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1 **A whole-cell bioreporter assay for quantitative genotoxicity**  
2 **evaluation of environmental samples**

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23 **Abstract**

24 Whole-cell bioreporters have emerged as promising tools for genotoxicity evaluation,  
25 due to their rapidity, cost-effectiveness, sensitivity and selectivity. In this study, a  
26 method for detecting genotoxicity in environmental samples was developed using the  
27 bioluminescent whole-cell bioreporter *Escherichia coli recA::luxCDABE*. To further  
28 test its performance in a real world scenario, the *E. coli* bioreporter was applied in two  
29 cases: i) soil samples collected from chromium(VI) contaminated sites; ii) crude oil  
30 contaminated seawater collected after the Jiaozhou Bay oil spill which occurred in  
31 2013. The chromium(VI) contaminated soils were pretreated by water extraction, and  
32 directly exposed to the bioreporter in two phases: aqueous soil extraction (water phase)  
33 and soil supernatant (solid phase). The results indicated that both extractable and soil  
34 particle fixed chromium(VI) were bioavailable to the bioreporter, and the solid-phase  
35 contact bioreporter assay provided a more precise evaluation of soil genotoxicity. For  
36 crude oil contaminated seawater, the response of the bioreporter clearly illustrated the  
37 spatial and time change in genotoxicity surrounding the spill site, suggesting that the  
38 crude oil degradation process decreased the genotoxic risk to ecosystem. In addition,  
39 the performance of the bioreporter was simulated by a modified cross-regulation gene  
40 expression model, which quantitatively described the DNA damage response of the *E.*  
41 *coli* bioreporter. Accordingly, the bioluminescent response of the bioreporter was  
42 calculated as the mitomycin C equivalent, enabling quantitative comparison of  
43 genotoxicities between different environmental samples. This bioreporter assay  
44 provides a rapid and sensitive screening tool for direct genotoxicity assessment of

45 environmental samples.

46 **Keywords:** Genotoxicity; Seawater; Soil; Simulation; SOS model; Whole-cell

47 bioreporter

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## 67 **1 Introduction**

68 As many anthropogenic contaminants are released into the environment, genotoxins  
69 are of great concern as they are potentially dangerous to the natural environment and  
70 human health (Shin, 2010). Chemical analysis can only quantify the total amount of  
71 chemicals within the samples, however suffering from high cost, and time-consuming  
72 and laborious operation. Moreover, chemical analysis does not directly provide  
73 integrated genotoxic effects or information on the bioavailability of various  
74 contaminants in complex environmental media (Shin et al., 2005; Jiang et al., 2016).  
75 A microbial whole-cell bioreporter typically combines a promoter–operator region in  
76 a bacteria host, which acts as the sensing device, with a reporter gene encoding for an  
77 easily detectable protein (Robbens et al., 2010). The unique feature of ‘whole-cell’ is  
78 that living microbial cells are used to obtain the bioavailable effects of a stimulus (Gu  
79 et al., 2004). Without the need of precise chemical characterization, whole-cell  
80 bioreporters are compact, portable, cost-effective and simple to use, providing an  
81 alternative approach for evaluating the general impacts of individual or mixed  
82 chemicals (Vollmer and Dyk, 2004; Nagata et al., 2010). Normally, whole-cell  
83 bioreporters are classified into two categories. One is responsive to specific toxicity  
84 pathways and induced in the presence of specific compounds or their analogues with  
85 similar structure, such as alkanes (e.g., alkane degradation pathway) (Sticher et al.,  
86 1997; Wang et al., 2016), naphthalene (e.g., naphthalene degradation pathway)  
87 (Neilson et al., 1999), polycyclic aromatic hydrocarbons (e.g., phenanthrene  
88 mineralization) (Tecon et al., 2009) and mercury (mercuric resistant regulatory

89 pathway) (Rasmussen et al., 2000). The others can be induced by general toxicity  
90 pathways, including stressful conditions such as DNA damage (Vollmer et al., 1997;  
91 Min et al., 1999; Biran et al., 2009), membrane damage (Bechor et al., 2002) and  
92 oxidative damage (Lee and Gu, 2003). Bacterial SOS response is a global response  
93 to DNA damage in which the cell cycle is arrested and DNA repair and mutagenesis  
94 are induced (Radman and Prakash, 1973; Little and Mount, 1982). RecA is essential  
95 in the SOS response of *Escherichia coli*, responsible for DNA repair/maintenance via  
96 homologous recombination (Horii et al., 1980). Therefore, the *recA*-based whole-cell  
97 bioreporters are widely used for measuring general toxicity, capable of detecting not  
98 only the levels but also mechanisms of DNA damage (Sørensen et al., 2006; Ron,  
99 2007), including DNA cross-linking and delayed DNA synthesis, alkylation and  
100 hydroxylation of DNA (Min and Gu, 2003; Chen et al., 2008). As most genotoxins are  
101 inducers of the SOS response (Quillardet et al., 1982), the *recA*-based bioreporter  
102 assay is introduced in genotoxicity assessment of environmental samples.

103 The use of living microorganisms as the sensing elements of a whole-cell  
104 bioreporter has several advantages over other assays such as enzymes, antibodies, or  
105 sub-cellular components based tests (Shin, 2010). Firstly, microorganisms can be  
106 genetically modified using mature protocols and are easily prepared by simple  
107 cultivation in relatively inexpensive media (Yu et al., 2006; Yagi, 2007). Secondly, a  
108 correlation between genotoxicity as measured by microbial bioassays and  
109 carcinogenicity in mammals has been found (Josephy et al., 1997), indicating  
110 whole-cell reporters can help in diagnosing the health risks of genotoxins to some

111 extent. However, the microbial bioassay still suffers from a lack of eukaryotic  
112 metabolic enzyme systems (Lah et al., 2007), leading to uncertainties in extrapolating  
113 the genotoxic potency of one chemical from bacteria to eukaryotic cells, especially  
114 humans. Therefore, the whole-cell bioreporter assay cannot replace the role of direct  
115 measurement of carcinogenic effects in animals or humans, but still can be feasibly  
116 employed as a cost-effective and preliminary screening tool to assess ecotoxicity in  
117 environmental samples, particularly prior to well-established techniques (Alhadrami  
118 and Paton, 2013).

119 Although many whole-cell bioreporters are developed to sense the presence of  
120 specific chemicals or general toxicity, the majority of them are used still in laboratory  
121 proofs of concept (van der Meer and Belkin, 2010). In most cases, toxicities of  
122 chemicals in water samples or water extractions are evaluated by the bioreporter assay  
123 (Nagata et al., 2010; Zeinoddini et al., 2010; Axelrod et al., 2016). Recently, an *E.*  
124 *coli* bioreporter recApr–Luc2 was built to detect the genotoxicity of heavy metals in  
125 recycled ashes for livestock diets and evaluate their risks entering human food chain  
126 (Sanchezvicente et al., 2016). Nevertheless, the development of whole-cell  
127 bioreporters which are feasible in more complex environmental media (e.g., soils and  
128 seawater) is still challenging (van der Meer and Belkin, 2010; Michelini et al., 2013),  
129 as bioreporter sensitivity and chemical bioavailability are influenced by  
130 environmental variables (He et al., 2010; Jiang et al., 2015). Many attempts are made  
131 to overcome such barriers, and a limited number of bioreporters have been  
132 successfully applied in soils, seawater and groundwater (He et al., 2010; Zhang et al.,

133 2012a; Yoon et al., 2016). Moreover, new techniques such as magnet-nanoparticles  
134 functionalization (Zhang et al., 2011; Jia et al., 2016) and microchip (Cortés-Salazar  
135 et al., 2013) are also developed to enhance bioreporter performance in complex  
136 environmental media.

137 The quantification of genotoxicity via a bioreporter assay has been conducted  
138 using two approaches. Taking the bioluminescent bioreporter as an example, the first  
139 approach compares the induced bioluminescent signals over time for different  
140 concentrations of target genotoxins to a negative control. Here, the parameter defined  
141 as the relative luminescent unit is derived, as the most commonly used quantitative  
142 method in bioreporter assays (Gu and Chang, 2001; Ore et al., 2010; Zeinoddini et al.,  
143 2010). By taking the end-point bioluminescence at time  $t$  as a function of the  
144 concentration series, the genotoxicity of unknown samples can therefore be quantified  
145 by interpolating their bioluminescent signals using the calibration curve. The other  
146 approach is to develop an analytical model for a whole-cell bioreporter to simulate  
147 their behaviors based on the quantitative SOS response of DNA damage inducible  
148 genes. Daniel *et al.* (2010) develops an analytical model of a whole-cell  
149 bioluminescent bioreporter, with an input signal (toxin concentration) and an output  
150 signal (bioluminescent light). The model is characterized by three measurable sets of  
151 parameters: the biosensor effective rate constant, the total number of emitted photons  
152 and the biosensor reaction order, verified for the three DNA damage inducible  
153 promoters, including *recA*, *katG* and *micF*. Recently, a gene cross-regulation model is  
154 developed to simulate the SOS response of the *A. baylyi* bioreporter (Zhang et al.,

155 2012b). The model takes into consideration the dynamic variation in free RecA and  
156 single-stranded DNA (ssDNA)-bound RecA proteins, and the background expression  
157 of *luxCDABE* gene, correlating the input signal (genotoxin concentration) and output  
158 signal (bioluminescent light) with three empirical parameters: SOS response  
159 coefficient, genotoxicity coefficient and cytotoxicity coefficient. Although the  
160 mechanisms of *recA* gene induction and SOS response are similar in *E. coli* and  
161 *Acinetobacter baylyi* (Whitworth and Gregg-Jolly, 2000; Dolph et al., 2001; Hare et  
162 al., 2006), this mode has not been applied for *E. coli* bioreporter yet.

163 In the present study, a bioluminescent whole-cell bioreporter (Jiang et al., 2016)  
164 was employed to evaluate the genotoxicities and bioavailabilities of mitomycin C  
165 amended soils and seawater, which demonstrated the dose-effect relationships in both  
166 environments. Two case studies were further conducted on the bioreporter's response  
167 to chromium(VI) (Cr[VI]) contaminated soils and crude oil contaminated seawater.  
168 These two cases were chosen for the following reasons: i) Cr(VI) and crude oil are  
169 representatives of inorganic (e.g. heavy metals) and organic chemicals respectively  
170 and have high contamination levels in many regions of China and worldwide (Jacobs  
171 and Testa, 2005; Gao et al., 2015); ii) Cr(VI) and crude oil are known as genotoxins  
172 but with different mechanisms of DNA damage (Cohen et al., 1993; Mielżyńska et al.,  
173 2006), and it is therefore of great concern and importance to study the genotoxicity  
174 equivalent across different contaminants and environmental media for their impacts  
175 on ecosystems; iii) soil and seawater, with high turbidity and salinity, are more  
176 complex environmental media compared with laboratory conditions and freshwater.

177 For the first time, we modified the cross-regulation model in *A. baylyi* to predict the  
178 quantitative response of the *E. coli* bioreporter to environmental genotoxins.  
179 Investigations on bioreporter performance in soils and seawater are beneficial for  
180 overcoming the barriers of complex environmental media and expanding the  
181 application of bioreporters from the laboratory to potential *in situ* monitoring.

182

## 183 **2 Materials and Methods**

### 184 2.1 Preparation, sampling and chemical analysis of soil and seawater samples

185 Artificial Cr(VI)-contaminated soils were prepared by mixing standard soils (Chinese  
186 soil standards GBW07403, GSS-3) with potassium dichromate solution, and air-dried  
187 for three days, followed by sieving through a 20-mesh screen. The artificially  
188 contaminated soils contained 5.2 mg Cr(VI)/g soil dry weight. A series of 0, 10, 20, 50,  
189 100 and 200 mg artificial soil samples were individually mixed with 5 mL ultrapure  
190 water to form a soil/water slurry with Cr(VI). The soil to water ratio had limited  
191 effects on the bioluminescence intensity (Zhang et al., 2012a).

192 Cr(VI)-contaminated soil samples were taken from five sites (Henan, Hubei,  
193 Shandong, Jiangsu and Liaoning Provinces) in China. Soil samples were air-dried for  
194 three days at room temperature and sieved through a 20-mesh screen. The soil  
195 properties including pH, organic matter content, cation exchange capacity, particle  
196 size distribution, total/available nitrogen, phosphate and potassium were measured  
197 according to previously described methods (Jiang et al., 2014). The soils were  
198 pretreated using two methods to compare the bioavailability and genotoxicity of

199 Cr(VI) in different phases. The soil/water slurry (solid phase) was prepared by mixing  
200 200 mg of each soil sample with 5.0 mL ultrapure water and sonicated for 300 s. The  
201 soil supernatant (water phase) was obtained by shaking the soil/water slurry at 200  
202 rpm for 24 h at room temperature and centrifuged at 10000×g for 15 min to remove  
203 soil pellets.

204 Crude oil contaminated seawater samples were collected along the coastline of  
205 Jiaozhou Bay, where a severe oil spill occurred on 22<sup>nd</sup> November 2013. Due to the  
206 leakage from underground oil pipelines, over 1000 km<sup>2</sup> of pavement was  
207 contaminated. Part of the crude oil entered Jiaozhou Bay along with the rainwater  
208 pipeline, resulting in the contamination of approximately 3,000 km<sup>2</sup> of seawater. The  
209 seawater samples were taken from five sites (Figure 4) on Day 1, Day 3, Day 7 and  
210 Day 50 after the oil spill, and directly stored at 4°C for further genotoxicity  
211 assessment and chemical analysis. Total petroleum hydrocarbons (TPHs) and  
212 polycyclic aromatic hydrocarbons (PAHs) are the predominant contaminants in crude  
213 oil (Fathalla, 2007; Gao et al., 2015), which were analyzed following the Gas  
214 Chromatography-Flame Ionization Detector (GC-FID) and Gas Chromatography-  
215 Mass Spectrometer (GC/MS) methods as described in US EPA 8015B (USEPA,  
216 1996b) and US EPA 8270C (USEPA, 1996a), respectively.

## 217 2.2 Preparation of the *Escherichia coli* bioreporter for genotoxicity assessments

218 The *E. coli* bioreporter for genotoxicity assessment was prepared according to a  
219 modified protocol by Kim and Man (Kim and Man, 2003) and optimized in our  
220 laboratory (Jiang et al., 2016). In brief, the bioreporter cells were transferred into 10

221 mL of fresh LB medium supplemented with 100 mg/L ampicillin (LBA medium) and  
222 incubated at 37°C with shaking at 150 rpm for 6 h. The bioreporter suspension was  
223 diluted 1:25 in fresh LBA for use, and the optimal initial optical density at 600 nm  
224 ( $OD_{600}$ ) for genotoxicity assessment was approximately 0.06 (Jiang et al., 2016). Two  
225 microliters of soil/water slurry or soil supernatant was directly mixed with 198  $\mu$ L  
226 bioreporter suspension for bioluminescence detection, whilst the ratio for seawater  
227 detection was 20  $\mu$ L of seawater with 180  $\mu$ L of bioreporter suspension. Deionized  
228 water and mitomycin C amended soil/seawater samples were used as the negative and  
229 positive controls, respectively. To prepare the mitomycin C amended soils, a series of  
230 mitomycin C solutions at different concentrations were added to the soils in glass  
231 tubes, and thoroughly mixed using a vortex (Song et al., 2014). The mitomycin C  
232 amended seawater was acquired by dissolving a series of mitomycin C concentrations  
233 in artificial seawater mineral salt medium (Jiang et al., 2016).

234 The bioluminescent intensity and  $OD_{600}$  of the bioreporter were measured every  
235 15 min, with a Spectra M5 Plate Reader (Molecular Devices, California, USA), for 6  
236 h at 37°C. Cell viability was estimated by colony-counting on LB agar plates. Briefly,  
237 bioreporter cells exposed to different samples were collected at different time points  
238 and serially diluted. The 100  $\mu$ l of dilution was spread on a LB agar plate and  
239 incubated overnight (14-16 h) at 37°C for cell counting. All treatments were carried  
240 out in biological triplicates.

241 2.3 Data analysis and model simulation

242 The induced bioluminescence of the bioreporter was calculated by averaging the  
 243 monitored bioluminescent intensity from 150 and 180 min. The induction ratio was  
 244 evaluated by dividing the induced bioluminescence by that of the negative control  
 245 (non-induced). The bioavailability of genotoxins was calculated as the fraction of  
 246 genotoxins detected by the bioreporter assay, divided by their total concentrations in  
 247 the environmental samples. All statistical analyses were performed using SPSS 17.0.  
 248 One-way ANOVA was employed to evaluate the statistical significance of differences  
 249 and variance (p-value<0.05).

250 Based on the similar mechanisms of *recA* gene induction and SOS response  
 251 between *E. coli* and *Acinetobacter baylyi* (Dolph et al., 2001; Hare et al., 2006), we  
 252 modified the cross-regulation model in *A. baylyi* (Zhang et al., 2012b) to predict the  
 253 quantitative response of *E. coli* bioreporter to genotoxins. Different from the response  
 254 of regulator to specific chemicals (Zhang et al., 2012b), the DNA damage response in  
 255 the present study is simplified as five steps: alkylation/methylation of DNA,  
 256 formation of ssDNA, cleavage of LexA repressor dimers, LexA repressor's  
 257 self-cleavage, and expression of DNA damage inducible genes (Al-Anizi et al., 2014;  
 258 Jia et al., 2016). Induction of the bioreporter is shown in Equation 1.

$$SOS_{r,s} = 1 + \frac{k_{ssDNA} \cdot k_{SLSR}}{2(1 + k_{ssDNA})} [LSR]_{total} \cdot \frac{[genotoxin]}{(K_{genotoxicity} \cdot k_{genotoxicity} \cdot k_{ssDNA} \cdot K_{SLSR})^{-1} + [genotoxin]} \cdot (1 - K_{cytotoxicity})$$

259 (1)

260 Here,  $K_{genotoxin}$  is defined as the methylation rate of double-stranded DNA.  
 261 Damaged double-stranded DNA results in a certain amount of ssDNA with the  
 262 synthesis rate ( $k_{genotoxin}$ ). The recognition of ssDNA by RecA consequently causes

263 the cleavage of LexA-like SOS repressor ( $LSR$ ,  $\text{cell}^{-1}$ ), where  $dLSR$  ( $\text{cell}^{-1}$ ) and  
 264  $sLSR$  ( $\text{cell}^{-1}$ ) represent the  $LSR$  dimer (SOS box repressor) and monomer (SOS box  
 265 activator), respectively.  $[LSR]_{total}$  ( $\text{cell}^{-1}$ ) represents the total amount of SOS  
 266 repressor, and  $k_{ssDNA}$  is the cleavage reaction constant of  $LSR$  dimer.  $K_{dLSR}$  and  
 267  $K_{sLSR}$  determine the dynamic equilibrium of  $LSR$  dimer and monomer.  
 268  $[genotoxin]$  ( $\text{cell}^{-1}$ ) refers to the number of genotoxins inside the cells, and  $SOS_{r,s}$   
 269 represents the induced SOS response ratio. The SOS response coefficient is defined as  
 270  $K_{genotoxin} \cdot k_{genotoxin} \cdot k_{ssDNA} \cdot k_{sLSR}$ , which demonstrates the synergetic effects of  
 271 DNA damage, ssDNA recognition and SOS box promotion.  $K_{Genotoxicity}$  is the  
 272 genotoxicity coefficient, representing  $\frac{k_{ssDNA} \cdot k_{sLSR}}{2 \cdot (1 + k_{ssDNA})} \cdot [LSR]_{total}$ .

273 Cytotoxicity is also taken into consideration in the cross-regulation model as the  
 274 response of bioreporters is a synergistic effect of both genotoxicity and cytotoxicity.  
 275 Cytotoxicity is simulated in accordance with the inhibition effects of cytotoxic  
 276 compounds on protein activities, as described in Equation 2 with the cytotoxicity  
 277 coefficient ( $k_{cytotoxicity}$ ).

$$278 \quad \text{Cell activities} = \frac{k_{cytotoxicity}^{-1}}{k_{cytotoxicity}^{-1} + [genotoxin]} \quad (2)$$

279 Three parameters are involved in the cross-regulation model: SOS response  
 280 coefficient, genotoxicity coefficient and cytotoxicity coefficient. By fitting the  
 281 experimental data with nonlinear regression in SPSS, the coefficients were obtained  
 282 for a calibration curve correlating genotoxin (e.g., mitomycin C) concentration with  
 283 the induction ratio. For unknown environmental samples, the induction ratio of the  
 284 bioreporter was first obtained, followed by interpolation using the calibration curve to

285 obtain the mitomycin C equivalent, thus resulting in the quantitative evaluation of  
286 genotoxicity among different environmental media.

287

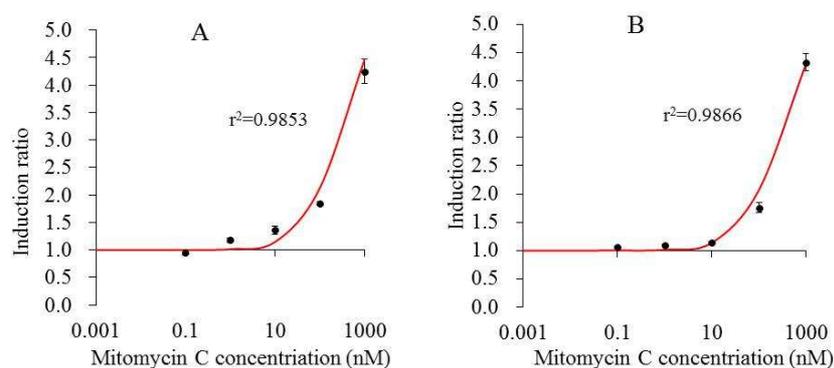
### 288 **3 Results and Discussion**

#### 289 3.1 Modelling of the *E. coli* bioreporter response to artificial samples

290 The *E. coli* bioreporter demonstrated a sensitive dose-effect response to mitomycin C  
291 amended soils and seawater, ranging from 0.1 nM to 1  $\mu$ M (Figure S1). The negative  
292 control expressed a consistent baseline of bioluminescence. The bioluminescence  
293 intensity of the bioreporter increased with mitomycin C concentration, and peaked at  
294 approximately 180 min. The detection limit for mitomycin C was 1 nM in both soils  
295 and seawater. Mitomycin C concentrations up to 1  $\mu$ M did not affect cell viability  
296 (Jiang et al., 2016).

297 The bioreporter was non-responsive until 120 min for soil and 180 min for  
298 seawater. The SOS process is not momentary, instead involving the processes of  
299 alkylation/methylation of DNA, formation of single-stranded DNA, cleavage of LexA  
300 repressor dimers, LexA repressor's self-cleavage and expression of DNA damage  
301 inducible genes. Only afterwards, the expression of bioluminescent *luxCDABE* gene  
302 is triggered for mRNA transcription and protein translation (Michel, 2005). Besides,  
303 the responsive time is relevant to DNA damage mechanisms. Min et al. (1999)  
304 demonstrated that direct DNA damage reagents can immediately induce  
305 bioreporter response, but indirect DNA damage takes more than 100 min. Due  
306 to the similar mechanism of DNA damage response between *A. baylyi* and *E. coli*

307 (Hare et al., 2012), the cross-regulation model was modified to simulate the *E. coli*  
 308 bioreporter's response to mitomycin C. In the present study, the induction ratio was  
 309 calibrated against mitomycin C, and the experimental data fitted well with the  
 310 cross-regulation model (Figure 1).



311  
 312 **Figure 1.** The dose-effect relationship of the induction ratio of the *E. coli* bioreporter to  
 313 mitomycin C (from 0 to 1  $\mu\text{M}$ ) amended soils (A) and seawaters (B). The solid black dots  
 314 represent the relative bioluminescence (RB) of the bioreporter induced by mitomycin C, and  
 315 the red line is the fitting curve of the cross-regulation model. Error bars indicate the standard  
 316 deviations of the replicates.

317 Two key parameters ( $K_{Genotoxicity}$  and SOS response coefficient) were  
 318 introduced in this model as cytotoxicity was negligible when mitomycin C  
 319 concentration was below 1  $\mu\text{M}$ . A comparison of  $K_{Genotoxicity}$  and SOS response  
 320 coefficient of mitomycin C between different environmental media is shown in Table  
 321 1. From Equation (1) and dimensional homogeneity, the unit of SOS response  
 322 coefficient ( $K_{genotoxin} \cdot k_{genotoxin} \cdot k_{SSDNA} \cdot k_{SLSR}$ ) is reciprocal to genotoxin unit

323 (nM/L). The SOS response coefficients in deionized water (0.004 L/nM), soil (0.003  
324 L/nM) and seawater (0.003 L/nM) did not significantly differ, indicating the identical  
325 mechanism of mitomycin C-induced SOS response process in different environmental  
326 media, including the integrated effects of DNA damage, ssDNA recognition and SOS  
327 box promotion. Nevertheless, the values of  $K_{Genotoxicity}$  were significantly reduced  
328 in soils (4.5) and seawater (4.3) compared with that in deionized water (12.5). For soil  
329 samples, the lowered  $K_{Genotoxicity}$  was possibly attributed to the relatively lower  
330 bioavailability of mitomycin C caused by the complicated soil-cell-chemical  
331 interactions, which was consistent with previous studies (He et al., 2010; Violante et  
332 al., 2010; Weng et al., 2010). For the seawater samples, high salinity may explain the  
333 decline in bioluminescent signals and the decreased  $K_{Genotoxicity}$ . Moreover, for a  
334 given sample with unknown genotoxicity, the mitomycin C equivalent can be derived  
335 by interpolating its induction ratio using the calibration curve in the same  
336 environmental medium, enabling the comparison of genotoxicities between different  
337 environmental samples with mitomycin C as the standard. Mitomycin C was chosen  
338 as the standard genotoxin in the present study as its DNA damaging mechanism has  
339 been established to be alkylation (Abraham et al., 2006), and it has been extensively  
340 used as a model genotoxin in many studies (Vollmer et al., 1997; Polyak et al., 2001;  
341 Tani et al., 2004; Aranda et al., 2013). It is clear that no single genotoxin can cover all  
342 the DNA damaging mechanisms inducing the bioreporter response, and one  
343 environmental sample with an undefined composition possibly contains more than  
344 one DNA-damaging reagent with different genotoxic mechanisms. However, the

345 calculated mitomycin C equivalent gives a detailed insight into the contents of  
 346 mitomycin C-like reagents in environmental samples. DNA-damaging reagents with  
 347 distinct genotoxic mechanisms such as N-methyl-N'-nitro-N-nitrosoguanidine  
 348 (MNNG) and 4-Nitroquinoline N-oxide (4-NQO) can also be employed for the  
 349 calibration curve and genotoxicity quantification.

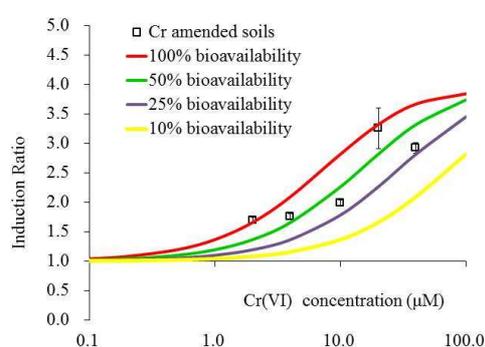
350 **Table 1** Comparison of  $K_{Genotoxicity}$  and SOS response coefficient of mitomycin C in  
 351 different environmental media.

Environmental media	$K_{Genotoxicity}$	SOS response coefficient (L/nM)	Reference
Soil	4.5	0.003	(Jiang et al., 2016)
Seawater	4.3	0.003	This study
Water	12.5	0.004	This study

### 352 3.2 Response of the bioreporter to Cr(VI) contaminated soils

353 The genotoxicity and bioavailability of Cr(VI) in artificially contaminated soils were  
 354 assessed by the *E. coli* bioreporter (Figure 2, blank squares). We introduced a simple  
 355 ultrasonic pretreatment (300 s) to prepare the soil/water slurry, which was  
 356 subsequently mixed with the bioreporter cells directly. All the artificially  
 357 contaminated soils positively induced the bioreporter within 3 h. The concentration of  
 358 Cr(VI) in soils ranged from 2 to 20  $\mu\text{mol/L}$ , and higher concentrations of Cr(VI)  
 359 induced stronger bioluminescent signals of the bioreporter, showing a dose-effect  
 360 relationship. Cr(VI) has been demonstrated to induce proteomic changes in  
 361 *Pseudomonas aeruginosa* (Kilic et al., 2010), as well as inhibiting light emission of a  
 362 luminescent bacteria (Villaescusa et al., 1997). At the highest Cr(VI) concentration  
 363 (40  $\mu\text{M}$ ), the cell viability of bioreporter was not significantly affected from cell count

364 (Figure S2), but a significant reduced induction ratio was observed, possibly  
 365 attributed to the inhibition of protein synthesis. The assay allowed direct contact  
 366 between the bioreporter cells and soil particles, enabling the detection of contaminants  
 367 in free water, bound water and soil-fixed fractions (He et al., 2010; Ore et al., 2010;  
 368 Zhang et al., 2010; Song et al., 2014).



369  
 370 **Figure 2.** The response of the *E. coli* bioreporter to artificial chromium contaminated soils.  
 371 The white squares refer to the induction ratio of the *E. coli* bioreporter. The red line  
 372 represents the fitting curve of the cross-regulation model to simulate the bioreporter's  
 373 response to chromium toxicity with 100% bioavailability, followed by different levels of  
 374 chromium bioavailability such as 50% (green line), 25% (purple line) and 10% (yellow line),  
 375 respectively. Error bars indicate the standard deviations of the replicates.

376 Concentration of Cr(VI) in contaminated sites could be up to several hundred to  
 377 several thousand (100 to 10000) mg/kg (Jiang et al., 2014; Ogunkunle et al., 2014).  
 378 And as regulated by EPA, the total Cr (no regulation for Cr[VI]) for generic soil  
 379 screening levels at contaminated site is 390 mg/kg for ingestion, and 2.0 mg/kg for  
 380 migration to groundwater (USEPA, 1996). We therefore consider 2.0 mg/kg as the  
 381 background concentration of Cr, and the generic contamination level of Cr(VI) in

382 soils ranges from 390 mg/kg to 10000 mg/kg. According to the protocols described in  
383 the present study (in section 2.1), the detection range of the bioreporter for Cr(VI)  
384 ranges from 260 mg/kg to 2600 mg/kg. Thus, the bioreporter assay is capable of  
385 covering routine detection of Cr(VI).

386 Bioavailability is a parameter to evaluate the percentage of contaminants which  
387 are accessible and detectable by the bioreporter assay. Some fractions of contaminants  
388 may interact with environmental media, such as soil particles, and become  
389 unavailable to living microorganisms. Therefore, in some cases, environmental  
390 samples with the identical levels of contaminants might have different toxicities. The  
391 cross-regulation model was also used here to simulate the bioreporter's response to  
392 the bioavailable fraction of Cr(VI) in soils (Figure 2). The red line (100% of  
393 bioavailability) assumes that all Cr(VI) in the soils is bioavailable to the bioreporter,  
394 and its genotoxicity can be detected by the bioreporter assay. Meanwhile, the 50% of  
395 bioavailability curve simulates the response of the bioreporter when only 50% of  
396 Cr(VI) can be sensed. Lower simulated Cr(VI) bioavailability (50%, 25% and 10%)  
397 leads to a shift in the calibration curves towards higher chromium values. The actual  
398 induction of the bioreporter (shown as blank squares) was significantly lower than the  
399 red line, indicating that a minor fraction of Cr(VI) was bioavailable to positively  
400 induce the bioreporter response. The induction ratios of the bioreporter to artificially  
401 contaminated soils were all located within the lines of 25% and 100% bioavailability,  
402 which was possibly explained by the complex interactions between soil particles,  
403 microorganisms and Cr(VI) (Flynn et al., 2002; Ivask et al., 2002). The SOS response

404 coefficient (0.125 L/ $\mu$ M) and genotoxicity coefficient  $K_{Genotoxicity}$  (3.3) for Cr(VI)  
 405 in the present study were derived from the bioreporter's response to Cr(VI) in the  
 406 aqueous phase (data not shown). These parameters remained stable for all the  
 407 calibration curves, which was similar to previous research (Jia et al., 2016).

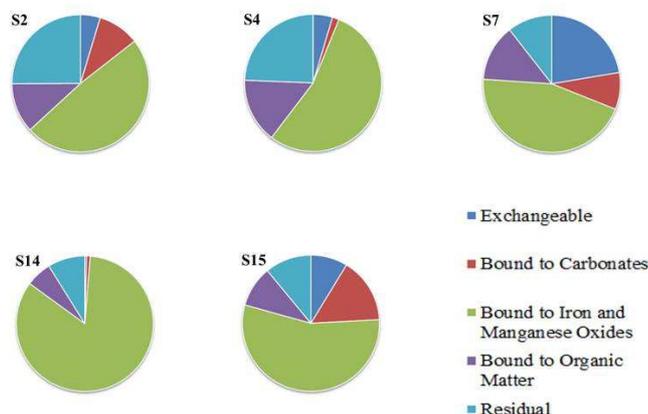
408 The bioreporter assay was then used to evaluate the genotoxicities of real  
 409 Cr(VI)-contaminated soils taken from five contaminated sites in China (Figure S3).  
 410 Contamination levels in the soil samples are listed in Table 2. The soils were  
 411 pretreated in either soil/water slurry or soil supernatant, and the induction ratios of the  
 412 bioreporter were measured for both treatments. Mitomycin C equivalents were  
 413 calculated based on the calibration curves, which showed significant differences in  
 414 soil/water slurry and soil supernatant (Table 2). The bioavailable Cr(VI) in soil  
 415 supernatant was only a minor fraction of that in soil/water slurry, which varied  
 416 between 6.16% and 30.79%, indicating that most chromium was fixed on soil  
 417 particles and showed greater genotoxicity. For soil samples with Cr(VI) concentration  
 418 less than 1000 mg/kg dry soil weight, the bioreporter was significantly induced  
 419 without affecting cell viability from the results of cell count (data not shown). For  
 420 soils S7 and S8 which were heavily contaminated with Cr(VI), cell viability of the  
 421 bioreporter was inhibited in both soil/water slurry and soil supernatant, suggesting  
 422 that the genotoxicity in these soils was more than 1  $\mu$ M of mitomycin C equivalent.  
 423 **Table 2** Mitomycin C equivalent of Cr(VI) contaminated soil samples using different  
 424 pretreatment methods.

Sample No.	Induction ratio	Mitomycin C equivalent	Induction ratio (soil)	Mitomycin C equivalent (soil)	Percentage of Cr(VI) (slurry/supernatant, %)	Cr(VI) concentration
------------	-----------------	------------------------	------------------------	-------------------------------	--	----------------------

	(soil/water slurry)	(soil/water slurry, nmol/L)	supernatant)	supernatant, nmol/L)		(mg/L)
S4	1.92±0.06	77.09±2.50	1.89±0.04	18.95±0.45	24.58	111.91
S15	2.21±0.09	110.33±4.37	2.09±0.09	23.62±1.05	21.41	122.68
S6	1.96±0.05	81.36±2.17	2.15±0.11	25.05±1.30	30.79	388.51
S13	3.57±0.14	399.48±15.79	2.77±0.14	40.79±2.69	10.21	390.43
S2	2.50±0.08	150.00±4.73	1.45±0.08	9.23±0.90	6.16	499.35
S14	2.20±0.11	109.09±15.50	1.80±0.11	16.91±0.44	15.50	499.35
S7	1.78±0.05	>1000	1.78±0.05	>1000	26.16	7088.57
S8	2.04±0.08	>1000	1.74±0.08	>1000	17.25	7505.02

425 Interestingly, the response of the bioreporter to soil samples with similar  
426 contamination levels (S4/S15, S6/S13, S2/S14, S7/S8) was different, possibly due to  
427 different bioavailability or forms of chromium. We further analyzed different forms of  
428 chromium in soils using the Tessier method (Tessier et al., 1979). The composition of  
429 chromium showed notable differences (Figure 3). Chromium bound to iron and  
430 manganese was the predominant form in all soils. Residual chromium in a stable form  
431 was less accessible to the bioreporter to activate genotoxicity response. For example,  
432 the lower residual chromium in soil S15, compared with that in soil S4, led to a higher  
433 genotoxic response of the bioreporter. More exchangeable and carbonate-bound  
434 chromium in soil S2 also contributed to more genotoxicity than that in soil S14. The  
435 Cr(VI) forms have a causal relationship with different processes related to soil  
436 properties, including dissolution, ion exchange, sedimentation, complex formation,  
437 oxidation and reduction (Roberto Terzano et al., 2007; Zhao et al., 2009). Therefore,  
438 soil properties influence the occurrence, transportation and fate of chromium in the  
439 soil environment (Lubomir Simeonov, 2008), of which the most important ones are

440 identified as soil acidity, cation exchange capacity and soil organic matter (Wazne et  
 441 al., 2007). For instance, the acidity of soil solution (pH) determines the possibility of  
 442 an equilibrium transition of different forms of chromium, from steadily bound forms  
 443 with humic substances to water soluble ion forms and slightly bound exchangeable  
 444 forms on clay colloids (Lubomir Simeonov, 2008). In the present study, the ANOVA  
 445 test showed that the soil pH had significant effects on the bioreporter response  
 446 ( $p < 0.05$ ).

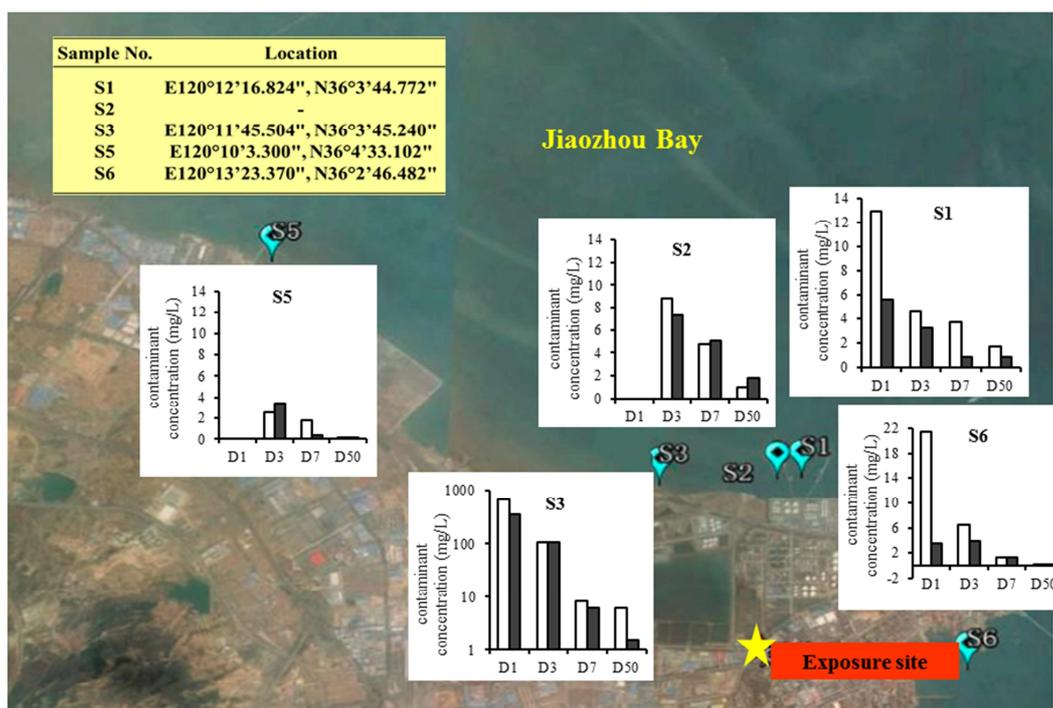


447  
 448 **Figure 3.** Different forms of chromium in soil samples (Tessier method).

### 449 3.3 Response of the bioreporter to crude oil contaminated seawater

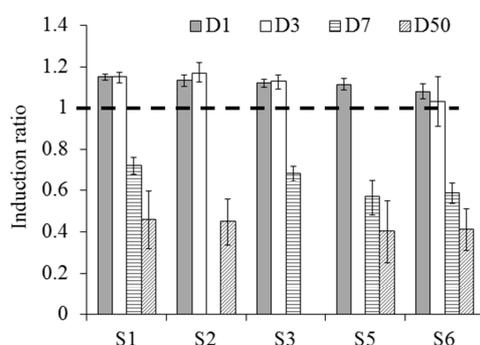
450 The sampling sites of seawater samples taken from Jiaozhou Bay are shown in Figure  
 451 4, and they were collected on Day 1, Day 3, Day 7 and Day 50 after the oil spill for  
 452 genotoxicity evaluation. The predominant contaminants in the seawater samples were  
 453 total petroleum hydrocarbons (TPHs) and polycyclic aromatic hydrocarbons (PAHs),  
 454 and the contamination levels declined over time (Figure S4). TPHs and PAHs  
 455 concentrations at Day 50 after the oil spill decreased to less than 10% of the original

456 contamination levels (Day 1). Seawater S3 was the most contaminated point with  
 457 TPHs and PAHs concentrations as high as 685.45 mg/L and 351.36 mg/L,  
 458 respectively, and alkanes (C<sub>13</sub>-C<sub>26</sub>), naphthalene and phenanthrene were the dominant  
 459 contaminants (Figure S5). A sudden increase in contaminant concentrations was  
 460 observed on Day 15 after the oil spill in seawater S1 and S2, suggesting the migration  
 461 of crude oil from point S3 to S1 and S2. Contaminants were naturally attenuated  
 462 through the dilution, spreading, dispersion, evaporation and emulsification effects of  
 463 seawater, along with the artificial oil-absorbing felt and other emergency measures  
 464 taken (Fathalla, 2007; Zhang et al., 2013).



465  
 466 **Figure 4.** The sampling sites in Jiaozhou Bay. Total petroleum hydrocarbons (TPHs, white  
 467 bar) and total polycyclic aromatic hydrocarbons (PAHs, black bar) in seawater samples (1, 3,  
 468 7 and 50 d after the oil spill) are shown in small pictures.

469 The induction ratios of the *E. coli* bioreporter to seawater samples are shown in  
 470 Figure 5. All the samples positively induced the bioreporter response on Day 1 and  
 471 Day 3 after the oil spill. With regard to the contaminant concentrations, the detection  
 472 limit of the bioreporter to crude oil contaminated seawater was at the mg/L level. The  
 473 bioluminescent intensities of the bioreporter were dramatically lowered in seawater  
 474 samples taken on Day 7 and Day 50 after the oil spill, however, cell viability was not  
 475 inhibited. This is possibly due to the mild inhibitory effects or cytotoxicity of oil  
 476 degradation products after Day 7, as crude oil is still capable of releasing toxic  
 477 compounds into seawater by photo-oxidation when they are extensively degraded  
 478 (Maki et al., 2001).



479  
 480 **Figure 5.** The induction ratio of the *E. coli* bioreporter to seawater samples collected from  
 481 Jiaozhou Bay (1, 3, 7 and 50 d after the oil spill). The black dashed line marks the induction  
 482 ratio of 1.0. The bioreporter was significantly induced by seawater samples. Error bars  
 483 indicate the standard deviations of the replicates.

484 Mitomycin C equivalents in seawater samples on Day 1 and Day 3 were

485 calculated using the calibration curve shown in Figure 1B (Table 3). Mitomycin C  
 486 equivalents in seawater S3 and S6 showed a significant decline over time. In contrast,  
 487 although a slight decrease in contaminant concentrations was observed in seawater S1  
 488 and S2 by chemical analysis (Figure S4), the genotoxicity test showed higher  
 489 mitomycin C equivalents of S2 on Day 3 compared with Day 1. This was possibly  
 490 caused by the integrated effects of various oil degradation products detected by the  
 491 bioreporter.

492 **Table 3** Mitomycin C equivalent in oil contaminated seawater.

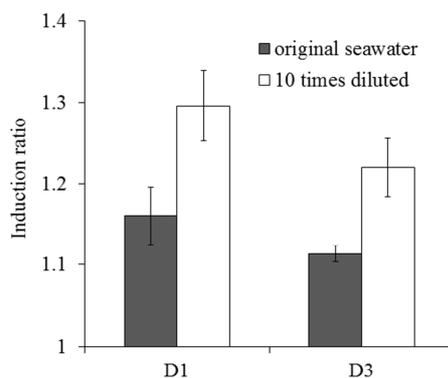
Sample No.	Mitomycin C equivalent (nmol/L)
D1-S1	$10.77 \pm 0.19^b$
D1-S2	$9.65 \pm 0.15^b$
D1-S3	$\sim 222.1 \pm 7.40^a$
D1-S5	$8.09 \pm 0.08^b$
D1-S6	$5.64 \pm 0.04^b$
D3-S1	$10.72 \pm 0.09^b$
D3-S2	$12.52 \pm 0.19^b$
D3-S3	$\sim 160.15 \pm 4.83^a$
D3-S5	-
D3-S6	$2.14 \pm 0.03^b$

493 Note: <sup>a</sup>The mitomycin C equivalents were calculated according to the induction ratios in  
 494 Figure 6.

495 <sup>b</sup>The mitomycin C equivalents were calculated according to the induction ratios in Figure 5.

496 As cell viability was inhibited in the original seawater S3 (1 d and 3 d after the  
 497 oil spill, data not shown), seawater S3 was further diluted 10 times with  
 498 uncontaminated background seawater S8 (N36°5'24.72", E120°29'40.92", not marked  
 499 in Figure 4). The induction ratio of the bioreporter to the 10-times diluted S3 sample

500 was significantly increased, without any inhibition of cell viability. Therefore, the  
501 mitomycin C equivalent of the original seawater S3 was estimated using the 10-times  
502 diluted sample (Table 3). Regardless of the possible changes in contaminant forms,  
503 dilution eliminated the inhibition of cell viability caused by high concentrations of  
504 contaminants and allowed genotoxicity quantification of heavily contaminated  
505 samples. Our result therefore suggested that dilution may be used to roughly estimate  
506 heavily contaminated samples. The bioreporter assay provides as an appropriate  
507 first-step monitoring system and an alternative to chemical analysis (Harms et al.,  
508 2006).



509  
510 **Figure 6.** Induction ratio of the *E. coli* bioreporter to seawater S3 (1 d and 3 d after the oil  
511 spill) with different dilution factors (1 and 10). Error bars indicate the standard deviations of  
512 the replicates.

513

#### 514 **4 Conclusion**

515 This study established a whole-cell bioreporter assay for genotoxicity assessment of

516 real environmental samples in harsh conditions, including soils and seawater. As no  
517 currently available biological assay can provide detailed or precise information on the  
518 chemical composition of environmental samples, the whole-cell bioreporter assay  
519 provides a possible answer to the question of whether targeted samples have  
520 genotoxicity potentially possessing threats to ecosystems or microorganisms.  
521 Moreover, the whole-cell bioreporter assay coupled with the modified  
522 cross-regulation model and mitomycin C equivalent enables the quantification and  
523 comparison of genotoxicities between various environmental samples. This assay can  
524 provide suggestions for subsequent chemical analysis to determine the precise type  
525 and concentrations of genotoxins.

526

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- ◆ A bioreporter evaluates genotoxicity and bioavailability of environmental samples.
- ◆ The bioreporter is used in real world scenario for risk assessment.
- ◆ A gene regulation model is derived for SOS-based bioreporters.
- ◆ The gene regulation model enables quantitative genotoxicity assesement.