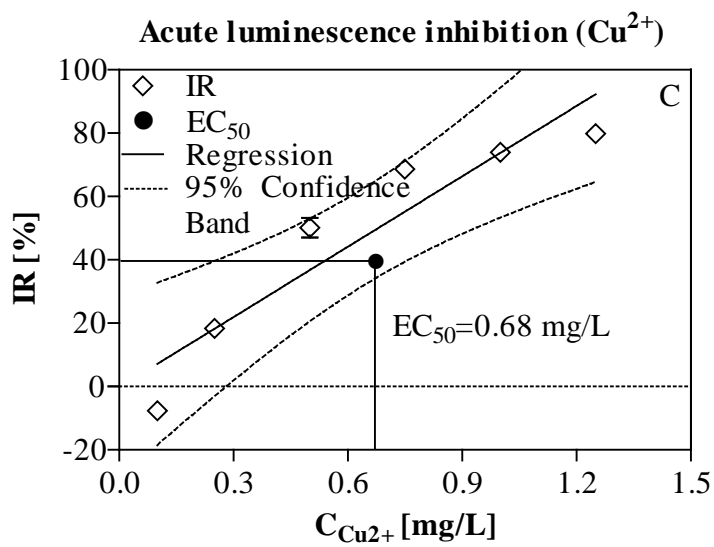




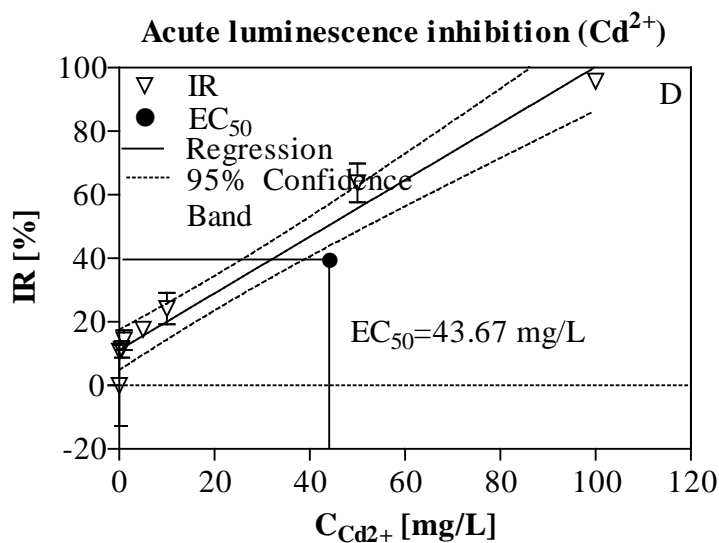


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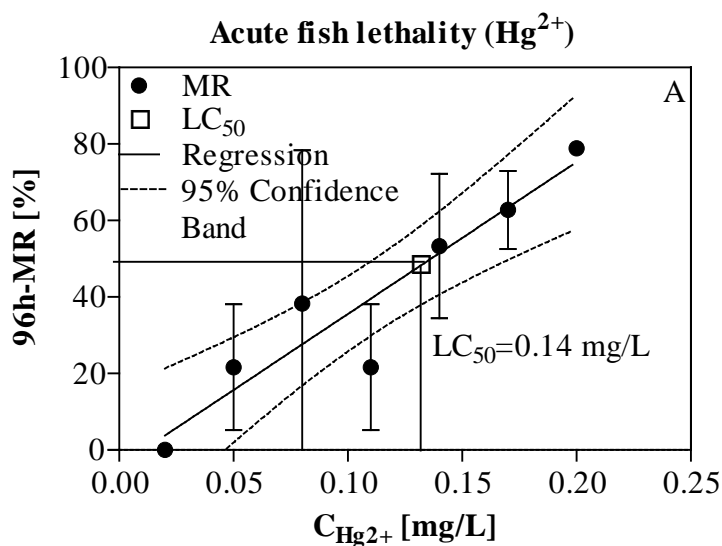
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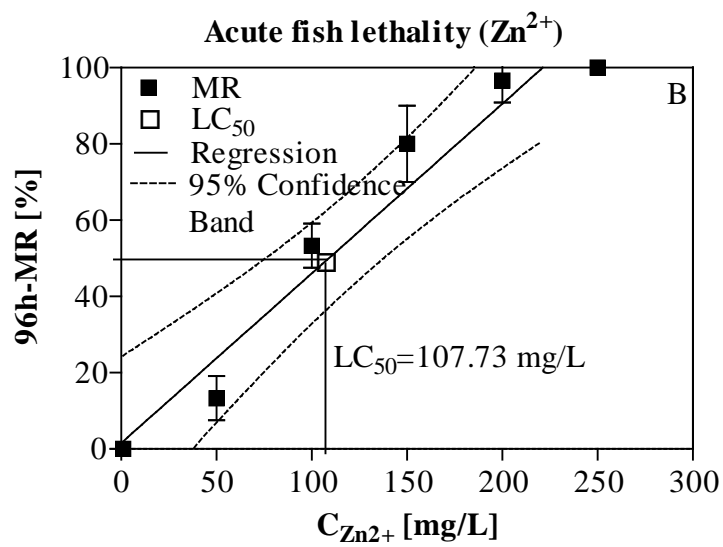


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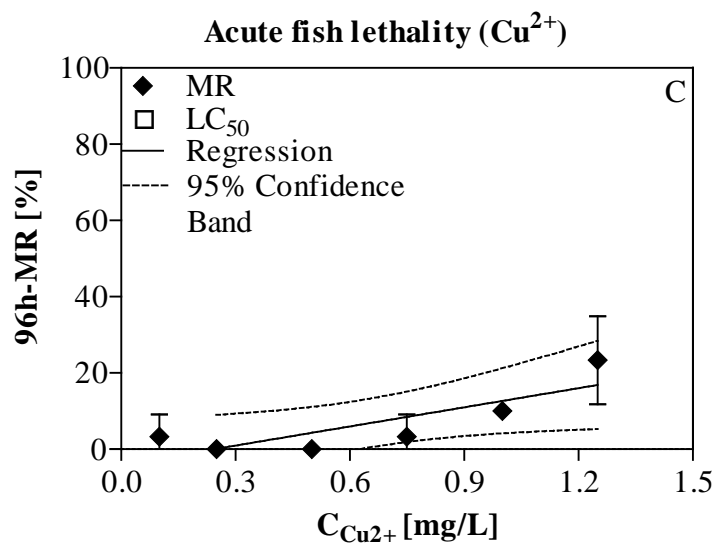


**Fig. 2.** IR of *A. baylyi* Tox2 under different concentrations of  $\text{Hg}^{2+}$  (A),  $\text{Zn}^{2+}$  (B),  $\text{Cu}^{2+}$  (C) and  $\text{Cd}^{2+}$  (D). IR, luminescence inhibition ratio.  $\text{EC}_{50}$ , median effective concentration. Regression=Fitting curve. Each point represents a mean value $\pm$ SD of three replicates.

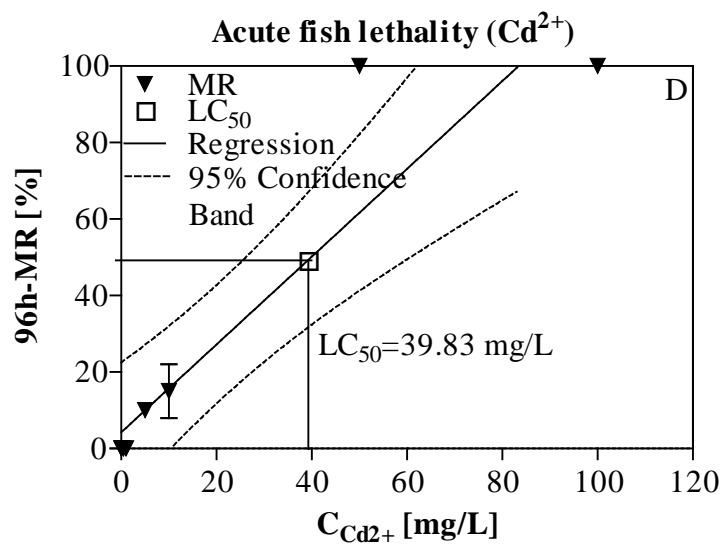




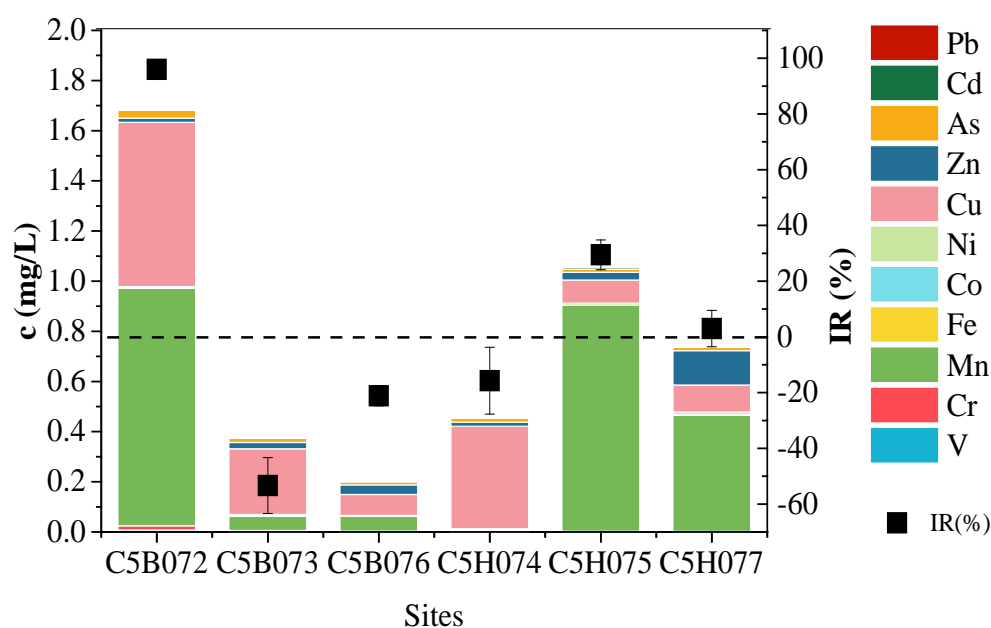
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**Fig. 3.** 96h-MR of *M. chulae* under different concentrations of Hg<sup>2+</sup> (A), Zn<sup>2+</sup> (B), Cu<sup>2+</sup> (C), and Cd<sup>2+</sup> (D). MR, mortality ratio. LC<sub>50</sub>, median of the lethal concentration. Regression=Fitting curve. Each point represents a mean value±SD of three replicates.



**Fig. 4.** The concentrations of heavy metals (bar) in the collected seawater samples, and their corresponding IRs to *A. baylyi* Tox2; Left Y indicates the content (mg/L) of heavy metals in the seawater sample; Right Y axis indicates the IRs of *A. baylyi* Tox2 exposed to the samples. Each point represents a mean value $\pm$ SD of three replicates. Vertical bar denotes a standard error. Dash line indicates where IR equals 0.

516 **Tables**

517 **Table 1**

518 Pearson correlation analysis between LBTA using Tox2 and fish exposure method using *M. chulae* in the cytotoxicity detection of heavy metal  
519 amended seawaters

Toxicants	Test	Regression	$r^2$	P	EC <sub>50</sub> or LC <sub>50</sub>	Pearson Correlation	Sig. (2- tailed)	N
	Species				(95% Confidence Intervals)			
Hg <sup>2+</sup>	Tox2	y=590.4x-18.99	0.92	<0.01	0.12(0.10-0.13)	0.913(**)	0.004	7
	<i>M. chulae</i>	y=397.5x-4.20	0.89	<0.01	0.14(0.09-0.19)			
Zn <sup>2+</sup>	Tox2	y=0.31x+27.74	0.87	<0.01	71.81(70.63-72.99)	0.972(**)	0.001	6
	<i>M. chulae</i>	y=0.45x+1.52	0.94	<0.01	107.73(98.83-116.63)			

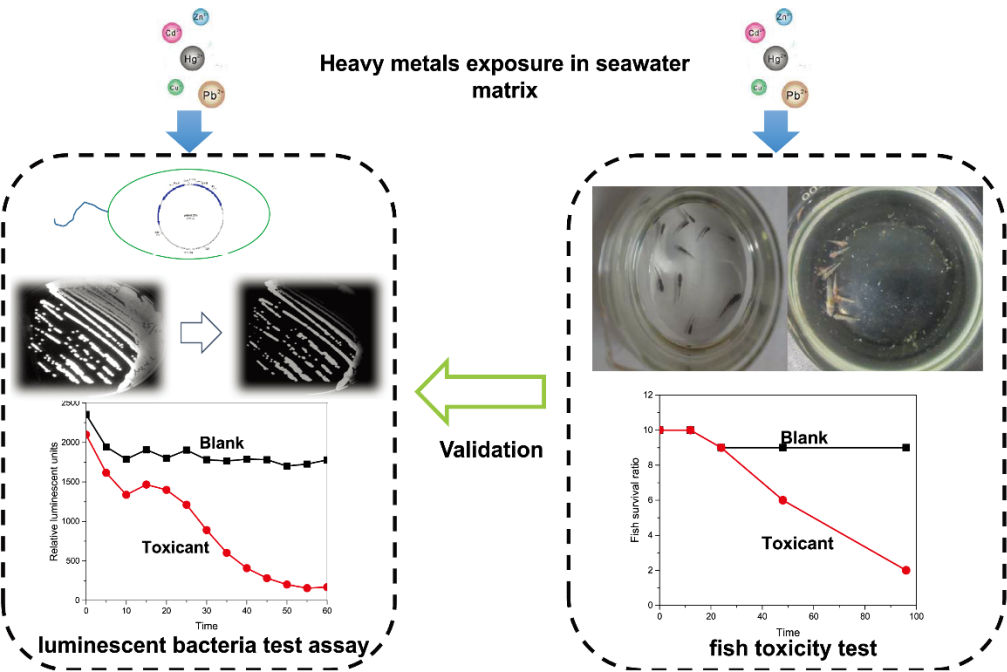
Cu <sup>2+</sup>	Tox2	y=74.04x-0.30	0.88	<0.01	0.68(0.63-0.73)	0.588	0.220	6
	<i>M. chulae</i>	y=16.72x-4.06	0.68	<0.05	/			
Cd <sup>2+</sup>	Tox2	y=0.89x+11.13	0.97	<0.01	43.67(37.05-50.29)	0.956(**)	0.000	8
	<i>M. chulae</i>	y=1.15x+4.20	0.86	<0.01	39.83(38.60-41.06)			

520 / indicates the corresponding EC<sub>50</sub> or LC<sub>50</sub> could not be calculated. \*\* indicates that correlation is significant at the 0.01 level (2-tailed). N:

521 the degree of freedom

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523      Graphical Abstracts



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