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Application of a bacterial whole cell biosensor for the rapid detection of cytotoxicity in heavy metal contaminated seawater

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Abstract

A toxicity biosensor *Acinetobacter baylyi* Tox2 was constructed with the host strain *A. baylyi* ADP1 harboring a new and high-copy-number plasmid pWH1274_lux, and was applied to detect the cytotoxicity of heavy metal contaminated seawater. The cassette *luxCDABE* was site-specifically inserted after and controlled by constitutively expressed promoter *Ptet* on pWH1274_lux so the bioluminescence intensity of the biosensor reduces in proportional to the concentrations of toxic compounds exposed. *A. baylyi* Tox2 exhibits tolerance to salinity, hence is applicable to seawater samples. *A. baylyi* Tox2 and *Mugilogobius chulae* were exposed to different concentrations of heavy metals (Hg^{2+}, Zn^{2+}, Cu^{2+}, and 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. This suggests that the performance of *A. baylyi* Tox2 is comparable to the conventional fish toxicity test in terms of cytotoxicity detection of metal contaminated seawater. Furthermore, *A. baylyi* Tox2 was used to evaluate cytotoxicity of field-collected seawater samples. 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 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; *Mugilogobius chulae* ; *Acinetobacter baylyi*;

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, 1987). In many cases, heavy metals such as Hg^{2+}, Zn^{2+}, Cu^{2+}, and 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, making it potentially a good model strain for molecular cloning (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). Metabolic versatility, easy cloning, and robustness to environmental change make *A. baylyi* ADP1 ideal host for biosensor construction (Kathryn et al., 2011). A genotoxicity biosensor based on *A. baylyi* ADP1 has previously been developed to detect the genotoxins in contaminated soil and groundwater (Song et al., 2009, Song et al., 2014, Jiang et al., 2015), and has been reported more robust than genotoxicity biosensors derived from *E. coli* in terms of viability, maintenance, and storage (Song et al., 2009). The *A. baylyi* ADP1 derived genotoxicity biosensor was also proved to be functional in marine environment (Zhang et al., 2013). 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, 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±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 the *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 used the *A. baylyi* Tox2 biosensor to rapidly detect the cytotoxicity of contaminated seawaters from the 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'-gcggatccATGACTAAAAAAATTTCATTCATATTAACGG-3' and the reverse primer: 5'-gcggatccTCAACTATCAAACGCTTCGGTTAAGCTTAAAGCAC-3' that included *BamHI* 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 *BamHI*, ligated together (New England Biolabs, UK) and transferred into competent cells of *E. coli* JM109 (Promega, UK). The resulting plasmid pWH1274_lux contained promoterless

112 *luxCDABE* under the control of a constitutively expressed promoter. The plasmid
113 pWH1274_lux was then transferred into *A. baylyi* ADP1 by gene transformation
114 (Wang et al., 2012a) to make *A. baylyi* Tox2.

115 *2.2. Chemicals*

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

123 *2.3. Preparation of test solutions containing heavy metals*

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

127 Stock solutions were prepared separately by dissolving 0.1 g of Hg^{2+}, Zn^{2+}, Cu^{2+},
128 and Cd^{2+} ($HgCl_2$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, and $CdCl_2 \cdot 2.5H_2O$) in 100 mL of
129 distilled water. The following range of concentrations of test solutions were prepared
130 by diluting with artificial seawater: Hg^{2+} (0.20 mg/L, 0.17 mg/L, 0.14 mg/L, 0.11
131 mg/L, 0.08 mg/L, 0.05 mg/L, and 0.02 mg/L); Zn^{2+} (200.00 mg/L, 150.00 mg/L,
132 100.00 mg/L, 50.00 mg/L, and 1.00 mg/L); Cu^{2+} (1.25 mg/L, 1.00 mg/L, 0.75 mg/L,

133 0.50 mg/L, 0.25 mg/L, and 0.10 mg/L), and Cd^{2+} (100.00 mg/L, 50.00 mg/L, 10.00
134 mg/L, 5.00 mg/L, 1.00 mg/L, 0.50 mg/L, 0.10 mg/L, and 0.05 mg/L).

135 *2.4. Sampling of coastal seawaters containing heavy metals*

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

144 *2.5. Bioassays of artificial seawaters containing heavy metals by A. baylyi Tox2 and*
145 *M. chulae*

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

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

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

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

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

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

2.6. Chemical analysis and bioassays of the contaminated seawater samples by A. baylyi Tox2

The collected seawater samples were filtered through a 0.22 μm pore-size membrane filter, acidified by nitric acid before the measurement of heavy metals by

ICP-MS (Agilent ICP-MS 7500a, America). A tuning solution was used to tune the ICP-MS to obtain the best analysis requirements for double charge, oxide, sensitivity, and resolution. The radio frequency power was 1, 350 W. The flow rates were 15.0 $L \cdot min^{-1}$ for the plasma gas, 1.18 $L \cdot min^{-1}$ for the carrier gas, and 1.0 $L \cdot min^{-1}$ for the auxiliary gas. The sampling depth was 6.5 mm, the spray chamber temperature was 2.0 °C, the sample uptake rate was 1.00 $mL \cdot min^{-1}$, the acquisition mode was the quantity, the integration time was 0.5 s, the dwell time was 30 ms, and the number of replicates was 3.

Environmental calibration standard solutions with concentrations of 0.0, 10.0, 20.0, and 100.0 $\mu g \cdot L^{-1}$ were measured with internal standard solution containing 50.0 $\mu g \cdot L^{-1}$ 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 maintain consistency, the salinity of the field collected seawaters was adjusted to 30 by adding solid sodium chloride to the samples to eliminate its effect on the 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 Hg^{2+}, Zn^{2+}, Cu^{2+}, and Cd^{2+} and exhibited a significant correlation with the following concentrations of Hg^{2+}(0.02 mg/L～0.20 mg/L), Zn^{2+} (1 mg/L～250 mg/L), Cu^{2+} (0.1 mg/L～1.25 mg/L), and Cd^{2+} (0.05 mg/L～100 mg/L) (Fig. 2 and Table 1). 0.20 mg/L of Hg^{2+} exerted the maximum inhibitory effect on *A. baylyi* Tox2, whilst 250 mg/L of Zn^{2+}, 1.25 mg/L of Cu^{2+}, and 100 mg/L Cd^{2+} produced 92.3%, 79.9%, and 95.7% inhibition respectively. The sensitivity of the *A. baylyi* Tox2 biosensor was higher than that in previously reported animal tests, with levels as low as 0.02 mg/L of Hg^{2+}, 1 mg/L of Zn^{2+}, 0.2 mg/L of Cu^{2+}, and 0.05 mg/L of 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 Hg^{2+} started having a lethal effect on *M. chulae* at 96 h, with 0.20 mg/L of Hg^{2+} causing a 96 h-MR of 78.8%. A Zn^{2+} concentration of 50 mg/L began to have a lethal effect on *M. chulae* at 96 h, whilst 250 mg/L of Zn^{2+} caused 100% MR after 96 h. No fish mortality was observed at less than 0.5 mg/L of Cu^{2+} after 96 h with 1.25 mg/L of Cu^{2+} only having a limited lethal effect on *M. chulae* (23.3%). 5.0 mg/L of Cd^{2+} started having a lethal effect on *M. chulae* at 96 h, with 50 mg/L of 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 an cytotoxicity test for heavy metals in seawater (the P values were all <0.01, except that of 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

241 concentration of Cu in C5H075 (0.09 mg/L) was much lower than that in C5B072
242 (0.65 mg/L). The corresponding IR of C5H075 was also much lower than that of
243 C5B072, suggesting that Cu might be a key factor exerting cytotoxicity on *A. baylyi*
244 Tox2. The concentration of total heavy metals in C5H077 (0.74 mg/L, including 0.46
245 mg/L of Mn, 0.14 mg/L of Zn, and 0.11 mg/L of Cu) was much lower than those in
246 C5B072 and C5H075. Therefore, C5H077 exhibited slight cytotoxicity, with the IR of
247 *A. baylyi* Tox2 only being 3.0%, indicating heavy metal mixtures at such
248 concentrations cause limited cytotoxicity to *A. baylyi* Tox2. In addition, C5B073,
249 C5B076, and C5H074 had lower concentrations of total heavy metals, with Cu being
250 the predominant species in the heavy metal mixtures (0.26 mg/L, 0.08 mg/L, and 0.41
251 mg/L, respectively). However, the luminescence intensity of *A. baylyi* Tox2 was
252 increased in these three samples.

253

254 **4. Discussion**

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

263 luciferase for bioluminescence (Meighen, 1991). Hence, the stronger the toxicity, the
264 lower the intensity of bioluminescence.

265 *4.1. Comparison of A. baylyi Tox2 with other Acinetobacter cytotoxicity bioreporters*

266 *Acinetobacter* sp. is a broadly distributed environmental microorganism, unlike the
267 popular biosensor host of *E. coli* which is a gut micbrobe, *Acinetobacter* hosting
268 biosensors are suitable for the detection of environment samples containing organic
269 and inorganic pollutants in a more environment related context (Song et al., 2009).
270 Another bioreporter *A.* sp. DF4/PUTK2 had previously been used in the detection of
271 heavy metals in wastewater (Desouky, A.E.H., et al., 2006). As both being
272 cytotoxicity bioreporters developed on the chasis of *Acinetobacter* sp., *A. baylyi* Tox2
273 and *A.* sp. DF4/PUTK2 share similarities but also exhibit differences. The major
274 differences between the two are : 1) the plasmid pUTK2 in strain DF4 was created by
275 conjugal mating of *Alcaligenes eutrophus* A5 carrying plasmid pSS50 with *E. coli*
276 HB101 harboring the *lux* transposon Tn4431 (Abd-El-Haleem et al., 2006). Hence,
277 the cassette *luxCDABE* was randomly inserted in pSS50, and the promoter controlling
278 the inserted *luxCDABE* was random and might not be a strong one. The plasmid
279 pSS50 mediates the mineralization of 4-chlorobiphenyl (Hooper et al., 1989), which
280 gives strain DF4 an extra catabolic burden when only the expression of luminescence
281 is required. In addition, the plasmid pSS50 displays some homology with the mercury
282 resistance transposon (Burlage et al., 1990), so it might be problematic with sensing
283 Hg toxicity. In contrast, the toxicity biosensor Tox2 harbors a new and
284 high-copy-number plasmid pWH1274_lux, with the cassette *luxCDABE*

285 site-specifically inserted after and controlled by *Ptet* which gives it stable and
286 constitutive expression. Besides, the backbone of pWH1274 doesn't encode any
287 protein related to mineralization of 4-chlorobiphenyl or heavy metal resistance. 2) the
288 feasibility of strain DF4/PUTK2 was not verified by any other toxicity method. In
289 contrast, a good correlation between Tox2 detection and traditional fish toxicity test
290 has been established in this study, which will help to translate biosensor detection data
291 to traditional animal toxicity data. 3) strain DF4/PUTK2 works under a salinity of 10.
292 In contrast, the strain Tox2 has been applied to seawater, demonstrating that the
293 sensing is functional in seawater and salty environment. In addition, comparison of
294 the EC_{50} of Cu, Zn, and Cd (for DF4/PUTK2: ~20 mg/L, NA, and NA; for Tox2: 0.68
295 mg/L, 71.81 mg/L, and 43.67 mg/L) between the two biosensors (Table S9) also
296 indicates that strain Tox2 is much more sensitive to the heavy metals tested than strain
297 DF4/PUTK2.

298 *4.2. Feasibility of LBTA using A. baylyi Tox2 to detect the cytotoxicity of heavy metals*
299 *in seawater samples*

300 In this study, we compared *A. baylyi* Tox2 and a fish (*M. chulae*) exposure
301 experiment to detect cytotoxicity caused by heavy metals in artificial seawater.
302 Detection of cytotoxicity by the LBTA method is mainly based on the interference or
303 inhibition of cell metabolic activity, whilst detection of cytotoxicity by the fish
304 exposure experiment is mainly based on the effects of pollutants on the survival of the
305 fish. Hence, there are some differences between the two methods due to their different
306 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). Hg^{2+} and Cd^{2+} inhibit most organisms and *A. baylyi* Tox2 EC_{50} values of Hg^{2+} (0.12 mg/L) and Cd^{2+} (43.67 mg/L) were similare to *M. chulae* LC_{50} values of Hg^{2+} (0.14 mg/L) and 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 LC_{50} value of Zn^{2+} detected by *M. chulae* (107.73 mg/L) was just 1.5 times that of the EC_{50} value detected by *A. baylyi* Tox2 (71.81 mg/L), but almost two order of magnitudes higher than that of the EC_{50} value detected by *Vibrio fischeri* (3.5 mg/L) (Codina et al., 2000; Fulladosa et al., 2005). Moreover, the LC_{50} value of Zn^{2+} (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 EC_{50} value detected by *A. baylyi* Tox2. This indicated that the cytotoxicity of 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 the LBTA method using *V. fischeri*. Hence, LBTA based on this new biosensor could be a potential alternative to the Microtox test using *V. fischeri* especially when applied to cytotoxicity detection of seawater.

The cytotoxicity of Cu^{2+} is very strong, because 0.0167 mg/L of Cu^{2+} could inhibit 50% of the larvae of *Chironomus tentans* at first-instar in soft water (Gauss et al., 1985). Besides, 2.0 μg/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 EC_{50} value of Cu^{2+} detected by the LBTA

329 method using *A. baylyi* Tox2 was 0.68 mg/L, whilst the LC_{50} value of Cu^{2+} detected
330 by *M. chulae* could not be obtained because of its low MR. Since the *M. chulae* in this
331 test showed a relatively high tolerance to Cu^{2+}, it may not be a good model animal for
332 copper toxicity in seawaters. Therefore, the sensitivity of *A. baylyi* Tox2 was much
333 higher than that of *M. chulae* in the detection of cytotoxicity of Cu^{2+}.

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

339 *4.3. Correlation between LBTA using A. baylyi Tox2 and chemical analysis of the field*
340 *collected seawaters*

341 The cytotoxicity of a field collected sample is the summed adversely biological
342 impact derived from all the pollutants in the sample. Therefore, it's always difficult to
343 ascribe the toxicity to one or several specific components amongst all the pollutants in
344 the sample. The cocktail effect make this even more complex. In the field study, there
345 was a significant correlation between the toxicity of the field collected samples and
346 the total concentration of heavy metals according to Pearson correlation analysis (at
347 0.01 level, 2-tailed) (Fig. 4 and Table S10).

348 C5B072 and C5H075 exhibited remarkable cytotoxicity, and chemical analysis
349 showed that the total concentration of heavy metals in these two samples were higher

than the others. Moreover, Pearson analysis between chemical analysis and biosensor data showed that the toxicity was most likely derived from Mn, Fe, and Pb (Table S10). Therefore, the significant inhibition of the field collected seawaters to *A. baylyi* Tox2 might due to the combined toxicity (Beyer et al., 2014) of all the heavy metals in these two samples. In contrast, IR of *A. baylyi* Tox2 exposed to the other three samples (C5B073, C5B076, and C5H074) were less than 0 which might be ascribed to antagonistic mixture effects (Balistrieri and Mebane, 2014; Tipping and Lofts, 2013).

4.4. Prospects for the application of LBTA using A. baylyi Tox2 in a 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. The Microtox test using *V. fischeri* showed no relationship with PAH exposure concentrations in Gulf of Mexico seawater whilst, 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 methods (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, a salt-tolerant bioreporter *A. baylyi* Tox2, harboring a new and high-copy-number plasmid pWH1274_lux, 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 and sensitive biosensor to evaluate the cytotoxicity of field collected seawater.

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395

396 **Appendix A. Supplementary data**

397 Supplementary data related to this article can be found here.

398

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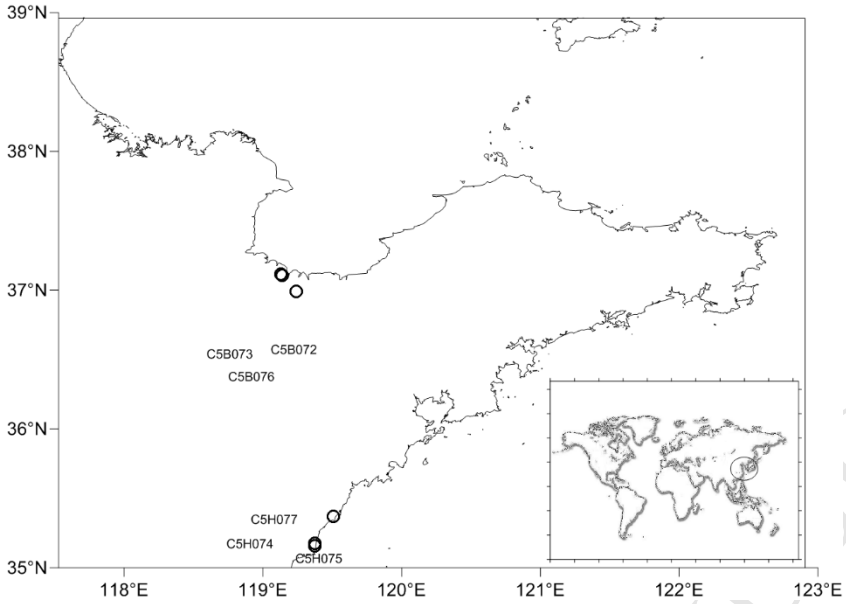
531 **Figures**



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

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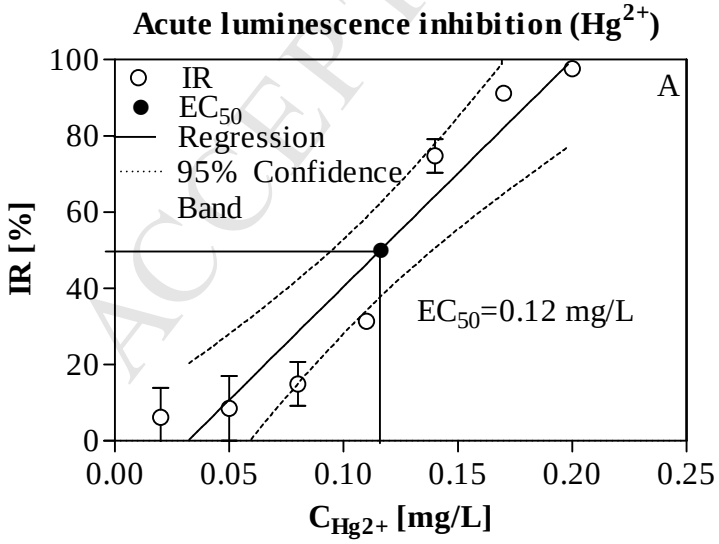

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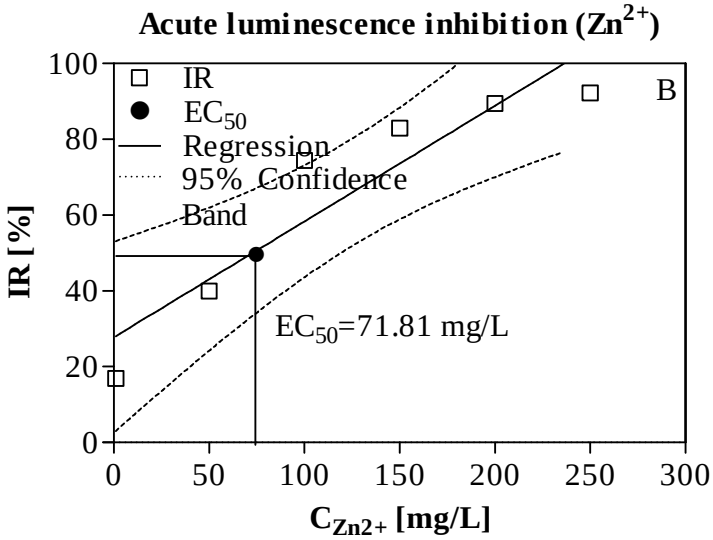

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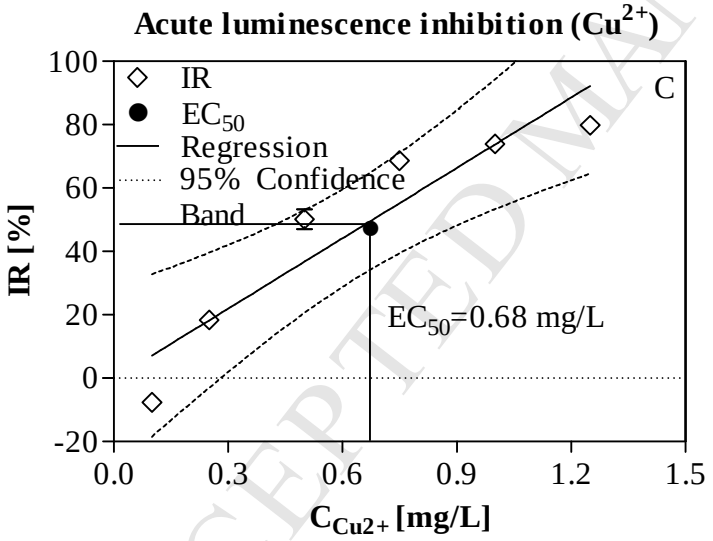

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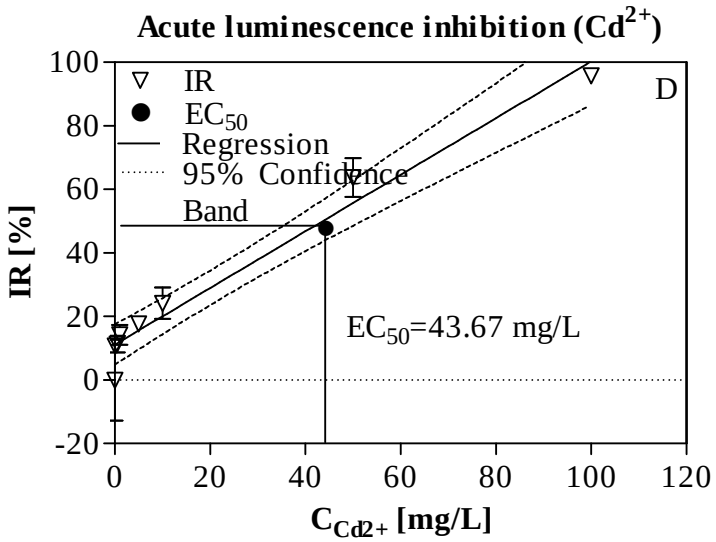

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542 **Fig. 2.** IR of *A. baylyi* Tox2 under different concentrations of Hg^{2+} (A), Zn^{2+} (B), Cu^{2+}
543 (C) and Cd^{2+} (D). IR, luminescence inhibition ratio. EC_{50}, median effective
544 concentration. Regression=Fitting curve. Each point represents a mean value±SD of
545 three replicates.

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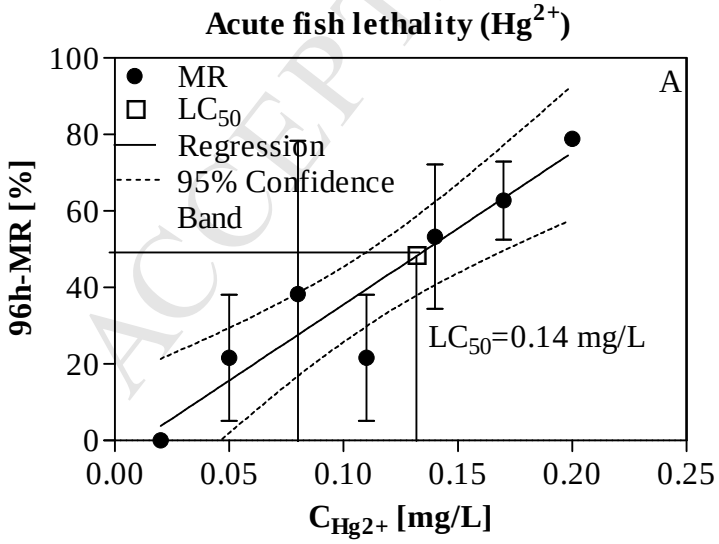

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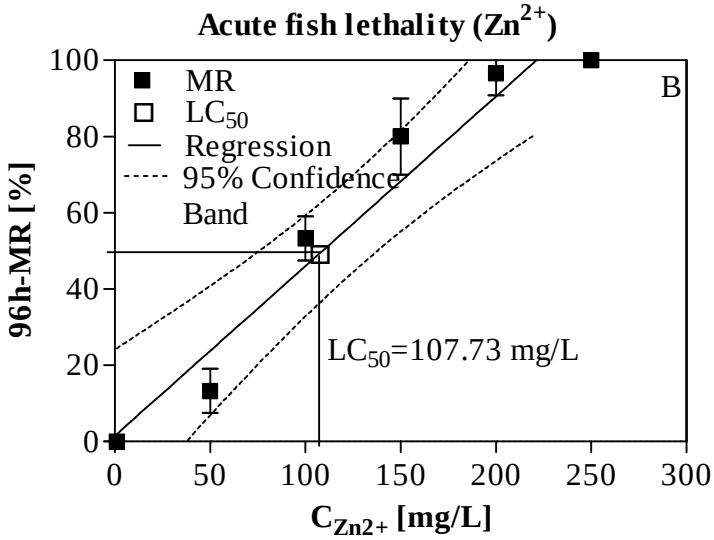

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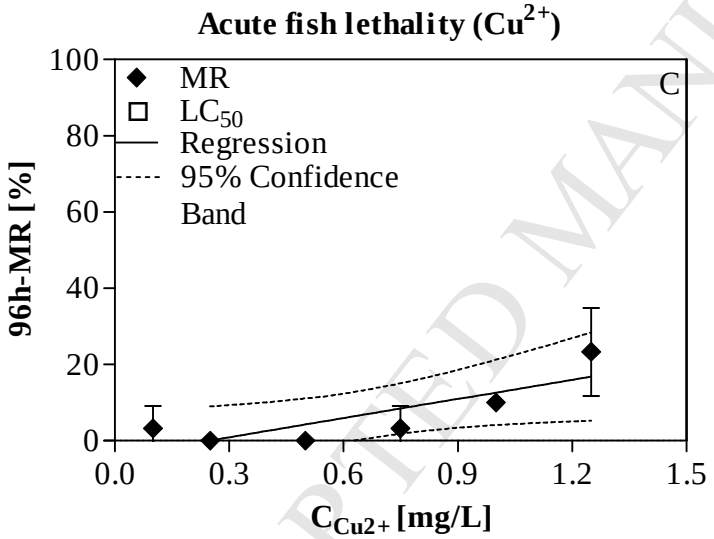

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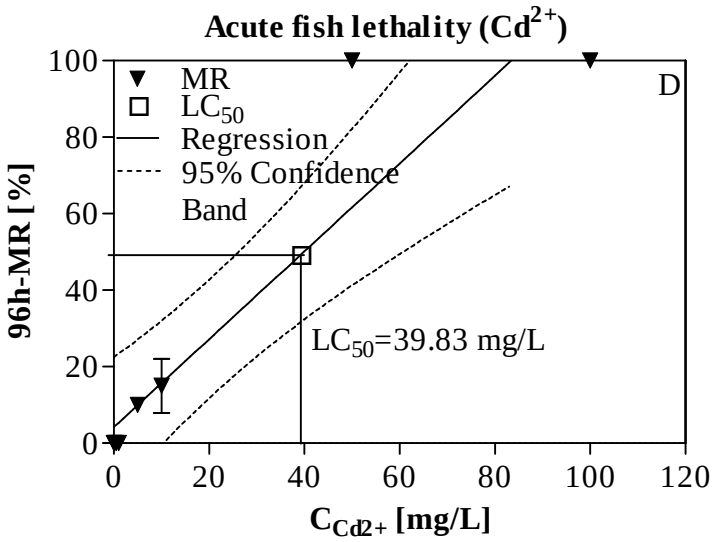

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551 **Fig. 3.** 96h-MR of *M. chulae* under different concentrations of Hg^{2+} (A), Zn^{2+} (B),

552 Cu^{2+} (C), and Cd^{2+} (D). MR, mortality ratio. LC_{50}, median of the lethal concentration.

553 Regression=Fitting curve. Each point represents a mean value±SD of three replicates.

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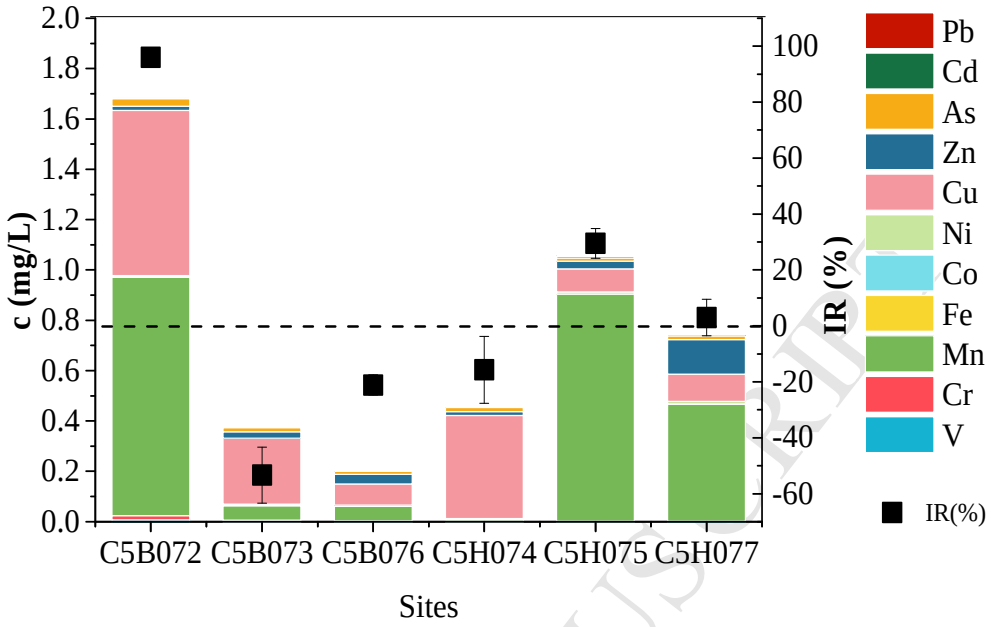

Fig. 4. The concentrations of heavy metals (bar) in the collected seawater samples, and their corresponding IRs to *A. baylyi* Tox2; Left Y indicates the content (mg/L) of heavy metals in the seawater sample; Right Y axis indicates the IRs of *A. baylyi* Tox2 exposed to the samples. Each point represents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

568 **Tables**

569 **Table 1**

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

571 amended seawaters

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

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

572 / indicates the corresponding EC_{50} or LC_{50} could not be calculated. ** indicates that correlation is significant at the 0.01 level (2-tailed). N:

573 the degree of freedom

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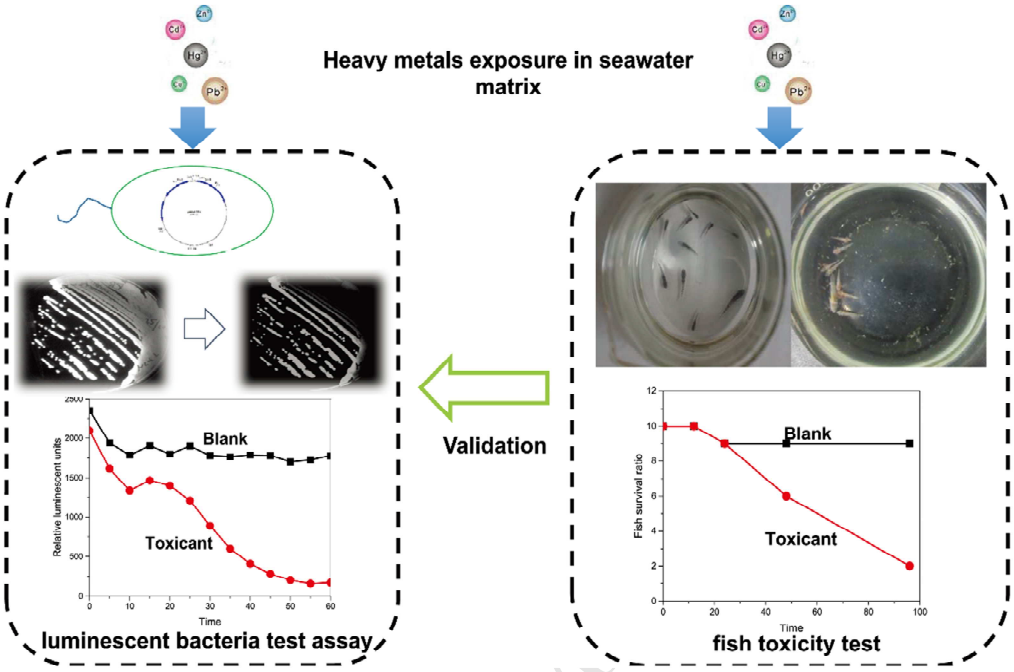
575 Graphical Abstracts



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Highlights

- a biosensor *Acinetobacter baylyi* Tox2 was developed for rapid toxicity detection
- The biosensor performs well in artificial seawater amended by heavy metals
- The toxicity by LBTA is in good agreement with that of the animal toxicity test
- We applied *A. baylyi* Tox2 to detect the toxicity of *in situ* contaminated seawaters
- The toxicity by LBTA is in good agreement with the chemical analysis