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

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Abstract

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

environmental samples.

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

1 Introduction

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

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

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

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

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

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

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

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

2 Materials and Methods

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

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

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

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

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

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

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

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

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

2.3 Data analysis and model simulation

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

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

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

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

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

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

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

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

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

3 Results and Discussion

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

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

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

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

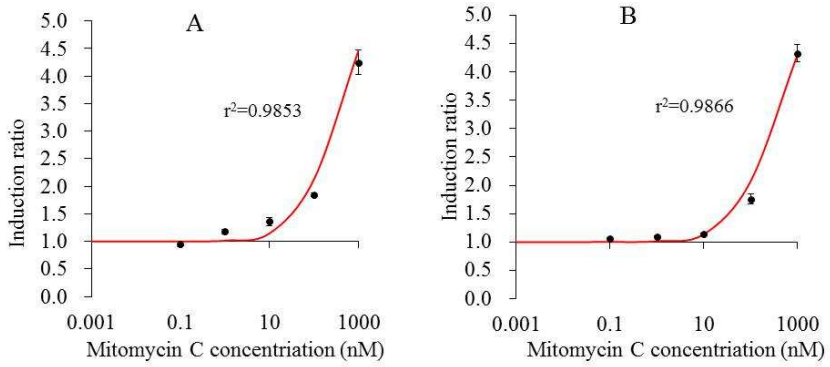


Figure 1. The dose-effect relationship of the induction ratio of the *E. coli* bioreporter to mitomycin C (from 0 to 1 μM) amended soils (A) and seawaters (B). The solid black dots represent the relative bioluminescence (RB) of the bioreporter induced by mitomycin C, and the red line is the fitting curve of the cross-regulation model. Error bars indicate the standard deviations of the replicates.

Two key parameters ($K_{Genotoxicity}$ and SOS response coefficient) were introduced in this model as cytotoxicity was negligible when mitomycin C concentration was below 1 μM. A comparison of $K_{Genotoxicity}$ and SOS response coefficient of mitomycin C between different environmental media is shown in Table 1. From Equation (1) and dimensional homogeneity, the unit of SOS response 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 μmol/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 μM), the cell viability of bioreporter was not significantly affected from cell count

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

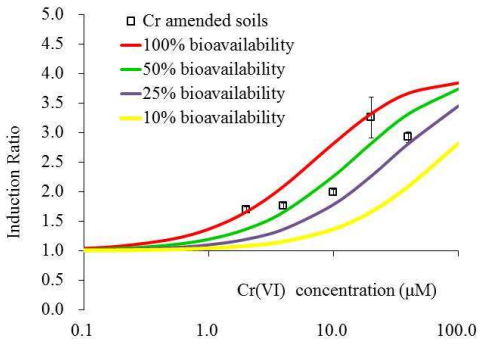


Figure 2. The response of the *E. coli* bioreporter to artificial chromium contaminated soils. The white squares refer to the induction ratio of the *E. coli* bioreporter. The red line represents the fitting curve of the cross-regulation model to simulate the bioreporter's response to chromium toxicity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

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

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

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

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

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

Table 2 Mitomycin C equivalent of Cr(VI) contaminated soil samples using different 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

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

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

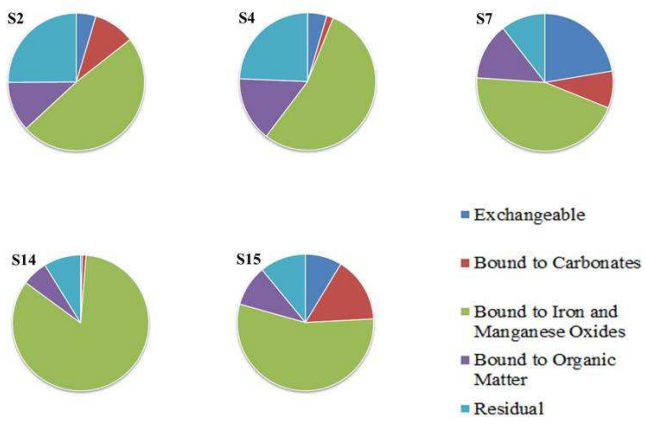
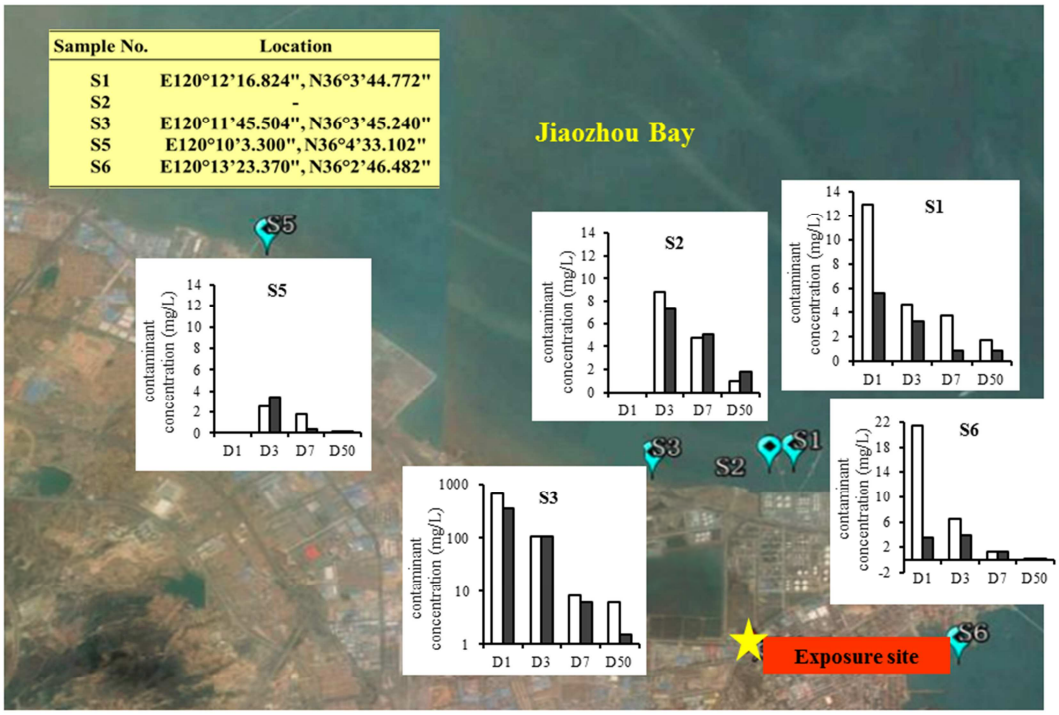


Figure 3. Different forms of chromium in soil samples (Tessier method).

3.3 Response of the bioreporter to crude oil contaminated seawater

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

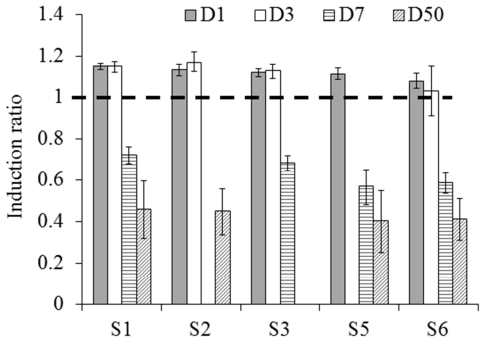
456 contamination levels (Day 1). Seawater S3 was the most contaminated point with
457 TPHs and PAHs concentrations as high as 685.45 mg/L and 351.36 mg/L,
458 respectively, and alkanes (C_{13}-C_{26}), naphthalene and phenanthrene were the dominant
459 contaminants (Figure S5). A sudden increase in 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.

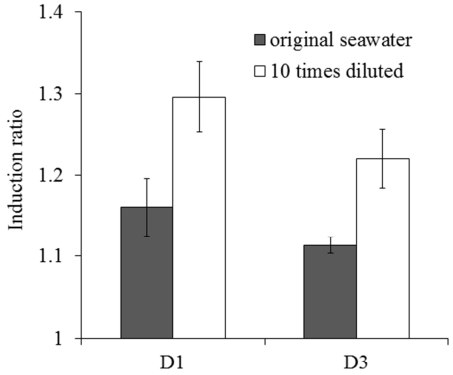
Sample No.	Mitomycin C equivalent (nmol/L)
D1-S1	10.77±0.19[b]
D1-S2	9.65±0.15[b]
D1-S3	~222.1±7.40[a]
D1-S5	8.09±0.08[b]
D1-S6	5.64±0.04[b]
D3-S1	10.72±0.09[b]
D3-S2	12.52±0.19[b]
D3-S3	~160.15±4.83[a]
D3-S5	-
D3-S6	2.14±0.03[b]

493 Note: [a]The mitomycin C equivalents were calculated according to the induction ratios in
494 Figure 6.

495 [b]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

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

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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 assessement.