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A gas reporting whole-cell microbial biosensor system for rapid on-site detection of mercury contamination in soils

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ABSTRACT

As optical reporting elements, fluorescent proteins are extensively used in whole-cell microbial biosensors. However, the use of these optical reporters is limited in opaque media such as soil. This study described a method utilizing gas as a reporting signal that could be used for the rapid on-site detection of mercury in soil. In this biosensor, the MerR protein could capture mercury ions and then bind the promoter of the *efe* gene to initiate the synthesis of the ethylene (C_2H_4)-forming enzyme that produced the gas. The research showed that the mercury ion concentrations could be converted into C_2H_4 gas signals, which were quantified using a handheld C_2H_4 sensor. By optimizing the biosensor to improve its anti-interference ability in the system, it could detect mercury ion concentrations in the soil ranging from 0.2 to 20 mg/kg within 45 min, effectively reflecting whether the mercury pollution in the soil exceeded the limit standard. This study provides a simple, inexpensive, and portable method for the on-site detection of soil pollutants.

1. Introduction

In recent decades, mercury pollution has occurred extensively in soil, water, and even the atmosphere due to the expansion of industrialization. The mercury in soil and water can be absorbed and enriched by corn and vegetables, causing severe harm to the health of animals and humans. The development of effective detection methods will promote the management and remission of mercury pollution (Bruins et al., 2000; Giller et al., 1998, 1999; Müller et al., 2001). Biosensors provide a simple, fast, and cheap method for the detection of heavy metals. Of all the available types of biosensors, whole-cell microbial biosensors have received particular attention because they can achieve the specific detection of bioavailable mercury (Bontidean et al., 2004; Rasmussen et al., 2000; He et al., 2014), which is accepted to be a better indicator of risks than total chemical load (Peijnenburg et al., 2002; Shuttleworth and Cerniglia, 1995).

Generally, a whole-cell microbial biosensor possesses a genetic circuit composed of sensor genes and reporter genes. Several whole-cell microbial biosensors have been constructed for the detection of mercury using the MerR protein as a sensor, while employing fluorescence and bioluminescence as reporters (Cai et al., 2018; Mahbub et al., 2017; Priyadarshi et al., 2012; Selifonova et al., 1993), enabling the visualization of the detection results. However, these visual reporters can only be used in transparent materials, which is a limitation that prevents the use of whole-cell biosensors in many opaque mediums, such as soil (Cheng et al., 2018). In some studies, the researchers extract the mercury compounds from the soil using water, after which the leachable water fraction is submitted to the biosensors for a mercury detection assay (Bontidean et al., 2004; Rasmussen et al., 2000; He et al., 2014), (Cai et al., 2018; He et al., 2010; Ivask et al., 2002; Liao et al., 2006; Mahbub et al., 2017; Priyadarshi et al., 2012; Selifonova et al., 1993), (Guo et al., 2019). Some of the procedures to extract mercury from the soil are complex and challenging to perform. More importantly, the extraction rates are challenging to keep consistent in different soil samples. Consequently, the detection results can not fully reflect the actual mercury ion concentrations in the soil.

Gas reporters is an alternative reporting system for whole-cell microbial biosensors. Different from visual reporters, gas reporters enable

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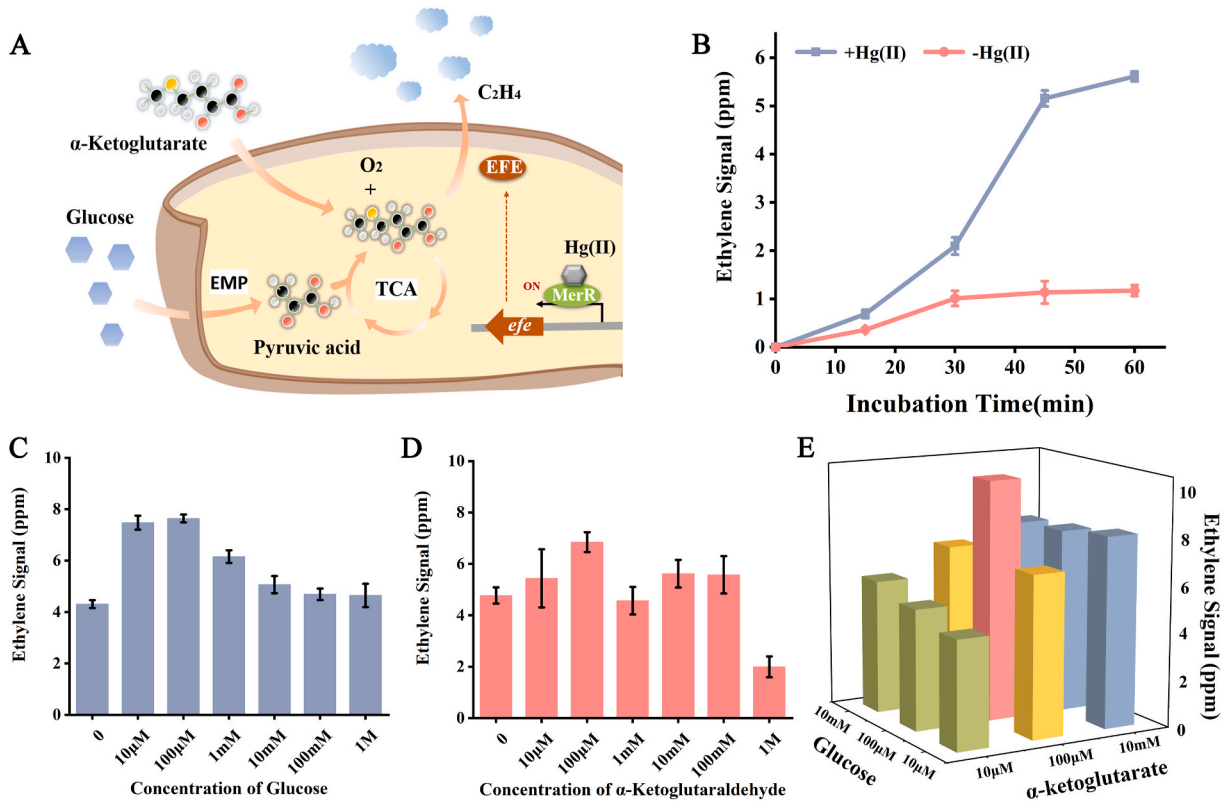

Fig. 1. The construction and optimization of the iEFE biosensor. (A) The principle of the iEFE biosensor. The MerR protein captures mercury ions in the cells, then binds to the promoter region of the *efe* gene and promotes the expression of the C_2H_4-forming enzyme that catalyzes C_2H_4 production. Exogenous glucose produces pyruvic acid through Embden-Meyerhof-Parnas (EMP) pathway, and pyruvic acid enters the tricarboxylic acid cycle (TCA) to produce α-ketoglutarate. Then α-ketoglutarate will react with oxygen to produce C_2H_4, CO_2 and H_2O through EFE enzyme. These substrates can increase C_2H_4 production. (B) The iEFE biosensor was incubated with 100 μM mercury solution or mercury-free water. (C–E) The effect of the exogenous addition of glucose (C), and α-ketoglutarate (D), and both (E) to the detection system on the C_2H_4 yield of iEFE biosensor.

the nondestructive monitoring of targets in opaque materials since gas can automatically escape from the materials (Borek et al., 2016; Cheng et al., 2016; Liu et al., 2019). Gas reporters have been applied to monitor the quorum sensing of gram-negative bacteria in soil (Cheng et al., 2018). In this study, the biosensor used methyl bromide (CH_3Br) and C_2H_4 dual gas reporters, with the former indicating the concentration of quorum signaling molecules, and the latter denoting the cell viability of the biosensor cells while normalizing the CH_3Br signals. However, due to the low gas production and complex gas composition, this biosensor system must use gas chromatography (GC) and mass spectrometry (MS) to analyze the results, limiting its on-site use.

The objective of this study is to construct a gas reporting biosensor system for the on-site detection of mercury in opaque materials by converting the mercury signal into a C_2H_4 signal. After improving the C_2H_4 production, a handheld C_2H_4 sensor can competently detect the C_2H_4 signal. Introducing a constitutive gas reporting biosensor into the mercury detection system enhances the anti-interference ability. This biosensor system can realize the on-site detection of bioavailable mercury in the soil in 45 min without the need for large instruments.

2. Materials and methods

2.1. Bacterial strain, oligonucleotides, and chemicals

Escherichia coli (E. coli) DH5α was used as chassis cells for all the plasmids in this study. The genes and oligonucleotides used in this study are listed in Table S1, Table S2 and Table S3. The genes were synthesized by Genewiz Inc. (South Plainfield, USA), while the oligonucleotide synthesis and plasmid sequencing were conducted by Ruibio Biotech (Beijing, China). The $HgCl_2$ (99.5%) was purchased from Xiya Reagent (Shandong, China).

2.2. Construction of the whole-cell mercury biosensor

The endonuclease digestion site modified with pENTR/D-TOPO (Thermo Fisher, Waltham, USA) was used as a plasmid backbone. To construct the mercury inducing iEFE biosensor, the *merR* gene (Brown et al., 2003) with promoter P479 was inserted into the plasmid backbone using *Xho*I/*Hin*dIII restriction enzyme digestion and a T4 ligation reaction. Then the *efe* gene (Fukuda et al., 1992a), which was codon-optimized, was inserted into the plasmid backbone together with the MerR-binding promoter (Brocklehurst et al., 2003), using *Kpn*I/*Xho*I restriction enzyme digestion and a T4 ligation reaction. To construct the constitutive cEFE biosensor, three candidate constitutive promoters, namely PJ23119, P637, and P699 (Brewster et al., 2012; Lucks et al., 2011), were respectively used as the promoter for the *efe* gene, and they were separately inserted into the plasmid backbone using *Kpn*I/*Hin*dIII. *E. coli* DH5α transformed with the constructed plasmids was cultured in Luria-Bertani (LB) broth, and stored with 50% glycerol at -80 °C until used.

2.3. The iEFE biosensor for the detection of mercury ion concentrations in solutions and soil

The biosensor cells were cultured until the OD_{600} reached 0.8 (Fig. S1). Then, 2.5 mL of the culture was mixed with 2.4 mL of fresh medium, and 100 μL of substrate solution (glucose and α-ketoglutarate), after which either 50 μL of the solution samples or 0.25 g of soil samples

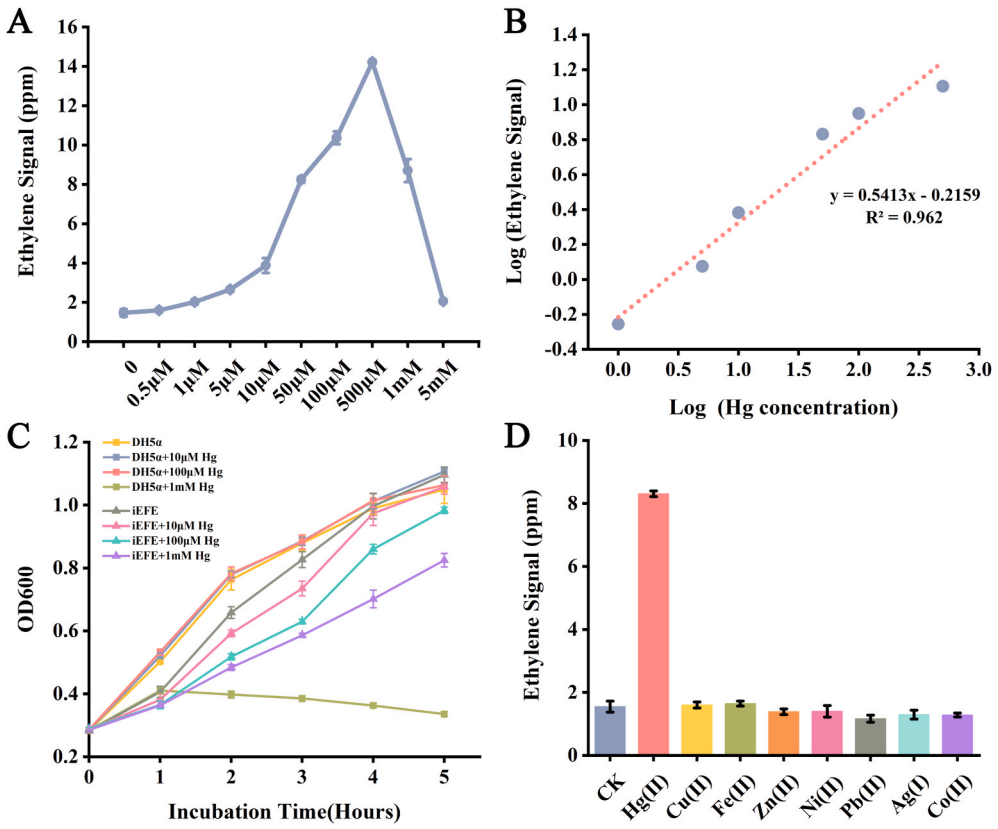

Fig. 2. The sensitivity and specificity of the iEFE biosensor. (A) The liquid detection system containing the iEFE biosensor was incubated with 50 μL mercury solution at different concentrations. (B) The logarithmic conversion of the detection range of the biosensor. (C) The growth curves of the iEFE biosensor and DH5α cells that were incubated in different concentrations of mercury ion solutions. (D) The iEFE biosensor was incubated with 50 μM of different kinds of heavy metal solutions.

were added to the detection system. After mixing thoroughly, the detection system was sealed in a vial with a rubber plug and incubated while shaking for 45 min. The C_2H_4 signal detection was conducted using a handheld C_2H_4 detector (Fig. S2) (Sundo Technology, uSafe3000), the probe of which was inserted into the rubber plug, and the C_2H_4 concentration above the liquid level in the vial was determined according to the manual. After the detection process, the biosensor cells were sterilized.

2.4. The iEFE/cEFE biosensor system for the detection of mercury ion concentrations in soil

A 0.5 g soil sample was suspended in 4.8 mL fresh medium. After thorough mixing, the suspension was evenly divided into two vials. Then, 2.5 mL of the iEFE biosensor culture (OD_{600} reached 0.8) and 100 μL of substrate solution were added to one vial, while 2.5 mL of the cEFE biosensor culture (OD_{600} reached 0.8) and 100 μL of substrate solution were added to the other. The vials were sealed with rubber plugs, and the subsequent detection process was the same as described previously.

2.5. Detection of mercury diffusion in soil

Mercury-free soil (not detectable by atomic fluorescence spectroscopy) was homogenized, dried, and divided into two portions. One portion was placed directly into a flowerpot, while the other portion was thoroughly mixed with activated carbon at a mass ratio of 10:1 and placed into another flowerpot. Then, 1 mL of 500 μM mercuric chloride solution was dropped in the center of the pot of soil. After 3 h at room temperature, soil samples were taken from different locations for mercury ion detection.

2.6. Data analysis

Each determination was repeated three times. The data were analyzed using one-way analysis of variance (ANOVA) followed by a *t*-test.

3. Results and discussion

3.1. Construction of the gas reporting whole-cell mercury iEFE biosensor

The MerR protein is a mercury-responsive transcription factor with a weak transcriptional inhibition without binding mercury ions and a strong transcriptional promotion after binding with mercury ions. The whole-cell iEFE biosensor was constructed by combining the mercury sensing effect of MerR with the expression of the *efe* gene in the *E. coli* DH5α cell chassis (Fig. 1A, Fig. S3). Furthermore, to obtain a preliminary evaluation of the function of the iEFE biosensor, 2.5 mL of the iEFE cultures (OD_{600} reached about 0.8) were mixed with 2.5 mL fresh medium, and incubated with 50 μL of the 100 μM mercury ions or mercury-free water. The C_2H_4 production of the iEFE biosensor was relatively low in the absence of mercury ion induction (Fig. 1B). However, in the presence of mercury ions, the biosensor produced a large amount of C_2H_4 in 45 min, after which the C_2H_4 production declined. These results indicated that the iEFE biosensor could convert mercury ion concentrations into C_2H_4 concentration signals. Furthermore, the iEFE biosensor exhibited responses to other inorganic mercury with different valences (Fig. S4).

Moreover, α-ketoglutarate and oxygen are the substrates for EFE-catalyzed C_2H_4 production (Fukuda et al., 1992b). Therefore, exogenous α-ketoglutarate or its upstream metabolite, glucose, were added to the detection system to improve the C_2H_4 signal efficiency of the iEFE

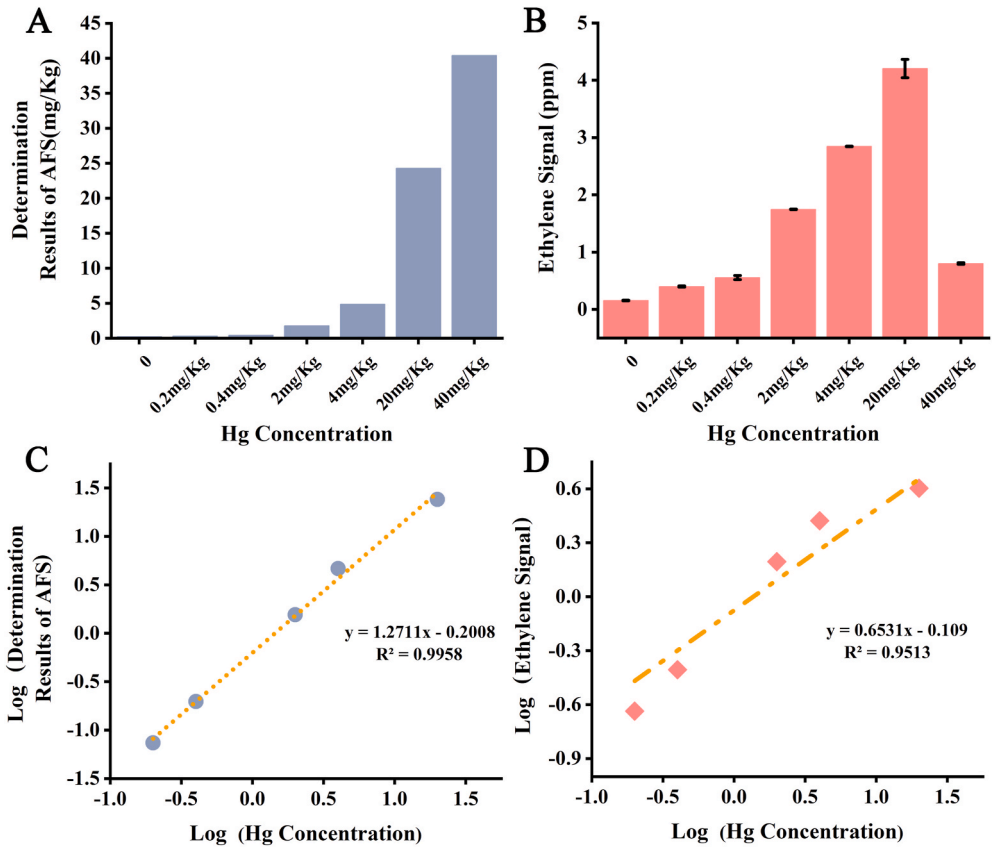

Fig. 3. A comparison of the determination results between AFS and the iEFE biosensor. (A) and (B) represent the results of the AFS and microbial sensor detection of mercury ions in the soil at concentrations of 0.2 mg/kg, 0.4 mg/kg, 2.0 mg/kg, 4.0 mg/kg, 20.0 mg/kg, and 40.0 mg/kg (C) and (D) represent the logarithmic conversion of the results mentioned above.

biosensor (Fig. 1A). The C_2H_4 yield tended first to increase and then decrease in conjunction with an increase in the concentration of the exogenously added glucose (Fig. 1C). The relationship between the concentration of the exogenously added α-ketoglutarate and C_2H_4 production was more complex (Fig. 1D), which may be because α-ketoglutarate is involved in many important metabolic pathways. The simultaneous addition of glucose and ketoglutarate synergistically increased C_2H_4 production, at an optimal combination of 100 μM glucose and 100 μM ketoglutarate (Fig. 1E). Compared with the iEFE biosensor without exogenous substrates, the C_2H_4 yield of the iEFE biosensor containing the optimal substrate combination increased about 2.1 times.

3.2. The assessment of the sensitivity and specificity of the iEFE biosensor

To evaluate the detection performance of the iEFE biosensor, the liquid detection system (2.5 mL iEFE cultures, 2.4 mL fresh medium, and 100 μL optimal substrate combination) were incubated with 50 μL of the mercury solution of different concentrations. When the concentration of the mercury solution reached 1 μM, the C_2H_4 production of the iEFE biosensor was significantly higher than that of the control group without mercury addition (Fig. 2A). The range of the iEFE biosensor in detecting the mercury ions was between 1 μM and 500 μM. After logarithmic conversion, there was a strong linear correlation ($R^2 = 0.962$) between the C_2H_4 production and the mercury ion concentrations within the detection range (Fig. 2B). Therefore, the mercury ion concentrations in this range can be calculated via C_2H_4 production. In order to verify whether the *efe* gene of the iEFE biosensor could be expressed to produce C_2H_4 in the presence of mercury ions, a qRT-PCR test was conducted and the results showed that the transcription level of *efe* had a linear relationship with the concentration of the mercury ions. The expression of *merR* gene was constant, which was consistent with our design (Fig. S5).

When the concentration of the mercury ions exceeded 500 μM, the C_2H_4 yield began to decrease. Excessive mercury ion concentrations, as well as excessive EFE expression and C_2H_4 production, can have a toxic effect on biosensor cells, which may be the reason why the sensor cannot accurately detect the concentration of mercury ions above 500 μM. The growth curve (Fig. 2C) of the sensor cells confirms this conjecture. When DH5α cells were incubated with either 10 μM or 100 μM mercury ions, the growth curve was not affected, while the cell growth was inhibited completely when they were incubated with 1 mM mercury ions. In contrast, the effect of the mercury ions on the iEFE cells were more moderate and gradual. The iEFE cells were more resistant to mercury ions at 1 mM than the DH5α cells due to the protective effect of the mercury-binding MerR protein (Qin et al., 2006). However, with an increase in the mercury concentration, the expression of EFE and the production of C_2H_4 increased, while the cell growth was gradually inhibited. Therefore, excessive mercury does have inhibitory effects on iEFE cell growth.

The iEFE biosensor shows excellent specificity for mercury ions. Copper ions (Cu^{2+}), ferrous ions (Fe^{2+}), zinc ions (Zn^{2+}), nickel ions (Ni^{2+}), lead ions (Pb^{2+}), silver ions (Ag^{+}), and cobalt ions (Co^{2+}) at concentrations of 50 μM were added to the detection system of the iEFE biosensor, respectively. The C_2H_4 signals induced by these metal ions were equal to or lower than that induced by the deionized water (Fig. 2D). Therefore, the detection of mercury ions by the iEFE biosensor is not disturbed by other heavy metal ions in the sample.

3.3. The gas reporting biosensor achieves the on-site detection of mercury contamination in soil

A series of soil samples, artificially contaminated with mercury ions

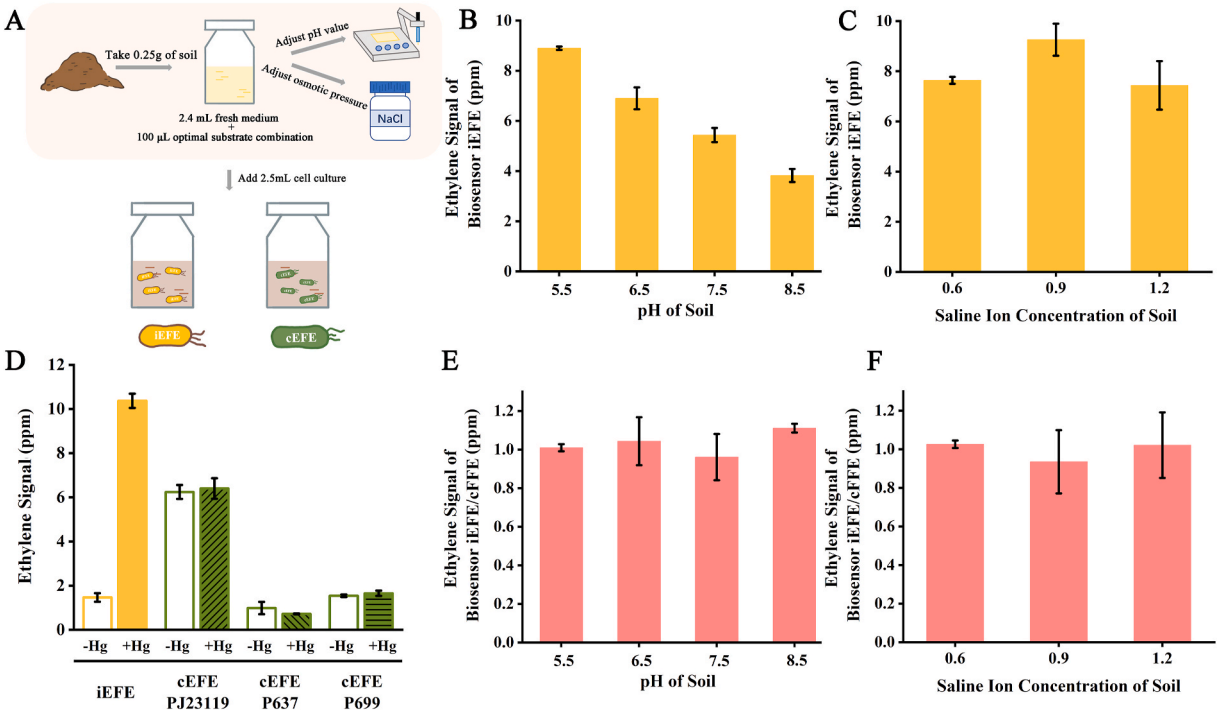

Fig. 4. The design of the iEFE/cEFE system for improving the anti-interference ability. (A) A flow chart for preparing soil samples to investigate the effect of pH and osmotic pressure on the cell viability and metabolic levels of biosensors. (B) and (C) represent the results of the pH and osmotic pressure in the soil on the iEFE biosensor induced by 500 μM mercury solution. (D) The iEFE biosensor and three cEFEs (cEFE-PJ23119, cEFE-P637, and cEFE-P699) were incubated with 500 μM mercury solution or mercury-free water. (E) and (F) denote the respective ratios of iEFE and cEFE during the determination of the pH and osmotic pressure induced by 500 μM mercury solution.

(0.2 mg/kg, 0.4 mg/kg, 2.0 mg/kg, 4.0 mg/kg, 20.0 mg/kg, and 40.0 mg/kg), were prepared to explore whether the iEFE biosensor could be used in soil (Fig. S6). The mercury ion concentrations in these samples were determined by both atomic fluorescence spectrometry (AFS) and the iEFE biosensor. The detection system of the iEFE biosensor for the soil included 2.5 mL iEFE cultures, 2.4 mL fresh medium, a 100 μL optimal substrate combination, and 0.25 g of the soil sample. Therefore, 0.2 mg/kg of mercury in the soil samples is approximately equivalent to 5 μM mercury ions in the liquid detection system. As shown in Fig. 3A, AFS exhibited excellent sensitivity and accuracy in detecting the mercury ion concentrations in the soil, also confirming the successful preparation of the artificially contaminated soil. The range of mercury ion concentrations in soil that the iEFE biosensor could respond to was 0.2–20 mg/kg (Fig. 3B). In addition, because the types of mercury detected by microbial sensors depend on the absorption of biological cells, the detection results of biosensors are more responsive to the level of biotoxicity of mercury ion contamination.

Many whole-cell biosensors for detecting mercury ions have been developed, and most of them use visual fluorescent proteins as reporting elements. Bontidean (Bontidean et al., 2004) used a fluorescent whole-cell biosensor to detect mercury contamination in the soil with the detection range of 80 μM-1 mM (3.2–40 mg/kg), but its minimum detection limit was higher than the limit standard of mercury ions in soil. A fluorescent whole-cell biosensor was employed to detect mercury ion pollution in environmental water samples, which responded to mercury ion concentrations of 10 nM–100 μM after 3 h of induction (Mohsen et al., 2017). In addition, Guo et al. (2020) reported a fluorescent whole-cell biosensor to detect mercury ion contamination in cosmetics with the detection range of 50 nM-10 μM after incubating for 2 h. In spite of their wide detection ranges, the detection time was a little long and large instruments were required. Compared with fluorescent whole-cell biosensors, the iEFE biosensor has no significant improvement at the detection limit. However, the detection range of the iEFE biosensor (5 μM–500 μM, or 0.2–20 mg/kg in soil) could perfectly cover the limit standard of mercury ions in soil (0.5–6 mg/kg). Besides, the iEFE biosensor can quickly and accurately determine whether mercury ion pollution in soil exceeds the limit standard in 45 min, and no large instruments were required.

3.4. *The introduction of a constitutive gas reporting cEFE biosensor into the mercury detection system improved its anti-interference ability*

The detection accuracy of whole-cell biosensors is closely related to their metabolic stability (Fig. 4A). Some of the properties in the soil, such as pH (Fig. 4B) and osmotic pressure (Fig. 4C), can affect the cell viability and metabolic level, which, in turn, exhibits non-negligible interference in the detection results. Therefore, to overcome this problem, a constitutive EFE-expressing biosensor, cEFE, was constructed to characterize cell viability. Three constitutive promoter candidates, namely P637, P699, and PJ23119, were used to control the expression of the *efe* gene (Fig. S3). None of the C_2H_4 production in any of the three biosensors was affected by the presence of mercury (Fig. 4D). The biosensor with PJ23119 as the promoter of the *efe* gene displayed higher C_2H_4 production and was therefore selected as the cEFE biosensor. Each soil sample was divided into two aliquots, one incubated with the iEFE biosensor and the other with the cEFE biosensor (Fig. 4A). Contrary to the detection result of the single iEFE biosensor, the C_2H_4 yield ratio of iEFE to cEFE remained relatively constant at different levels of soil pH (Fig. 4E) and osmotic (Fig. 4F) pressure. Therefore, this iEFE/cEFE dual-biosensor system can eliminate the effect of soil components on cell activity, and more accurately reflect the concentration of mercury ions.

3.5. *The iEFE/cEFE biosensor system used for monitoring the diffusion of mercury ions in the soil*

The iEFE/cEFE biosensor system can be widely applied in the

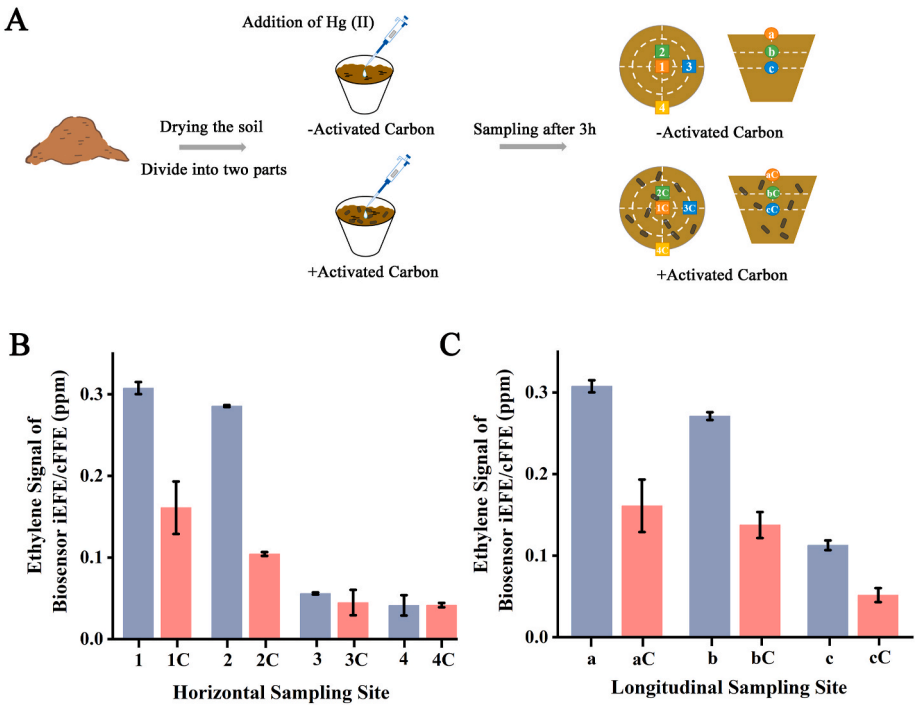

Fig. 5. Using the iEFE/cEFE biosensor system to study diffusion of mercury ions in soil. (A) A flow chart for studying the diffusion mechanism of mercury ions in soil. (B) The horizontal diffusion of mercury ions in the soil. (D) The results of the longitudinal diffusion of the mercury ions in the soil.

detection and mechanism research of mercury pollution in soil due to its simplicity, rapidity, low cost, and portability. One research topic that requires investigation is studying the diffusion mechanism of mercury ions in the soil, while exploring ways to slow down this process. As a case study, the effects of activated carbon on the diffusion rate of mercury ions in soil was analyzed using the iEFE/cEFE biosensor system (Fig. 5A). After dropping 1 mL of mercury solution at a concentration of 500 μM into the center of the soil in a pot, the mercury contamination rapidly diffused transversely and longitudinally within 3 h (Fig. 5B). The longitudinal diffusion occurred faster than the lateral diffusion, which could be attributed to the gravity of the solution. When activated carbon was added to the soil, the mercury ion concentration decreased at all monitoring points, indicating that activated carbon could adsorb mercury and reduce its bioavailability (Fig. 5C). As evidenced by the ratio of the mercury ion concentration at each monitoring point, as well as that at the center point, activated carbon can slow down the rate of both the lateral and longitudinal diffusion of mercury pollution. These results demonstrated the practical application value of the iEFE/cEFE biosensor system.

4. Conclusion

In this study, a novel method for the on-site detection of mercury pollution in the soil has been developed based on whole-cell biosensors. This method converts mercury content in soil into a C_2H_4 production signal and quantifies the results using a handheld detector. Since large instruments and complex pretreatment processes are not required, this method can be conducted at the sampling site, saving time and the cost of detection. This study has further demonstrated the significant potential of whole-cell biosensors in the field of on-site detection, while also emphasizing the essential role of synthetic biology in the design of the genetic circuits of the whole-cell biosensors.

CRediT authorship contribution statement

Yanger Liu: Methodology, Investigation, Resources, Writing - original draft. **Mingzhang Guo:** Methodology, Investigation, Resources. **Ruoxi Du:** Formal analysis. **Jiani Chi:** Method construction. **Xiaoyun He:** Supervision, Conceptualization. **Zixin Xie:** Data curation. **Kunlun Huang:** Project administration, Supervision. **Yunbo Luo:** Project administration, Writing - review & editing. **Wentao Xu:** Conceptualization, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2020.112660.

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