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Construction and application of a zinc-specific biosensor for assessing the immobilization and bioavailability of zinc in different soils

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ABSTRACT

The inducibility and specificity of different *czcRS* operons in *Pseudomonas putida* X4 were studied by *lacZ* gene fusions. The data of β-glycosidase activity confirmed that the *czcR3* promoter responded quantitatively to zinc. A zinc-specific biosensor, *P. putida* X4 (pczcR3GFP), was constructed by fusing a promoterless enhanced green fluorescent protein (*egfp*) gene with the *czcR3* promoter in the chromosome of *P. putida* X4. In water extracts of four different soils amended with zinc, the reporter strain detected about 90% of the zinc content of the samples. Both the bioavailability assessment and the sequential extraction analysis demonstrated that the immobilization of zinc was highly dependent on the physicochemical properties of soils. The results also showed that the lability of zinc decreased over time. It is concluded that the biosensor constitutes an alternative system for the convenient evaluation of zinc toxicity in the environment.

1. Introduction

Zinc compounds have been widely used in industry, agriculture, and medicine, leading to large-scale environmental contamination. Although zinc is an essential micronutrient that plays important roles in numerous physiological processes, excess zinc has significant toxicity and acts as a potent disrupter of biological systems (Blencowe and Morby, 2003). In soils and sediments, the mobile and bioavailable zinc fractions, rather than the total zinc, determine ecological and toxicological relevance because they have the greatest immediate environmental impact. Therefore, the development of rapid and sensitive monitoring systems for zinc mobility and bioavailability in the environment has become increasingly important. As a response to this need, many genetically modified microbial sensors have been developed.

In general, these biosensors rely on expression analysis of a reporter gene controlled by a promoter responsive to zinc. Ivask et al. (2002) employed the *Escherichia coli* MC1061(pzntRluc) biosensor to determine the bioavailable metal ions in contaminated soil and found that approximately 2% of the total zinc in the soil was available for this bacterial sensor in soil-water extracts. The detectable concentration range of *Synechocystis* sp. PCC6803 (pcoaTlux) for zinc was 1–3 μmol L^{-1}, and this reporter strain detected about 90% zinc content in the acetic acid extracts of contaminated soil (Peca et al., 2008). Bondarenko et al. (2008) demonstrated that there was no statistically significant difference among the bioavailable zinc amounts determined by six different biosensors. However, most of these biosensors are not specific to zinc, they respond to several non-target divalent heavy metal ions, such as Co^{2+} and Cd^{2+}, thus affecting the accuracy of zinc detection in the environment. Highly target-specific promoters may have good application potential for the development of zinc-specific bioreporters.

For environmental analysis, the most significant promoters are found in bacteria that survive in extreme environments contaminated by heavy metals or organic compounds (Rensing and Maier, 2003). The ability of bacteria to survive in a metal-contaminated environment is usually based on genetically encoded resistance systems, the expression of which is precisely regulated. Czc metal-resistance systems have been identified in *Cupriavidus metallidurans* CH34, *Pseudomonas aeruginosa*, and *Pseudomonas putida* (Caille et al., 2007; Grosse et al., 1999; Leedjarv et al., 2008). Although the genome of *P. putida* contains several *czcRS* operons (Canovas et al., 2003), no study has been reported regarding the function of different *czcRS* genes in the same strain.

In this study, the inducibility and specificity of different *czcRS* operons were studied by *lacZ* fusions, and then a zinc-specific

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Table 1
Strains and plasmids.

Strain or plasmid	Relevant characteristic (s)[a]	Reference
Strains		
E. coli		
DH5α	*supE*44 Δ*lacU*169 (Φ80*dlacZ*ΔM15) *recA*1 *endA*1 *hsdR*17 *thi*−1*gyrA*96 *relA*1	Invitrogen
C118λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX*74 *galE galK phoA*20 *thi*−1*rpsE rpoB argE* (Am) *recA*1 λ*pir*	Invitrogen
MM294	*endA thiA hsdR*17 *supE*44	
P. putida		
X4	Ap^r, Co^r, Zn^r, Cd^r, Cu^r, wild-type	(Fu et al., 2008)
X4(pczcR1lac)	X4 with a recombination between *czcR*1 promoter and *lacZYA*	This study
X4(pczcR2lac)	X4 with a recombination between *czcR*2 promoter and *lacZYA*	This study
X4(pczcR3lac)	X4 with a recombination between *czcR*3 promoter and *lacZYA*	This study
X4(pczcR3GFP)	X4 with a recombination between *czcR*3 promoter and *egfp*	This study
Plasmids		
pEGFP-C3	Km^r *egfp*	Takara
pRK2073	RK2 helper plasmid, Spe^r	(Hu et al., 2007)
pTA2	Cloning vector, Amp^r	Toyobo
pVIK112	*LacZYA*, Km^r, suicide vector	(Kalogeraki and Winans, 1997)
pVIKR1	*czcR1* fragment cloned in pVIK112	This study
pVIKR2	*czcR2* fragment cloned in pVIK112	This study
pVIKR3	*czcR3* fragment cloned in pVIK112	This study
pVIK212	Derivate of pVIKR3, containing a *gfp* gene instead of *lacZYA*	This study

[a] Co^r, Zn^r, Cd^r, and Cu^r indicate resistance to cobalt, zinc, cadmium, and copper, respectively; Amp^r, Km^r, and Spe^r stand for resistance to ampicillin, kanamycin, and spectinomycin, respectively.

biosensor was constructed based on *egfp* and the promoter of *czcR3*. Moreover, a laboratory incubation experiment with four different soils was conducted for 4 weeks. The main purpose of this work was to use the newly constructed biosensor coupled with atomic absorption spectrophotometer (AAS) to evaluate the effect of different soil types on the fixation and bioaccessbility of zinc. Sequential extraction analysis was also performed at 1 and 4 weeks after the start of the experiment to better understand the speciation of zinc in different soils.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are described in Table 1. *P. putida* X4 was isolated from waste soil around the Academy of Hubei Agricultural Sciences, Wuhan, China (Fu et al., 2008). For the construction and maintenance of plasmids, *Escherichia coli* strains DH5α and C118λ*pir* were used.

Bacteria were routinely grown in Luria-Bertani (LB) medium at 37 °C (*E. coli*) or at 28 °C (*P. putida*). When required, antibiotics were added at the following final concentrations: 100 μg mL^{-1} ampicillin and 50 μg mL^{-1} kanamycin. Tris-buffered medium (Mergeay et al., 1985) containing 0.1% glucose was used for testing promoter inducibility and biosensor fluorescence performance. Analytical grade reagents of $CuSO_45H_2O$, $CdCl_2H_2O$, $ZnCl_2$, $CoCl_26H_2O$, $Pb(NO_3)_2$, and $NiSO_46H_2O$ were prepared into 1.0 mol L^{-1} stock solutions and sterilized by filtration.

2.2. Soil samples and their preparation

Soil samples used in this study and their pertinent properties are shown in Table 2. Water solution of $ZnCl_2$ was added in the soils in order to reach the concentration of 800 mg kg^{-1}. The moisture content of the soils after treatment was 20%. The samples were incubated at 25 °C in the dark for different periods. At the selected times soil was air-dried before being analyzed. For bioavailability analysis, soil-water suspensions were prepared by mixing of 5 g dry soil with 50 ml MilliQ water and shaking at 28 °C for 24 h. Soil–water extracts were further prepared by centrifugation of the soil-water suspensions for 5 min at 12 000 g. Chemical fractions of zinc in soils were investigated using the procedure of Sposito et al. (1982). Four fractions were obtained: (1) soluble/exchangeable fraction, (2) organic-bound fraction, (3) inorganic precipitates, and (4) residual fraction. The concentration of zinc for each fraction was determined by AAS.

2.3. Homologous recombination for pczcR/lacZ and pczcR3/egfp fusions

The primer pairs used in this study are shown in Table 3. For DNA manipulation, standard DNA molecular cloning methods were used (Sambrook et al., 2001). The suicide fusion vector pVIK112 (Kalogeraki and Winans, 1997) was selected for constructing the LacZ fusion reporter systems. *czcR1*, *czcR2*, and *czcR3* fragments were amplified from *P. putida* X4 genomic DNA by PCR. The plasmid pVIKR1 containing a *czcR1* fragment was constructed as follows: the *czcR*1 fragment was cloned into pTA2 (Toyobo, Japan) and its orientation was confirmed by sequencing; after digestion with *Xba*I and *Kpn*I, the restriction fragment was cloned into pVIK112 to create pVIKR1. The same procedure was then employed to create plasmids pVIKR2 and pVIKR3. For the construction of the whole-cell reporter *P. putida* X4 (pczcR3GFP), *egfp* gene was amplified from pEGFP (Clontech Takara, Japan), and then ligated with the fragment containing the kanamycin resistance gene, RK2/RP4 *oriT*, R6K*ori*, and *czcR3* (amplified from pVIKR3), yielding pVIK212. These plasmids were introduced into *P. putida* X4 by triparental conjugation. The helper strain was *E. coli* MM294 with the helper plasmid pRK2073 (Hu et al., 2007). Recombinant strains were screened by their ability to grow in medium supplemented by ampicillin and kanamycin. They were further verified by PCR amplification and sequencing.

2.4. β-Galactosidase activity assay

The activity of β-galactosidase was measured in permeabilized cells according to the method described by Nies (1992), using *o*-nitrophenyl-D-galactoside (ONPG; 4 mg mL^{-1}) as the substrate. The unit 1 U was defined as the activity that forms 1 nmol of *o*-nitrophenol per minute at 30 °C.

2.5. Bioavailability tests and calculations

During the course of this work, soil-water extracts were analyzed. Water was chosen as an extractant because aqueous extracts of soils were analyzed for the

Table 2
Selected properties of examined soils.[a]

Soil type	Location	Depth (cm)	pH	OM (g kg^{-1})	CEC (cmol kg^{-1})	Zn (mg kg^{-1})
Brown soil (Alfisol)	Taishan, Shandong, China	0–20	6.3	42.4	15.3	120
Black soil (Mollisol)	Gongzhuling, Jilin, China	0–20	6.2	15.6	18.8	105
Cinnamon soil (Alfisol)	Gongyi, Henan, China	0–20	7.0	12.6	13.1	139
Red soil (Ultisol)	Chenzhou, Hunan, China	0–20	4.5	26.7	10.5	185

[a] Organic matter (OM), point of zero charge (PZC), cation exchange capacity (CEC), and total zinc were analyzed by $K_2Cr_2O_7$ digestion, Mehlich, NH4AcO method (Xiong, 1985) and acid digestion method (Zampella and Adamo, 2010), respectively.

Table 3
Primers used in this study.

Primer pair	Sequence(5′–3′)	Tm (°C)
czcR1	atg cca tac cct cag cca aac cgt acc caa cgc cac gaa	60
czcR2	cat cgt gtt tgc ctc gta ata ggt cca gtt cca ggt cag c	56.5
czcR3	att ggc ggg tcc tgt tag gtc cct gaa tgc cct gtg	55.5
gfp	gag cac tag t[b]tg act gaa tag gga aac agc tat gac cat ggt gag caa ggg cga g gtg aac tag ttt act tgt aca gct cgt cca tgc	59
vector[a]	gag cac tag tcg atg act aca cgg taa caa tc gtg aac tag tat aac tgc cgt cac tcc aa	53.5

[a] Primer pairs used to amplify DNA fragment containing the kanamycin resistance gene, RK2/RP4/*oriT*, R6K*ori*, and *czcR3* from pVIKR3.
[b] Restriction site of *Spe* I.

ecotoxicological effects and, in addition, water extractability describes the potential transfer of pollutants via the soil-water path (Kahru et al., 2005). Water, zinc solution with suitable concentration, and soil extracts were pipetted in separate parallels of tubes. Then an equal volume of the sensor bacteria suspended in 2× strength Tris-buffered medium was added into each tube. Fluorescence intensity was measured in a spectrofluorometer (RF 5310PC; Shimadzu, Kyoto, Japan) at an excitation wavelength of 490 ± 5 nm and an emission wavelength of 510 ± 5 nm after incubation. All the induction measurements were performed in duplicate.

Response of the sensor bacteria to zinc standards was calculated by following formula:

$$F_i = F_s - F_b \quad (1)$$

where F_i is zinc-induced fluorescence, F_s is the fluorescence measured in a standard solution, and F_b is the fluorescence measured in water. In soil-water extracts, the effect of soil matrix on bacterial fluorescence was taken into account. Thus, correction factor *CF* was calculated as described by Bondarenko et al. (2008).

$$CF = F_b/F_{sb} \quad (2)$$

where F_{sb} is fluorescence of the biosensor in extract of non-spiked soil. In water extracts of spiked soils, the induction of the sensor bacteria was calculated by taking into account of the *CF* as follows:

$$F_i = (F_{sa} - F_{sb}) \times CF \quad (3)$$

where F_{sa} is the fluorescence measured in water extract of spiked soil. The relative fluorescent intensity was calculated by dividing the zinc-induced fluorescence (F_i) by the optical density at 600 nm measured in each sample (Liao et al., 2006). In parallel to assays of soil-water extracts, zinc standard solutions were analyzed with the sensor bacteria to build standard calibration curve. The lowest detectable concentration of *P. putida* X4 (pczcR3GFP) for zinc was defined as the concentration that caused the fluorescence level twice exceeding that of the background (Ivask et al., 2002).

3. Results

3.1. Expression patterns and inducer specificities of czcR1, czcR2, and czcR3

Expression patterns and inducer specificities were determined by measuring the changes in LacZ activity. All promoters were tested for their inducibility in the presence of 0–180 μmol L^{-1} Zn^{2+}, Co^{2+}, Cu^{2+}, and Cd^{2+}. The expression patterns and inducer specificities of *czcR1* are shown in Fig. 1a. The *czcR1* transcripted in the absence of tested metal ions. The activity of LacZ that expressed under the promoter of *czcR1* dropped to 40% in the presence of 180 μmol L^{-1} Cd^{2+}, and to 34% after a 180 μmol L^{-1} Zn^{2+} treatment. The transcription of *czcR2* was induced by Cd^{2+}. The influence of the other tested metal ions was minimal (Fig. 1b). Fig. 1c shows that the transcription of *czcR3* was best induced by 60 μmol L^{-1} Zn^{2+}, but was unaffected by Co^{2+}, Cu^{2+}, and Cd^{2+}. In addition to four metal ions above, Pb^{2+} and Ni^{2+} were also tested for their impacts on *czcR3*. It was observed that the transcription of *czcR3* was also not induced by Pb^{2+} and Ni^{2+}.

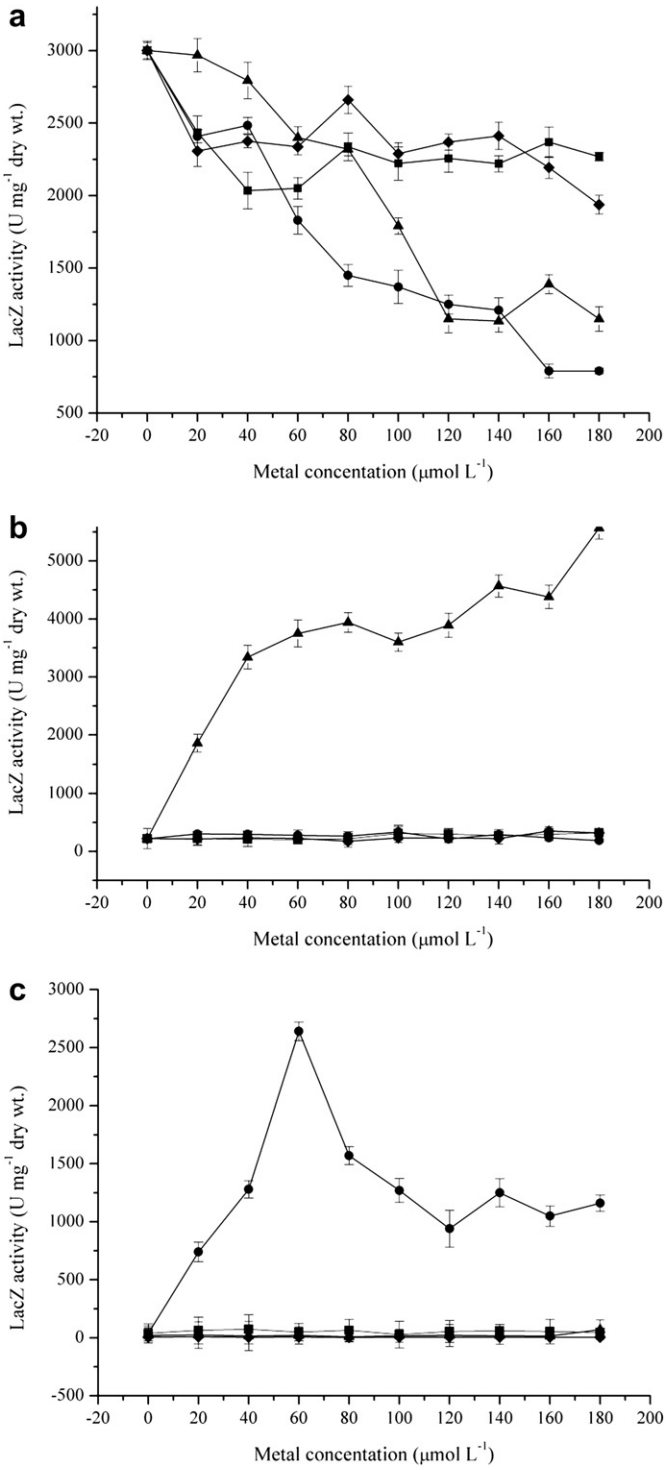

Fig. 1. Expression patterns and inducer specificities of a) *czcR1*, b) *czcR2*, and c) *czcR3*. LacZ activity was measured in permeabilized cells after induction with different concentrations of Cu^{2+} (■), Zn^{2+} (●), Cd^{2+} (▲), and Co^{2+} (◆) for 4 h. Error bars represent the standard deviations of three determinations.

3.2. Effect of induction time and initial cell number on zinc-induced fluorescence

The time-dependent induction of *P. putida* X4 (pczcR3GFP) in response to zinc was determined by exposing the cells to zinc for different time intervals. As shown in Fig. 2, the induction of

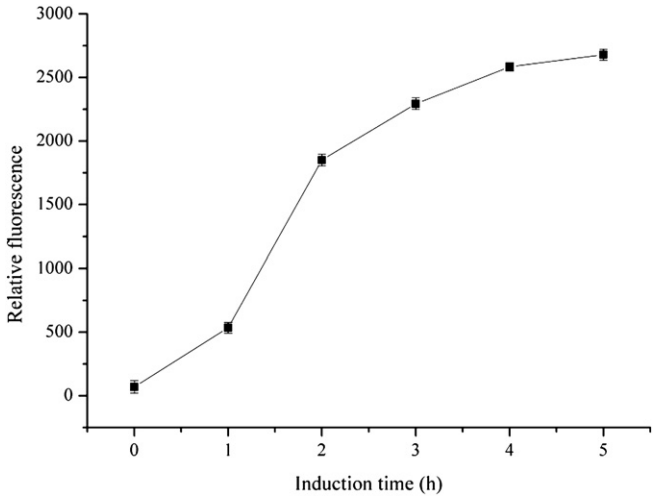

Fig. 2. Time-dependent induction of relative fluorescence with zinc. The biosensor cells were exposed to 60 μmol L^{-1} zinc, and the fluorescence intensity was determined after different exposure periods. The relative fluorescence was calculated as described in Bioavailability tests and calculations. The data presented here are the mean values of three independent experiments with the standard deviations.

fluorescence by zinc occurred in a time-dependent manner. As the time of induction increased, there was an increase in the relative fluorescence during the first 4 h of incubation. Although strong green fluorescence was observed after incubated the biosensor for 2 h, in order to improve the limit of detection, 4-h induction was chosen in this study.

Fluorescence intensity was also measured at different initial cell numbers (10^8–10^5 CFU mL^{-1}) in the presence of zinc at 28 °C and pH 7.0 (the condition for the highest cell growth rate) to evaluate the effects of initial cell number on the fluorescence response. Fig. 3 illustrates that the relative fluorescence of the biosensor increased as cell number decreased from 10^8 to 10^6 cells mL^{-1}. No further improvement was observed as cell density decreased from 10^6 to 10^5 cells mL^{-1}. The ideal density that results in the highest sensitivity of zinc detection by *P. putida* X4 (pczcR3GFP) was assumed to be 10^6 cells mL^{-1} (OD_{600} = 0.4). Rasmussen et al. (1997) proposed that the increase in sensitivity is attributed to a reduction in negatively charged groups and other ligands on the outer surface of the cell which may compete with the regulatory protein for the binding of inducer metal ions.

3.3. Sensitivity and dose–response of P. putida X4 (pczcR3GFP)

Mid-log phased bacteria cells were adjusted to 10^6 cells mL^{-1} (OD_{600} = 0.4), and then treated with zinc for 4 h to evaluate the dose–response relationship between zinc and relative fluorescence intensity. Fluorescence intensity and OD_{600} were measured as described in Bioavailability tests and calculations. The lowest detectable concentration of *P. putida* X4 (pczcR3GFP) for zinc was 5 μmol L^{-1} at 28 °C and pH 7.0. The dose–response relationship between zinc concentration and relative fluorescence intensity of *P. putida* X4 (pczcR3GFP) is shown in Fig. 4. The *P. putida* X4 (pczcR3GFP) responded to zinc with a detection range of 5–55 μmol L^{-1}. The relative fluorescence increased gradually with increasing concentration of zinc up to 55 μmol L^{-1}. At higher zinc concentrations, the fluorescence signal gradually decreased. Polynomial curve fitting method was used to model this nonlinear calibration curve. A ninth order polynomial approximation (R^2 = 0.99) was the best choice in all cases tested. The detailed equation is shown in Supplementary data.

3.4. Quantification of water-extractable zinc in soils

Water-extractable zinc in soil samples was measured by AAS and the biosensor strain. In non-spiked soils, water-extractable zinc was not detected by *P. putida* X4 (pczcR3GFP), thus water-extractable zinc only of zinc-treated soils is shown in Fig. 5. Zinc turned out to behave differently depending upon the type of soil. It rapidly bound to cinnamon soil particles; only 1.26% of total zinc was extracted within the first week of incubation. However, larger amounts of water-extractable zinc were always detected in red soil. After 3-week incubation, the rankings of extracted zinc for the soils were: red soil > brown soil > black soil > cinnamon soil. The amounts of water-extractable zinc also decreased as the time of incubation increased. Most of zinc was immobilized at the end of this experiment; only 1.10%, 1.83%, and 1.04% of zinc was

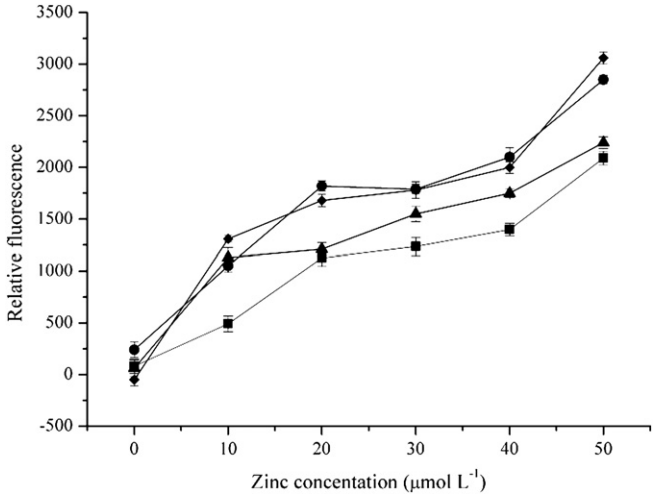

Fig. 3. Effect of initial cell number on the fluorescence performance of the biosensor in the presence of zinc. The relative fluorescence intensity was measured at different cell numbers (10^8, ■; 10^7, ▲; 10^6, ◆; and 10^5 CFU mL^{-1}, ●) in the presence of different concentrations of zinc at pH 7.0 and 28 °C. Data is presented as mean ± standard deviation of three independent measurements.

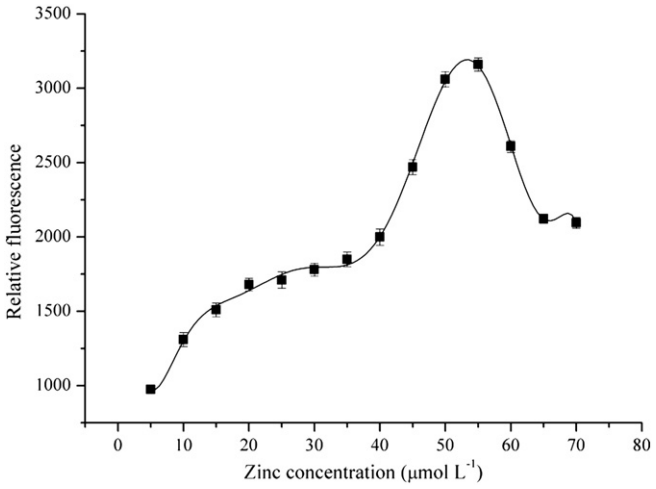

Fig. 4. Dose-dependent induction of relative green fluorescence by zinc and its approximation curve. Cells were incubated for 4 h in a Tris-buffered medium supplemented with zinc. Each point represents the mean of four parallels. The approximation curve with the highest R^2 value was calculated by least-squares regression using Origin.

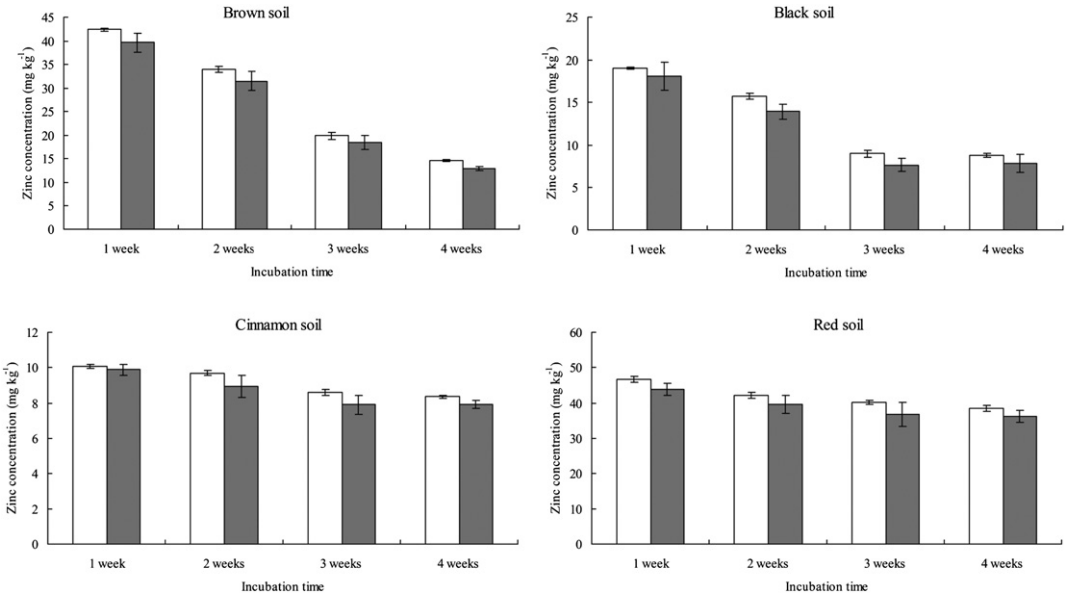

Fig. 5. Zinc concentrations in soil-water extracts measured by AAS (□) and *Pseudomonas putida* X4 (pczcR3GFP) (■). All the data are expressed as means ± standard deviations of three replicates.

extracted from black soil, brown soil and cinnamon soil, respectively. The test was continued for up to 4 weeks but only a slight decrease was found between the last two time points. In most cases, the results obtained by *P. putida* X4 (pczcR3GFP) and AAS showed a good correlation. The concentrations of zinc calculated from the fluorescence response represented an average of 92.2% of the AAS-determined zinc concentrations in the water extracts of red soil, brown soil, and cinnamon soil. There was a larger difference between AAS and the biosensor method in detecting zinc in black soil; the zinc concentrations measured by *P. putida* X4 (pczcR3GFP) only represented about 87.0% of the AAS-determined results.

3.5. Zinc speciation

Sequential extraction of the four amended soils that carried out at 1 and 4 weeks demonstrated that zinc distributed in different chemical forms. As shown in Fig. 6, nearly 63% of the total zinc was extracted as soluble/exchangeable form in brown soil, and 21% of zinc was extracted as inorganically-precipitated form, after spiking the soil for 1 week. Only 6% of the zinc was associated with the organic fraction. After 4 weeks of zinc amendment, the amount of zinc extracted in brown soil in the soluble/exchangeable form decreased significantly from 63% to 24% with an increase in organically-bound form from 6% to 39%. Meanwhile, 25% of the zinc

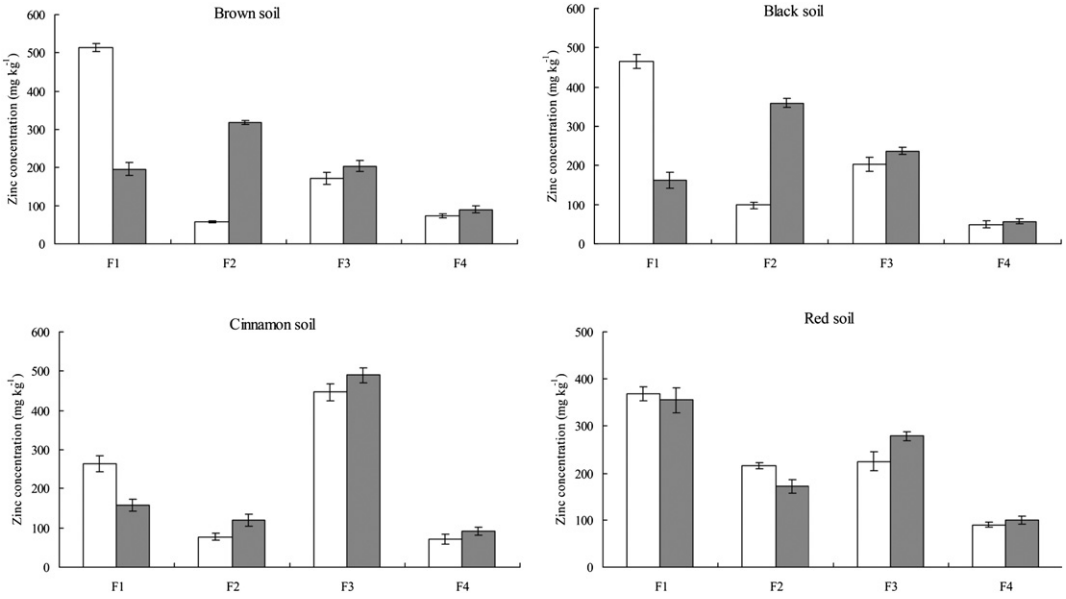

Fig. 6. Distribution of zinc (investigated at 1 week □ and 4 weeks ■) in four soil types contaminated with 800 mg kg^{-1} zinc. F1: soluble/exchangeable-Zn; F2: organically bound-Zn; F3: inorganically precipitated-Zn; F4: residual Zn. Values are means ± standard deviations of three replicates.

was detected in the inorganic fraction. A similar pattern of zinc speciation was also found in black soil. In the case of cinnamon soil, 52% and 57% of the total zinc were found in inorganic fraction at 1 and 4 weeks. Only 9% of the zinc was present in the organic fraction initially, which increased to 14% after 4 weeks of incubation time. In the red soil, the highest amount of zinc was extracted in the soluble/exchangeable fraction (41%), followed by inorganic fraction at 1 week. A decrease in the organically-bound zinc and an increase in the inorganically-precipitated zinc and the residual zinc were noticed after 4 weeks.

3.6. Deposition of strains and nucleotide sequences

P. putida X4 was deposited in the China Center for Type Culture Collection (CCTCC, http://www.cctcc.org/) with the accession number CCTCCM209319. The nucleotide sequences isolated in this study are available in the NCBI Genbank database: *czcR1* (HQ676126), *czcR2* (HQ676125), *czcR3* (HQ676124).

4. Discussion

4.1. The construction of the biosensor and its performance

Bacteria that inhabit metal-contaminated areas have developed various defense mechanisms to cope with metal stresses; mainly by regulating the expression of several subsets of genes involved in the defense response (Rosen, 2002). Studies on these metal response genes facilitate the selection of appropriate promoters applicable to the development of whole-cell based biosensors. In this study, three *czcR* genes found in *P. putida* were proved to have different inducibilities. A new fluorescent reporter strain inducible only by Zn^{2+} from a range of tested metal ions was constructed based on the *czcR3* promoter and *egfp*. The selection of a reporter gene is difficult because of many genes available. Most bacterial bioreporters are equipped with luciferase, beta-galactosidase, or fluorescent proteins because of the ease of measurement and the specificity of the signal given by the reporter protein (van der Meer et al., 2004). For the *lacZ* biosensor, the need for disrupting the cell membrane to permit entrance of the substrate prevents its use for real-time, nondestructive, in situ quantization. Although previous study showed that the detection by bioluminescence is much faster and more sensitive compared to fluorescence (Hakkila et al., 2002), the emission of bioluminescence is a very energy-consuming reaction which might cause a metabolic burden to bacteria expressing luciferase. In addition, zinc may interfere with bioluminescence reaction by forming nonspecific complexes with the active center cysteinyl residues of luciferase (Peca et al., 2008). Therefore, *egfp* was selected for this study.

Sensitivity, specificity, and stability are three important parameters in evaluating the performance of biosensors (He et al., 2010). Although the sensitivity of *P. putida* X4 (pczcR3GFP) for zinc was not better than the biosensors constructed by Peca et al. (2008), its lowest detectable zinc concentration reached 5 μmol L^{-1}. The detection range from 5 to 55 μmol L^{-1} Zn^{2+} matches the acceptable zinc concentration limit for drinking water (1.0 mg L^{-1} = 15.4 μmol L^{-1}, specified by legislation in China). More importantly, the biosensor described in this study was specific to zinc. Zinc detection will be more accurate despite the presence of other metals. In addition, the integration of the gene reporter system into the host chromosome ensured the stability of the biosensor even under nonselective conditions. The applicability of *P. putida* X4 (pczcR3GFP) for zinc detection has been tested using soil samples amended with zinc. The concentration of zinc detected by the reporter strain represented about 90% of the AAS-determined zinc concentration in soil-water extracts. Maybe a part of zinc was still not bioavailable.

4.2. Geochemical speciation of soil-Zn

In the bioavailability tests, cinnamon soil rapidly immobilized the zinc added in, while the soil-water extracts of red soil were always characterized by higher zinc levels compared with that of the other soils. The acidic pH of red soil likely played a key role in enhancing the zinc levels in soil solution. A decrease of zinc solubility with increasing pH has been reported by many authors (Zampella and Adamo, 2010). A large portion of zinc was extracted by water in brown soil and black soil after incubation for 1 week; however, most of zinc was immobilized 3 weeks later. Maybe less strong interactions were formed between zinc and these two soils at the beginning. Previous research investigating the kinetics of soluble metal sorption in soils has shown that the process can essentially be divided into two steps, with an initial stage of relatively rapid sorption followed by a secondary stage that can continue over weeks, months or even years (Donner et al., 2010). The fast adsorption is initiated by instantaneous retention to surfaces of solid particles via the formation of electrostatic or physical complexes driven by the concentration gradient from the solution phase to solid phase. Following the fast adsorption, a secondary slow transport from outer sphere complexes to reactive functional groups in the inner sphere of organic matter and minerals may occur (Saminathan et al., 2010). There were clear changes in the proportional distribution of zinc in all four studied soils after 4 weeks of zinc addition. Generally, the proportions of zinc associated with the weakly bound fractions (soluble/exchangeable) tended to decrease over time, with increases in the other more strongly binding forms (organically/inorganically bound). The magnitude of the decrease in soluble/exchangeable zinc in black soil and brown soil was close to that of the increase in organically-bound zinc after 4 weeks, indicating that transformation of soluble/exchangeable zinc to the organically-bound zinc may be the dominant process in these two soils.

In conclusion, this study demonstrates that the zinc-specific biosensor *P. putida* X4 (pczcR3GFP) constructed by linking *egfp* with *czcR*3 promoter could be a useful tool for the measurement of zinc toxicity in contaminated soils. In addition, differences of solubility and geochemical speciation of zinc between four different soil types were also observed. The results indicate that the soluble/exchangeable zinc decreased as a result of soil–metal interactions and soil aging. Therefore, the same amount of zinc does not imply equal negative impacts due to the speciation process. Bioavailability and motility must be considered while predicting the environmental risks from zinc-contaminated soils.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.envpol.2012.01.023.

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