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A Fast and Easily Parallelizable Biosensor Method for Measuring Extractable Tetracyclines in Soils

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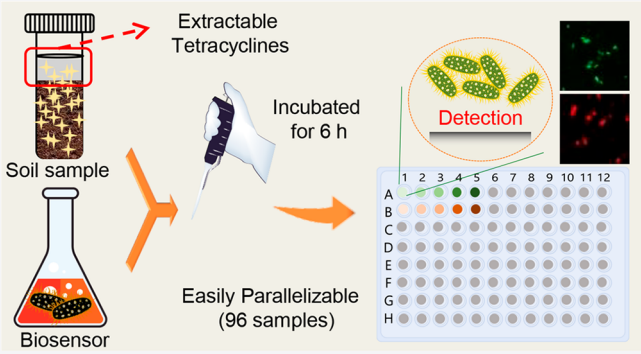
Supporting Information

ABSTRACT: Quantification of extractable antibiotics in soils is important to assessing their bioavailability and mobility, and ultimately their ecotoxicological and health risks. This study aimed to establish a biosensor method for detecting extractable tetracyclines in soils (Alfisol, Mollisol, and Ultisol) using whole-cell biosensors containing a reporter plasmid (pMTGFP or pMTmCherry) carrying fluorescent protein genes tightly controlled by tetracyclines-responsive control region (*tet*RO). This whole-cell biosensor method can simultaneously measure 96 or more samples within 6 h and is easily parallelizable, whereas a typical high-performance liquid chromatography (HPLC) method may require 7 times more of analysis time and much greater cost to achieve similar analytical throughput. The biosensor method had a detection limit for each of six tetracyclines between 5.32−10.2 μg/kg soil, which is considered adequate for detecting tetracyclines in ethylenediaminetetraacetic acid (EDTA) extracts of soils. Relative standard deviation was between 19.8−51.2% for the biosensor *Escherichia coli* DH5α/pMTGFP and 2.98−25.8% for *E. coli* DH5α/pMTmCherry, respectively, suggesting that *E. coli* DH5α/pMTmCherry was superior to *E. coli* DH5α/pMTGFP for detecting extractable tetracyclines in soils. This new, fast, easily parallelizable, and cost-effective biosensor method has the potential for measuring extractable concentrations of tetracyclines for a large number of soil samples in large-scale monitoring studies.



1. INTRODUCTION

Human activities such as manure spreading, biosolid application, and crop irrigation with treated wastewaters can substantially increase concentrations of antibiotics above their natural background levels in soils. Many discharged xenobiotic antibiotics are not naturally produced by soil microorganisms.[1−3] Thus, elevated levels of antibiotic residues in soils can alter microbial activities and community structures, increase the abundance of antibiotic resistance genes, potentially increase antibiotic-resistant infections in clinical settings, and therefore threaten human and animal health.[4−6] To tackle the challenge of antibiotic resistance on a large scale, fast and cost-effective analytical methods for measuring antibiotics in environmental samples are needed to accurately determine ecotoxicological and health risks of antibiotic residues in the environment, especially in the low- and middle-income countries where antibiotic resistance is proliferating.[7]

Tetracyclines are among the most commonly used groups of antibiotics in both human healthcare and animal agriculture (e.g., poultry, swine, and cattle), with an annual use of 1770 and 5186 tons, respectively, in China alone.[8] Thus, contaminated soils often have high tetracycline concentrations ranging from 0.30 to 5.6 mg/kg.[9−11] In soils, extractable fractions of antibiotics are often used to assess their bioavailability to biota and their mobility through soil profiles,[12−14] thus providing important information for assessing their environmental and health risks. A number of extractants have previously been used (such as methanol, acetonitrile, or their mixtures) often for analysis by high-performance liquid chromatography (HPLC). In addition, ethylenediaminetetraacetic acid (EDTA) solution is a mild extractant that is more suitable for whole-cell biosensors than organic solvents. It can complex metal ions to minimize their

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potential interference in analyses.[2,15−17] To date, the EDTA-extractable fraction of tetracyclines in soils has been determined by biosensor methods to help estimate ecotoxicological risks of soil-borne tetracyclines.[18−20] Indeed, high-efficiency analytical methods are a prerequisite for assessing contamination risks of antibiotics.[21,22] Analyses of antibiotic residues are typically performed by microbial toxicity assessment, immunoassays, and chemical analytical methods.[23−25] Bacterial toxicity tests (mainly using *Bacillus cereus* ATCC 11778 and *Aliivibrio fischeri*[26]) are generally conducted using overnight incubation and are thus time-consuming. These tests are also low in sensitivity and nonspecific to antibiotics of interest. Immunoassays are usually quite expensive.[27−29] Therefore, chemical analytical methods using advanced instrumentation such as HPLC and mass spectrometry (MS) have been widely used. These HPLC-MS methods could simultaneously analyze a range of chemicals and achieve low detection limits such as 0.20−3.80 μg/L in water and 0.5−1.0 μg/kg in soil for tetracyclines.[10,30,31] However, they are still quite time-consuming because samples must be analyzed in sequence (e.g., about 40 h for 96 samples) rather than in parallel. The HPLC methods also require substantial analytical expertise to overcome difficulties related to eluant selection and cleanup steps to remove the interferences of other constituents extracted from soils.[10,32] Thus, there is a great need to develop a fast, accurate, easily parallelizable, and cost-effective method for analyzing extractable antibiotics in soils.

It is hypothesized that whole-cell biosensor strains specific to particular antibiotics can meet this need.[33] Whole-cell biosensors can be developed by fusing reporter genes with an inducible gene promoter controlled by a repressor protein. When a specific antibiotic molecule binds to the repressor protein bound to the inducible promoter, the repressor protein is then detached from the promoter, which initiates the expression of reporter genes.[34−37] The whole-cell biosensors have been mainly utilized to detect tetracyclines in food such as cow milk,[16] poultry meat,[17] and fish[38] using microwell plate readers and luminescence with detection limits of 2−35 μg/L, 5−25 μg/kg, and 20−50 μg/kg, respectively. In addition, Hansen et al. used the whole-cell biosensors in combination with flow cytometry to analyze soil samples but needed overnight incubation.[39] Thus, the whole-cell biosensors could be further improved to decrease analytical time and complexity and improve analytical performance for wider adoption in environmental monitoring.

Therefore, this study aimed to first construct whole-cell biosensors specific to six types of tetracyclines (tetracycline, oxytetracycline, chlorotetracycline, deoxytetracycline, minocycline, and methacycline) and then establish a fast, easily parallelizable detection method for the extractable tetracyclines in soils. The whole-cell biosensors were constructed using GFP or monomeric red fluorescence protein (RFP), mCherry, as reporter protein.[40−42] The reporter protein-based biosensors were then used in combination with 96-microwell plates to allow for parallel measurements of EDTA-extractable tetracyclines in diverse soil samples (Alfisol, Mollisol, and Ultisol). This study provided a novel and promising approach for measuring extractable antibiotics in soils using the whole-cell biosensors.

2. MATERIALS AND METHODS

2.1. Chemicals and Soils. Chemicals used in this study are described in Text S1 and Table S1 of Supporting Information. Three soil samples collected from Harbin (Mollisol), Nanjing (Alfisol), and Yingtan (Ultisol) were used, representing three major soil orders from diverse geoclimatic zones covering some of most agriculturally productive areas in China. These soils were first spiked with tetracyclines as described in the Supporting Information and then used in subsequent experiments. Two field soils (Soil A and Soil B) nearby a livestock farm at Nanjing, China were also collected and used for method verification. Selected soil physicochemical properties are shown in Table S2.

2.2. Biosensor Plasmid Construction. Two plasmids encoding tetracyclines-inducible GFP and mCherry were first constructed. Briefly, *tet*M and *tet*RO, T7 terminator and reporter genes (*gfp* and *mCherry*) (Table S3) were amplified using primers on the homologous arm (Table S4 and S5), recombined, and inserted into two plasmids, which were used to develop two whole-cell biosensors named as *E. coli* DH5α/pMTGFP and *E. coli* DH5α/pMTmCherry. Detailed description is provided in the Supporting Information (Table S6). The expression of *gfp* and *mCherry* reporter genes to produce GFP and mCherry in responding to intracellular tetracyclines is tightly controlled by tetracycline-controlled transcriptional activation mechanism (*tet*RO) and is thus only specific to tetracyclines. The *tet*M gene was chosen because it can confer tetracycline resistance to *E. coli* cells in most circumstances by producing ribosomal protection proteins without changing intracellular tetracycline concentrations. The incorporation of *tetM* can also extend the range of detection to higher concentrations.

2.3. Biosensor Induction Test. Before developing the 96-microwell method, the fluorescence response of each biosensor at various concentrations of each tetracycline (0−10000 μg/L) was first measured after a 12 h incubation in the Lysogeny Broth (LB) medium. The temporal evolution of fluorescence for each biosensor was also determined by a fluorophotometer (Lengguang, Shanghai, China) at a 1.5 h interval over 12 h. The detailed procedure is provided in Text S1 of the Supporting Information. To further explore the fast response ability of these biosensors, a shorter incubation time (4.5 h) was tested for the fluorescence induction of *E. coli* DH5α/pMTGFP and *E. coli* DH5α/pMTmCherry by tetracycline at six concentration levels (0, 10, 50, 100, 1000, and 10000 μg/L). Afterward, fluorescence confocal images of biosensors were acquired by a Leica SP8 confocal microscope (Leica Co., Wetzlar, Germany).

2.4. Biosensor Method for Quantifying Tetracyclines in the LB Medium and Soil Extracts. Tetracycline-spiked LB and EDTA-extracts of soil samples (Alfisol, Mollisol, and Ultisol) were prepared as detailed in the Supporting Information. Biosensor cells were cultured exponentially for about 4−5 h at 37 °C in the LB medium (100 mg/L ampicillin) with vigorous shaking at 150 r/min, resulting in an $OD_{600\ nm}$ of 0.45−0.50. Biosensor cells were collected and resuspended in the LB medium with a phosphate buffer (100 mmol/L, pH 7.0) and 10% lactose. The cell suspension ($OD_{600\ nm}$ = 1.000) was divided into 1.00 mL and 1.50 mL vials, freeze-dried for 24 h, and then stored at −70 °C for later use.

To initiate the biosensor analysis, the freeze-dried cells were dispersed into 1 mL centrifuge tubes; this was followed by the addition of 0.500 μg/mL polymyxin B (PMB) which was revived for 1 h at 37 °C on a shaker at 150 r/min to increase the sensitivity of the biosensors to tetracyclines.[43] The

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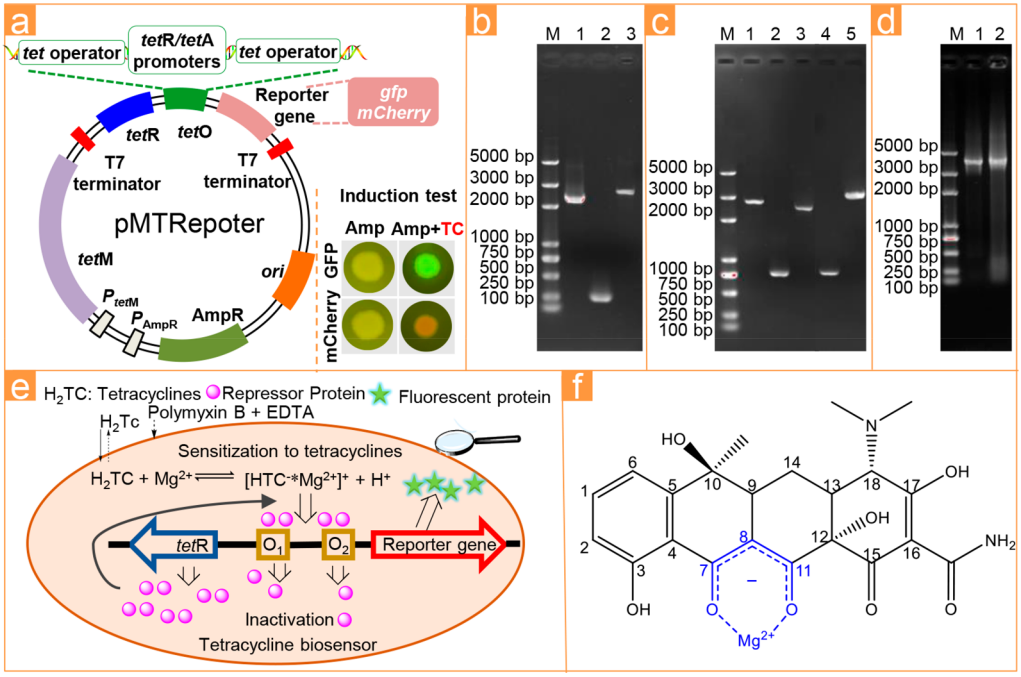

Figure 1. Construction of two recombinant plasmids. Schematic of the recombinant plasmid (pMTGFP and pMTmCherry) and description of fluorescence response of their corresponding bacteria (*E. coli* DH5α/pMTGFP and *E. coli* DH5α/pMTmCherry) induced by 50 mg/L tetracycline (Amp and TC are ampicillin and tetracycline, respectively.) (a) Agarose gel electrophoresis of fused gene fragment amplified by PCR (M-DNA marker DL5000, 1-*tet*M, 2-T7, 3-*tet*M-T7). (b) Agarose gel electrophoresis of cloned gene fragments that were amplified by PCR (M-DNA marker DL5000, 1-*tet*M-T7, 2-*tet*RO, 3-*ori*, 4-*gfp*, and 5-*mcherry-ori*). (c) Agarose gel electrophoresis of cloned genes verified by PCR (M-DNA marker DL5000, 1-pMTGFP partial fragment, 2-pMTmCherry partial fragment). (d) Genetic organization and mechanism of tetracycline-regulated TC-resistance determinant (e) and chemical structure of the tetracycline-Mg^{2+} complex occurring under physiological conditions (f).

combination of PMB and EDTA can sensitize the biosensors by pore formation in the bacterial outer membrane.[44,45] The sensitizing step took 1.5 h, after which the sensitized cells were divided into nontransparent 96-microwell plates in 100 μL aliquots. In advance, each microwell had 100 μL of either the LB medium or the soil extracts (including tetracyclines-free blank control samples) and this was mixed with 25 μL of 125 mmol/L EDTA in triplicates. The microplates were covered with a lid and incubated for 4.5 h at 30 °C and 300 r/min.[16,17] Fluorescence intensity was then measured by a SpectraMaxM5 microplate reader (Molecular Devices Co., Silicon Valley, US) and the equations of the standard curves between the tetracycline concentration and fluorescence were established. The detection limit of the whole-cell biosensors was determined by the previously established methods[46,47] according to $DL_{SE} = \frac{3SD}{S}$ where SD is the standard deviation of blank control and S is the slope of the standard curve, respectively. The linearity ranges of the whole-cell biosensors were calculated according to the r values of the standard curves greater than 0.800.[48] The obtained standard curves were then used for analyses of tetracyclines in spiked soils and contaminated field soils.

2.5. Using Whole-Cell Biosensor to Quantify the EDTA-Extractable Tetracyclines in Soils. In order to further verify this new method, it was applied for the detection of tetracyclines in spiked soils and contaminated field soils. Briefly, to extract the tetracyclines from the soils, 1 mL of preheated EDTA solution (25 mmol/L, 64 °C) was added to 1 g of each soil sample in triplicates. The mixture was then sonicated with 80 kHz ultrasound at 64 °C for 20 min, centrifuged for 10 min at ×5000 g, and filtered through a 0.2 μm filter. The filtered supernatant (i.e., the EDTA soil extract) was analyzed by the methods described above using the whole-cell biosensors. According to the standard curve of spiked EDTA soil extracts in the section Biosensor Method for Quantifying Tetracyclines in the LB Medium and Soil Extracts, the fluorescence intensity of tested samples obtained from the microplate reader was used to estimate the extractable tetracycline concentrations in soils. Heat-treatment of soils before the assay decreased the variations in background fluorescent signals and in tetracycline-induced fluorescence due to different soil matrices.[46,47] The extractable tetracycline concentrations in spiked and field-contaminated soil samples were also measured by the HPLC as described in the Supporting Information.

3. RESULTS

3.1. Biosensors Construction. A schematic of the recombinant plasmids (pMTGFP and pMTmcherry) used in this study is shown in Figure 1a. To construct these two biosensors for the detection of tetracyclines, the *tet*M-T7 gene (NO. 3 band of Figure 1b and NO. 1 band of Figure 1c), the *tet*RO gene (NO. 2 band of Figure 1c), the *ori* gene (NO. 3 band of Figure 1c), the *gfp* gene (NO. 4 band of Figure 1c), and the *mCherry-ori* gene (NO. 5 band of Figure 1c) were used to construct the biosensor plasmids by DNA homologous recombination. The *tet*M gene (NO. 1 band of Figure 1b) and the T7 gene (NO. 2 band of Figure 1b) were fused to obtain the *tet*M-T7 complement (Figure 1b). Additionally, the recombinant plasmid was verified by PCR with specific primers, and amplicon lengths were consistent with those expected (pMTGFP 4115 bp, NO. 1 band of Figure 1d; pMTmcherry 4110 bp, NO. 2 band of Figure 1d). As shown in

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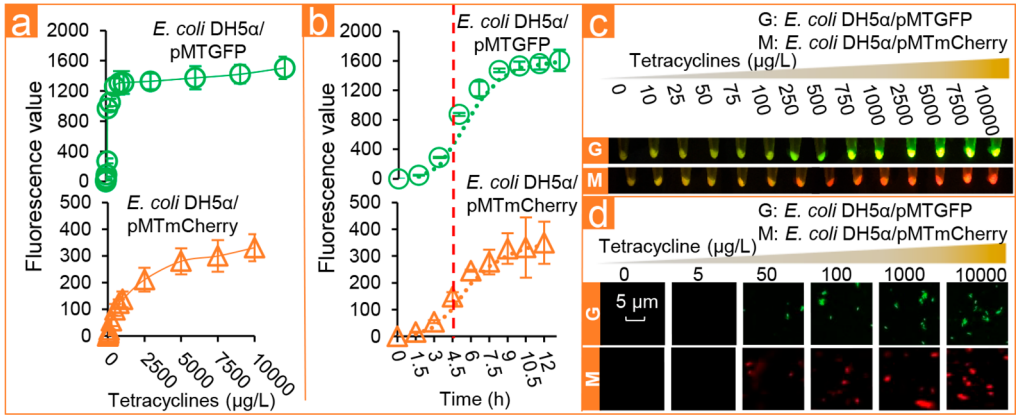

Figure 2. Images of two tetracyclines biosensors (*E. coli* DH5α/pMTGFP and *E. coli* DH5α/pMTmCherry). The concentration-dependent fluorescent response of biosensors induced by tetracycline (a). The temporal fluorescence of biosensors (b). Images of biosensors under blue light, and *E. coli* DH5α/pMTGFP and *E. coli* DH5α/pMTmCherry in the LB medium induced by various concentrations of tetracyclines (0, 10, 25, 50, 75, 100, 250, 500, 750, 1000, 2500, 5000, 7500, 10000 μg/L) (c). Confocal fluorescence microscopy images of *E. coli* DH5α/pMTGFP and *E. coli* DH5α/pMTmCherry induced by various concentrations of tetracycline in LB (d).

Table 1. Detection Parameters of Six Tetracyclines Measured by Biosensors in Soil Extracts (Alfisol, Mollisol, and Ultisol)

Soils extracts	TCs	*E. coli* DH5α/pMTGFP					*E. coli* DH5α/pMTmCherry				
		α (10^{-3})	r	*P* value	Detection limit (μg/L)	Linear range (μg/L)	α (10^{-3})	r	*P* value	Detection limit (μg/L)	Linear range (μg/L)
Alfisol	TC	5.28	0.876	1.95×10^{-4}	8.44	25-5000	24.8	0.889	1.68×10^{-3}	5.65	25-10000
	TC_1	5.63	0.906	2.32×10^{-3}	10.2	75-10000	26.5	0.938	1.02×10^{-3}	7.36	10-7500
	TC_2	3.53	0.856	5.44×10^{-5}	8.32	25-7500	22.7	0.945	6.91×10^{-4}	5.56	25-10000
	TC_3	3.32	0.907	4.82×10^{-5}	7.99	25-10000	15.1	0.945	6.91×10^{-3}	6.45	50-10000
	TC_4	9.66	0.903	5.70×10^{-4}	9.58	50-7500	29.0	0.953	6.67×10^{-5}	7.66	10-10000
	TC_5	7.97	0.868	1.05×10^{-4}	8.66	75-10000	30.6	0.941	7.27×10^{-4}	7.19	25-10000
Mollisol	TC	5.36	0.872	3.88×10^{-5}	8.68	10-5000	41.2	0.896	1.53×10^{-5}	5.78	10-5000
	TC_1	9.45	0.881	2.22×10^{-3}	9.25	10-10000	29.4	0.896	9.20×10^{-3}	7.68	10-10000
	TC_2	5.55	0.821	5.89×10^{-5}	8.63	25-7500	27.5	0.875	3.17×10^{-4}	5.88	10-10000
	TC_3	6.54	0.861	2.07×10^{-3}	8.23	10-7500	15.7	0.936	4.86×10^{-3}	6.77	50-7500
	TC_4	10.9	0.863	2.19×10^{-5}	9.98	25-5000	34.4	0.937	2.61×10^{-4}	7.85	50-7500
	TC_5	11.7	0.942	6.08×10^{-3}	8.69	25-2500	32.5	0.899	1.72×10^{-3}	7.28	50-10000
Ultisol	TC	3.71	0.862	4.20×10^{-4}	8.05	25-7500	28.8	0.938	1.21×10^{-3}	5.32	10-7500
	TC_1	6.41	0.898	2.16×10^{-5}	8.61	75-10000	19.5	0.922	6.63×10^{-3}	7.26	10-10000
	TC_2	3.55	0.893	7.76×10^{-5}	8.04	10-2500	19.8	0.922	3.80×10^{-3}	5.47	10-7500
	TC_3	2.31	0.927	6.48×10^{-5}	7.58	10-10000	15. 3	0.926	5.98×10^{-5}	6.22	75-7500
	TC_4	8.52	0.869	7.59×10^{-5}	9.36	10-7500	23.6	0.936	1.05×10^{-4}	7.37	10-10000
	TC_5	8.50	0.942	1.34×10^{-6}	8.22	50-10000	26.0	0.939	5.03×10^{-3}	7.09	50-10000

The general formula of the standard curve is as follows: $TC_{SEB} = 10^{\alpha F}$, α is shown in the table, the *P* values of all equations were < 0.010. r values are the correlation coefficient. TCs, TC, TC_1, TC_2, TC_3, TC_4 and TC_5 are tetracyclines, tetracycline, oxytetracycline, chlorotetracycline, deoxytetracycline, minocycline, and methacycline, respectively.

Figure 1a, *E. coli* DH5α strains containing a recombinant plasmid (pMTGFP or pMTmcherry) were induced by tetracycline and produced the fluorescent bacterial colony under ultraviolet light. The expression of the reporter gene is under the tight transcriptional control of the tetracycline repressor (*tetR*) on the plasmid (pMTGFP and pMTmcherry, Figure 1a,e). Only binding of $[MgTC]^+$ (Figure 1f, binding domains were marked in blue) to *tetR* abolishes DNA binding to two operator sites (*tet*O) and thus allows expression, importantly, of the divergently oriented reporter genes (Figure 1e).[49] The binding of tetracyclines-magnesium complex and *tetR* has a strong specificity, and no other antibiotics groups can induce *tetR* to end DNA binding to the *tetA* promoter.[50,51] Therefore, these two biosensors should be free of fluorescence interference from other groups of antibiotics.

3.2. Biosensor Fluorescence Response to Tetracyclines in LB Medium. The constructed biosensors had a dose−effect relationship with tetracyclines, and the fluorescence intensity of the biosensors increased nonlinearly with increasing tetracycline concentrations in the LB medium (Figure 2a). The time-dependent fluorescence of two biosensors was approximated by a sigmoidal function (Figure 2b). Longer incubations (up to about 12 h) resulted in higher accumulation of fluorescent proteins and thus potentially a lower detection threshold. The exposure to >250 μg/L tetracyclines for 12 h can induce *E. coli* DH5α/pMTGFP and *E. coli* DH5α/pMTmcherry to produce fluorescence that can be observed with naked eyes under blue light (Figure 2c). For a fast detection method, incubation time ideally should be shorter. Thus, on the basis of the above results, the incubation

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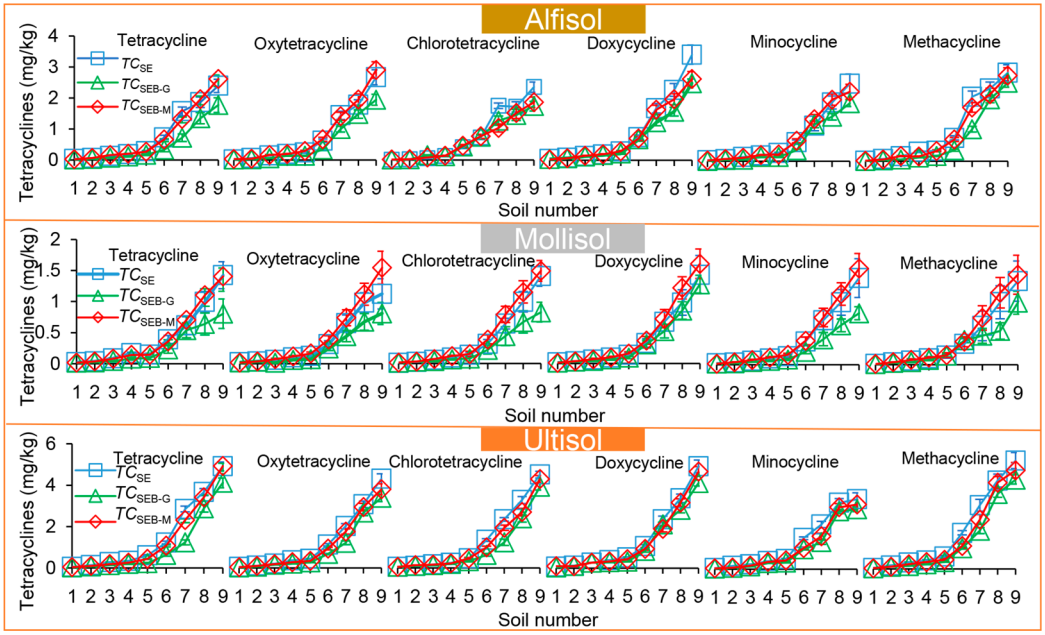

Figure 3. Concentrations of EDTA-extractable tetracyclines in soils by the chemical HPLC method and the biosensor method. TC_{SE}, TC_{SEB-G}, and TC_{SEB-M} are the concentrations measured by the HPLC method, *E. coli* DH5α/pMTGFP, and *E. coli* DH5α/pMTmCherry, respectively. Soil numbers (1−9) denote soil samples spiked with 0.10−10.0 mg/kg concentrations of tetracyclines.

time of 4.5 h at the middle of the sigmoidal curve was selected to expedite the analytical time. After 4.5 h of incubation, *E. coli* DH5α/pMTGFP and *E. coli* DH5α/pMTmcherry, exposed to >5 μg/L tetracycline, showed visible fluorescence in the concentration range of 10−10000 μg/L, as revealed by confocal fluorescence microscope images (Figure 2d). The two biosensors had a similar fluorescence response for other tetracyclines (Figures S3 and S4), suggesting the versatility of the biosensors to quantify various tetracyclines. Next, the new biosensors were first demonstrated to be capable of measuring tetracyclines in the LB medium as described in the Supporting Information (Figure S5 and Table S7).

3.3. Whole-Cell Biosensors for Quantification of Tetracyclines Added to Soil Extracts. We further obtained the standard curves of six tetracyclines in soil extracts using the constructed biosensors and calculated relevant method parameters. Taking the soil extracts of the Alfisoil as an example (Figure S6), there were significant correlations (P < 0.010) observed between biosensor fluorescence intensity and tetracycline concentrations in the EDTA-soil extracts. The logarithmic concentration of each tetracycline type in the soil extracts as a function of fluorescence intensity follows the equations

$$\log TC_{SEB} = \alpha F \quad (1)$$

or

$$TC_{SEB} = 10^{\alpha F} \quad (2)$$

where TC_{SEB}, F, and α are the concentration of each tetracycline in the soil extracts, the fluorescence intensity detected by the biosensors, and the slope of standard curves, respectively. Using the fluoresence intensity reading of the biosensors from the 96-microwell plate and eq 2, TC_{SEB} can be then calculated by F and α (Table 1) obtained from the standard curves.

This new method had comparable detection limits of tetracyclines and wide linearity ranges comparable to those of the HPLC (Table 1). For example, in the EDTA-extract of the Alfisol, good linearity ranges of 75−5000 and 50−7500 μg/L were found for *E. coli* DH5α/pMTGFP and DH5α/pMTmcherry (P < 0.010, Table 1) for six tetracyclines, respectively. *E. coli* DH5α/pMTmcherry had a slightly broader linearity range than *E. coli* DH5α/pMTGFP. The detection limits in the soil extracts were between 5.32 and 10.2 μg/L for all types of soils and tetracyclines, specifically 7.99−10.2, 8.23−9.98, and 7.58−9.36 μg/L for *E. coli* DH5α/pMTGFP and 5.56−7.66, 5.78−7.85, and 5.32−7.37 μg/L for *E. coli* DH5α/pMTmcherry in the EDTA-extracts of Alfisol, Mollisol, and Ultisol, respectively (Table 1). Overall, *E. coli* DH5α/pMTmcherry had a slightly lower detection limit than *E. coli* DH5α/pMTGFP in the soil EDTA-extracts. Taken together, this study established a fast, easily parallelizable detection method for tetracyclines in soil extracts using the whole-cell biosensors. Next, this method was used to analyze the EDTA-extractable tetracyclines in spiked and field soil samples.

3.4. Quantification of EDTA-Extractable Fraction of Tetracyclines in Soils Using Whole-Cell Biosensors. To evaluate the efficacy and performance of the whole-cell biosensor method, the measurement of the EDTA-extractable fraction of tetracyclines in spiked soil samples (TC_{SEB-G} and TC_{SEB-M}) by *E. coli* DH5α/pMTGFP and *E. coli* DH5α/pMTmCherry were compared to those (TC_{SE}) measured by conventional HPLC analysis[10] as described in the Supporting Information and illustrated in Figure S2. As shown in Figure 3, the TC_{SEB-M} values measured by *E. coli* DH5α/pMTmCherry were closer to TC_{SE} than the TC_{SEB-G} values measured by *E. coli* DH5α/pMTGFP. The line slopes for the measurements by *E. coli* DH5α/pMTGFP were significantly different with those of the HPLC method in the Alfisol and Mollisol soils (P < 0.05), whereas no significant difference for *E. coli* DH5α/pMTmCherry (P > 0.05) was found except for the detection of extractable deoxytetracycline in the Alfisol and methacycline in the Mollisol (Table S8). Taking the extractable fraction of tetracycline in the Alfisol soil as an example, TC_{SE}, TC_{SEB-G},

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Table 2. Method Performance for Measuring the Extractable Fraction of Tetracyclines in Spiked Soils by the Biosensors

Soils	TCs	*E. coli* DH5α/pMTGFP			*E. coli* DH5α/pMTmCherry			Chemical method	
		RSD (%)	DL (μg/kg)	DC (%)	RSD (%)	DL (μg/kg)	DC (%)	RSD (%)	DL (μg/kg)
Alfisol	TC	2.54–63.3(45.1)	8.44	1.88–25.9 (10.5)	3.25–23.1(8.89)	5.65	1.18–15.3 (8.09)	1.52–8.18 (2.84)	1.54
	TC_1	3.21–45.7(34.0)	10.2	4.74–34.2 (20.4)	2.05–37.2(18.6)	7.36	3.80–25.3 (13.4)	2.18–6.72 (4.49)	1.97
	TC_2	1.13–75.6(51.9)	8.32	0.121–24.5 (12.5)	0.982–45.2(22.2)	5.56	0.582–34.1 (18.8)	1.44–24.1 (14.0)	1.43
	TC_3	0.280–35.1(21.3)	7.99	0.899–56.0 (33.2)	3.13–20.7(9.91)	6.45	1.06–8.91 (6.17)	0.369–5.12 (2.33)	1.56
	TC_4	5.14–46.6(32.8)	9.58	3.19–33.9 (18.9)	0.575–5.32(2.98)	7.66	3.31–20.1 (11.2)	1.75–20.6 (9.02)	2.11
	TC_5	7.15–51.0(31.6)	8.66	2.78–31.1 (16.57)	1.59–11.8(8.60)	7.19	0.313–10.0 (4.46)	3.98– 15.8(10.1)	1.34
Mollisol	TC	3.45–53.3(21.5)	8.68	0.940–73.3 (46.2)	0.206–7.15(4.29)	5.78	0.435–37.1 (21.5)	0.732–29.4 (14.7)	2.63
	TC_1	4.44–61.1(33.9)	9.25	0.476–79.0 (47.7)	0.127–24.4(15.9)	7.68	0.943–48.5 (31.9)	4.61–23.8 (12.3)	3.84
	TC_2	3.27–61.4(42.7)	8.63	1.35–84.9 (53.2)	1.76–39.7(21.0)	5.88	0.975–68.1 (42.7)	1.43–14.5 (6.08)	2.23
	TC_3	2.88–57.8(44.8)	8.23	1.71–70.3 (40.8)	1.01–38.4(19.7)	6.77	1.06–22.1 (13.5)	3.36–33.1 (16.1)	2.47
	TC_4	1.08–48.7(19.8)	9.98	0.648–67.4 (39.0)	1.27–39.3(25.8)	7.85	0.608–19.8 (10.5)	0.29–17.2 (8.24)	3.10
	TC_5	0.787–34.6(19.5)	8.69	0.091–67.9 (33.3)	5.34–46.1(25.6)	7.28	0.273–8.88 (4.37)	1.92–23.7 (13.0)	3.00
Ultisol	TC	5.68–33.9(26.2)	8.05	1.61–13.9 (6.77)	1.81–5.67(4.29)	5.32	1.00–12.1 (4.66)	0.900–21.1 (9.77)	0.907
	TC_1	3.54–55.4(39.8)	8.61	0.448–22.3 (9.85)	3.97–32.1(17.8)	7.26	3.26–9.26 (4.58)	7.56–15.8 (13.0)	1.52
	TC_2	5.29–67.0(49.7)	8.04	3.32–41.8 (20.6)	1.56–26.4(15.9)	5.47	1.12–23.3 (10.7)	0.998–20.3 (11.1)	1.06
	TC_3	5.66–35.6(19.2)	7.58	2.22–44.9 (23.3)	0.275–23.8(7.30)	6.22	1.80–10.4 (5.26)	0.115–13.3 (7.02)	1.29
	TC_4	8.98–63.5(43.7)	9.36	0.372–36.1 (17.0)	0.444–22.7(11.3)	7.37	0.223–8.26 (4.05)	0.426–11.4 (7.08)	1.73
	TC_5	0.588–53.5(39.1)	8.22	0.446–26.1 (11.1)	0.100–12.3(5.93)	7.09	0.226–10.0 (5.17)	0.077–2.70 (1.59)	1.37

TCs, TC, TC_1, TC_2, TC_3, TC_4 and TC_5 are total tetracyclines, tetracycline, oxytetracycline, chlorotetracycline, deoxytetracycline, minocyline, and methacycline, respectively. RSD, DC, and DL are relative standard deviation, the deviation between the values determined by the chemical HPLC method and the biosensor method, and detection limit, respectively.

and $TC_{SEB\text{-}M}$ were 0.028 ± 0.002, 0.031 ± 0.003, and 0.035 ± 0.003 mg/kg for Soil 1, and 2.39 ± 0.217, 1.77 ± 0.134, and 2.61 ± 0.237 mg/kg for Soil 9, respectively (Figure 3). A linear correlation exists between the $TC_{SEB\text{-}G}$ and $TC_{SEB\text{-}M}$ values and the TC_{SE} values (Table S8). The total concentration of tetracyclines (TC_{STC}) in spiked soils had significant linear correlations ($R^2 > 0.900$, $P < 0.05$) with the $TC_{SEB\text{-}G}$ and $TC_{SEB\text{-}M}$ values (Figure S7).

The concentrations of the EDTA-extractable tetracyclines (TC_{SEB}) in soils, measured by the biosensors, deviated from the TC_{SE} measured by the HPLC method, and the deviation (DC) was calculated as $DC = \frac{TC_{SE} - TC_{SEB}}{TC_{SE}} \times 100\%$ (TC_{SE} denotes the EDTA-extractable tetracyclines in soils measured by the HPLC method). Table 2 also shows that the mean DC values for *E. coli* DH5α/pMTGFP were less than 51.9% for the Alfisol, 44.8% for the Mollisol, and 49.7% for the Ultisol, whereas the mean DC values for *E. coli* DH5α/pMTmCherry were less than 22.2% for the Alfisol, 25.8% for the Mollisol, and 17.8% for the Ultisol, respectively. The mean values of relative standard deviation (RSD) of the extractable tetracyclines in the spiked soils measured by *E. coli* DH5α/pMTGFP were 24.5–51.9%, 19.5–44.8%, and 19.2–49.7%, and those measured by *E. coli* DH5α/pMTmCherry were 2.98–22.2%, 4.29–25.8%, and 4.29–17.8% for the Alfisol, the Mollisol, and the Ultisol, respectively (Table 2). The DC and RSD values for the extractable tetracyclines in the test soils measured by *E. coli* DH5α/pMTmCherry were lower than those measured by *E. coli* DH5α/pMTGFP.

Additionally, the RSD values (2.98–25.8%) for the extractable tetracyclines measured by *E. coli* DH5α/pMTmCherry were close to those of the HPLC method (1.59–16.1%, Table 2). However, those measured by *E. coli* DH5α/pMTGFP (19.2–51.9%) were substantially greater than those by the HPLC method. The detection limits of the EDTA-extractable tetracyclines in soils was calculated as $DL = \frac{DL_{SE} \times 1\ mL}{1\ g}$ (DL_{SE} is the detection limit of tetracyclines in soil EDTA-extracts). The detection limit (5.32–10.2 μg/kg, Table 2) of the biosensor method was greater than that of the HPLC method (0.91–3.84 μg/kg, Table 2).

The two biosensors were further tested for soils that were contaminated with tetracyclines due to waste amendment. Using the HPLC, the extractable tetracycline concentrations in Soil A and Soil B were 158.7 ± 44.0 and 249.0 ± 46.9 μg/kg, respectively (Table S9). The extractable tetracycline concentrations measured by *E. coli* DH5α/pMTmCherry were 146.3 ± 55.2 μg/kg for Soil A and 216.6 ± 47.8 μg/kg for Soil B, which were very similar to those by the HPLC method and greater than those by *E. coli* DH5α/pMTGFP (128.9 ± 64.3 μg/kg for Soil A and 169.9 ± 87.4 μg/kg for Soil B). The RSD values of the extractable tetracyclines in Soil A and Soil B for *E. coli* DH5α/pMTmCherry (20.2% and 37.7%) were close to the RSD values of the HPLC method (18.8% and 27.7%) and less than the RSD values for *E. coli* DH5α/pMTGFP (49.9% and 51.4%) (Table S9). The DC values of the extractable tetracyclines in two field soils measured by *E. coli* DH5α/pMTGFP were 18.8% for Soil A and 31.8% for Soil B, and those measured by *E. coli* DH5α/pMTmCherry were 7.81% for Soil A and 13.0% for Soil B (Table S9). The above results showed that the two biosensors can be used for measuring tetracycline residues in contaminated field soils, and *E. coli* DH5α/pMTmCherry had greater accuracy and precision than *E. coli* DH5α/pMTGFP.

4. DISCUSSION

The fluorescence responses of the two biosensors were linearly correlated to the logarithmic concentrations of tetracyclines (Figure 2a, Figure 3, and Figure S6), in agreement with the study of Baumann et al. and Stocker et al. on whole-cell biosensors.[52,53] Kalinin et al. also observed that the receptor expression level followed the logarithm of drug's ligand concentrations in *E. coli*,[54] resulting in the linear correlation of the cell sensibility with the logarithmic concentrations of chemical effectors. Deviation from the semilogarithmic

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regression line was observed at the low concentrations (Figure 3 and Figure S6), probably due to fluorescence interference of other background soil constituents at the low concentrations of tetracyclines as well as the bias of the high concentration data in the regression. This deviation is not unexpected for a linearity of 4 orders of magnitude and was also observed by Baumann et al. and Trang et al.[53,55] The r values of 0.820−0.953 were lower than that of the typical HPLC methods but comparable to the r values of other whole-cell biosensor methods (~0.939). Therefore, our standard curves were acceptable for the whole-cell biosensors.

Our constructed biosensors are also capable of estimating the bioavailable concentrations of tetracyclines extracted by EDTA. The bioavailable fraction of tetracyclines in soils is part of the total tetracyclines and directly impacts living organisms.[56] The unextractable tetracyclines in soils could be potentially bioactive[12] but are difficult to measure using the whole-cell biosensors as they cannot be easily extracted, and other organic solvent extraction may need further cleanup (thus increasing analytical time and cost). While it is not certain that the EDTA-extractable fraction of tetracyclines is fully bioavailable, it is a widely used indicator for estimating the bioavailability of tetracyclines in soils.[57,58] In fact, the significant correlations between the extracted tetracyclines measured by the HPLC and the biosensor method (Table S8) support the adequacy of this approach. The concentrations of EDTA-extractable tetracyclines measured by *E. coli* DH5α/pMTmCherry were closer to those measured by the HPLC method than those measured by *E. coli* DH5α/pMTGFP (Table 2, Table S8, and Table S9), suggesting that the EDTA-extractable tetracyclines using *E. coli* DH5α/pMTmCherry are a better indicator of the extractable and bioavailable fraction than that measured by *E. coli* DH5α/pMTGFP. The lower peformance of *E. coli* DH5α/pMTGFP may resulted from the fluoresncene interference of soil organic matter. Previous studies reported that the optimal fluorescence peaks of natural organic matter in soil were located at 200−500 nm excitation wavelength and 300−450 nm emission wavelength,[59,60] which are close to the detection wavelengths of green fluorescence (488 nm excitation and 511 nm emission) of *E. coli* DH5α/pMTGFP but well separated from red fluorescence of DH5α/pMTmCherry (587 nm excitation and 611 nm emission).[61] Thus, *E. coli* DH5α/pMTmCherry had better performance because its excitation and emission wavelengths had less background interference. Additionally, the precision of detection for tetracyclines in soil extracts by *E. coli* DH5α/pMTGFP is worse than that of *E. coli* DH5α/pMTmCherry (Tables 1 and 2). Therefore, the latter is a better choice for estimating bioavailable tetracyclines in soils.

Like any other method for chemically complex soil samples, the biosensor method provides the ability to estimate the ecotoxicological risks of tetracyclines in soils.[19,56] Our biosensor method builds on the existing extraction method but substantially increases the analysis throughput using the 96-microwell plate and reduces the analytical cost associated with advanced instrumentation such as HPLC and MS. The detection limits of our biosensors (5.32−10.2 μg/kg, Table 2) are far below acceptable daily intake (20 μg/kg) set by the US Food and Drug Administration (FDA) and the tolerance limits (>100 μg/kg) established by the European Medicines Agency[62] for the soil assessment of medicinal products. Furthermore, these detection limits were lower than those of the lux biosensor method for milk (2−35 μg/L)[16] and those of the bioluminescent sensor method for fish tissue (20−50 μg/kg).[38] Soil extracts may contain metal ions and other antibiotics that may influence the biosensor analyses by either complexing with tetracyclines and inhibiting biosensor cell growth, respectively. EDTA used in our method could suppress the interference of metal ions because complexation constants (log K) of Ca and Mg with EDTA (8.79 and 10.65) are much greater than those with tetracyclines (3.9 and 5.8), and metal ions would prefer complexing with EDTA over tetracyclines.[63,64] The potential interference of other antibiotics would be minimal if their extractable concentrations were less than the minimum inhibitory concentrations of *E. coli* (e.g., 160 μg/L for ciprofloxacin, 320 μg/L for ceftazidime, 500 μg/L for gentamicin, 250 μg/L for trimethoprim, and 2000 μg/L for colistin).[65,66] As extractable concentrations of antibiotics in soils vary substantially, other antibiotics may potentially inhibit the growth of the biosensor cells, which may be compensated by a longer incubation period. Overall, our biosensor method is considered sensitive and relatively inexpensive for determining tetracyclines in soils.

The HPLC methods have a comparable analytical performance for the analysis of tetracyclines in soils and groundwater. Hamscher et al.[10] reported the detection limit of <5 μg/kg for each tetracycline in soils applied with liquid manure, and Zhu et al.[31] obtained a detection limit of 0.20−0.28 μg/L for tetracyclines in groundwater. In addition, Schlüsener et al. reported that the detection limits using pressurized liquid extraction and LC-MS were 1.40, 1.40, 1.00, 5.30, and 0.60 μg/kg for erythromycin, oleandomycin, roxithromycin, salinomycin, and tiamulin in soils, respectively.[67] Thus, the detection limits (5.32−10.2 μg/kg) of our biosensor method were greater than those of the HPLC methods. Using *E. coli* DH5α/pMTmCherry, the results of RSD (2.98−25.8%) for the EDTA-extractable fraction of tetracyclines were similar to the precision of the HPLC method (2.1−28.8%).[10,68] Therefore, the two biosensors (particularly *E. coli* DH5α/pMTmCherry) can be used to accurately measure tetracyclines in soils. We should also note that the biosensor method was first tested in the LB media that were spiked with tetracyclines (Table S7), achieving the detection limits of 4.87−9.81 μg/L and a linear range (50−7500 μg/L). Thus, this biosensor method can potentially be used to measure tetracyclines in water samples.

As the biosensor methods use the 96-microwell plate (Figure S1), this method has substantially greater throughput than the HPLC method. When using larger (e.g., 384- or 1536-microwell plates) or multiple 96-microwell plates, the throughput of the biosensor method can be further increased, although autodispensing systems would be ideal for 384- or 1536-microwell plates. For one 96-microwell plate, the test can be performed within 6 h with little preparation (e.g., with readily available freeze-dried cells) compared to the HPLC method that requires at least 40 h for 96 samples.[69] HPLC or HPLC-MS has a single detection per run, but the biosensor method can rapidly and simultaneously detect >96 samples in parallel.[10,68] Additionally, HPLC requires a large volume of organic reagents for extraction and separation and has a high capital and operation cost.[70] The biosensor method, in contrast, does not use organic solvents and relies on more affordable fluorescence plate readers. With parallel analysis capability and low analytical cost, and no need for organic solvents, the biosensor method for the EDTA-extractable fraction of tetracyclines in soils may be more suitable and cost-

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effective than the HPLC method for large-scale monitoring studies especially in the low- and middle-income countries.

The biosensor method does have some limitations. The fluorescence response to the dose of each tetracycline was different (Table 1), and the biosensors cannot differentiate the responses among the six tetracyclines.[16,17] Therefore, the biosensor method cannot specifically measure the concentration of a particular tetracycline type. Also, the biosensors can only be used to measure tetracyclines, and the measurement of other antibiotics and organic contaminants would require the construction of new biosensors. In comparison with the LC-MS method that can use isotope-labeled internal standards to improve analytical accuracy, the whole-cell biosensors cannot differentiate isotope signatures of antibiotics and thus can only be improved by optimizing reporter proteins (e.g., GFP vs mCherry), sensitizing cells (using EDTA and polymyxin B), and incubation conditions and duration. Overall, the method development and testing reported herein describes a working approach that can be readily applied to developing suitable biosensors for the detection of many other antibiotics.

5. ENVIRONMENTAL IMPLICATIONS

To our knowledge, this is the first time that fluorescent biosensor strains have been used for fast and parallel quantification of extractable tetracyclines in soils. The biosensor method is inexpensive to use, can be performed quickly with little preparation, and has parallel analysis capability. The biosensor method had similar sensitivity and precision as those of the HPLC method. In particular, *E. coli* DH5α/pMTmCherry was more sensitive and accurate than *E. coli* DH5α/pMTGFP. Once validated with more soil types, the whole-cell biosensors have the potential of monitoring bioavailable and leachable fractions of tetracyclines in a large set of soil samples and thus facilitating the assessment of ecological and health risks from antibiotic pollution. This method is limited to the detection of tetracyclines, and similar approaches could be taken to develop biosensors for other antibiotics. Further work should be conducted in more diverse field soil samples by designing large-scale studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.9b04051.

Chemicals, strains, cultures, and soils; biosensor plasmid construction and induction test; preparation of tetracyclines-spiked LB and EDTA-extracts of soil samples; HPLC method for measuring tetracyclines in soil extracts; whole-cell biosensors for detection of tetracyclines in the LB medium; comparison of EDTA-extractable tetracyclines in soils determined by the HPLC and biosensor methods; diagram of the biosensor method for detecting tetracyclines; diagram of extraction and analysis of tetracyclines in soils; concentration-dependent fluorescent signal curve; images of biosensors under blue light; standard curves; correlations between extractable fractions measured by two biosensors and total concentrations of antibiotics; chemical structure and selected properties of six tetracyclines; physico-chemical property of Alfisol, Mollisol, Ultisol, Soil A, and Soil B; strains and plasmids used in this study; primers used; detection parameters of six tetracyclines; correlation parameters; extractable tetracycline quantitation (PDF)

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Notes

The authors declare no competing financial interest.

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