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Investigation on the stress response of microbes in acute toxicity assay

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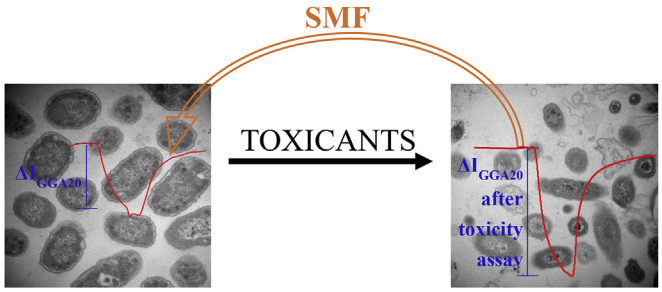
HIGHLIGHTS

- TOX-biosensor based on immobilized microbes was used for acute toxicity assay.
- Stress response of immobilized microbes was proved as a common phenomenon.
- The conditions and reasons that inducing stress response were investigated.
- Weak magnetic field can balance the activity of immobilized microbes.

GRAPHICAL ABSTRACT



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ABSTRACT

Acute toxicity assay by using microbes as bioindicators is an effective tool for reflecting the impact of water pollution on organisms. Until now, the suspended state of microbes is still mainly adopted, because the immobilized state of microbes will cause the poor stability of toxicity assay due to the microbes being reused. Here, an electrochemical biosensor based on biofilm was established for toxicity assay (TOX-biosensor), and the stress response of immobilized microbes in biofilm was demonstrated as the main factor that resulting in instability of the TOX-biosensor, and it can be induced by both of dose and time accumulation. The intensity of stress response for measuring a toxicant absence of organic substance was triple of that presence, indicating extracellular substances can be used for microbial repair and result in the decrease of the consumption of endogenous substance. Herein, a weak magnetic field (WMF) was employed to balance the activity of biofilm in the present TOX-biosensor, and a low intensity of WMF 10 GS was demonstrated to have the function for weakening stress response. Under this condition, the signals of biosensor can be kept stable for a month and the recovery time of dormant TOX-biosensor can be shorted from 50 h to 10 h. Its mechanism may be associated with the special magnetic field inside the organisms and the charge transfer happened in the life activity process.

1. Introduction

Today, large numbers of pollution were released into the water environment, which can be accumulated through the food chain, and resulting in amplified threaten for animal and human [1–3]. The toxicity assays, mainly based on chemical and biological methods, have received attentions in recent decades [4,5]. Comparing with chemical method, biological method is more useful because it can directly give the organism's response toward toxicants [6]. A mass of ling organisms, including luminescent bacteria, algae, fish and water flea, etc. had been successfully used as bioindicators in toxicity assays [7–12]. Among them, microorganism is the most often used for acute toxicity assays due to its

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good stability, high sensitivity, low cost and high detection speed [4,13]. Based on its metabolism can be affected by toxicants, some acute toxicity assays were carried out by measuring the change of electrons produced by microbial respiratory chain as that the concentration of electronic acceptor can be detected by electrochemical methods. But in associated reports, the suspended state of microbes was commonly used, and their cultured process was needed before each experiment, resulting in increased workload and test cost.

The application of biofilm was an alternative way for replacing the suspended microbes [14–16]. But until now, biofilm has not been applied in practice for acute toxicity assays. Its long-term stability was the limiting element, which will be caused by the reuse of immobilized microbes in a long time measurement. Firstly, toxic substances can cause the damage to microbes, thereby, the measured signals could not be correct [5]. Secondly, microbes may get the tolerance to toxic substances during the long time measurement, and resulting in non-sensitive response of microbes toward toxicants [17]. Beyond that, the physiological state of microbes was changed after adding toxicants, which will cause the self-repair of microbes, thus induce the stress response of microbes.

In order to solve the problem of immobilized microbes, the characteristics of microbial cells themselves had been taken into consideration. It is well known that the metabolic process of microbes is accompanied by a fixed amount of electron transfer. On the other hand, charges can be moved in magnetic field according to Lorentz forces [18,19]. Therefore treating by the magnetic field can offer a promising approach for adjusting microbial activity [20]. Generally, all of the organisms have their own magnetism, as well as special magnetic field inside the organisms [21], which can be affected by additional magnetic field [22,23]. So magnetic field would be a potential way for adjusting microbial activity. For example, the magnetic field can be used for enhancing the biodegradation ratio [24], improving the performance of MFCs [25], accelerating electron transfer at the electrode-liquid interface [25,26], and aggregating electroactive species in the anodic biofilm [27]. But the physiology of magnetic biologic effect on microbes is not completely understood. Here, the mechanisms of the interactions between magnetic field and living organisms reported by previous work were summarized:

(1) The changes in the gene expression can introduce the pleiotropic effects of biological functions of living organisms [28].
(2) Impact on cell's structure, such as membranes, protein and liquid crystals and etc. can directly make cells to reorganise their cytoskeletons to avoid external strain [29,30].
(3) The impact on the anisotropic magnetic susceptibility compounds of cells can carry out the properties change of living organisms, such as foldectures, magnetosomes or carboxysomes [31].
(4) The intermediate action for ion-radical pairs can produce biologically important molecules needed for cell growth, such as Zeeman and hyperfine (HFI) interactions [32].
(5) Changes in the properties and components of media, such as wetting, pH, increased coagulation rates, settling of sediments, crystallization, can carry out the effect on organisms' properties [33].
(6) The electrodynamic interaction can be connected to the Hall effect and Lorentz force [34].

In the present work, stress response of immobilized microbes toward toxicants was investigated, and the effects of magnetic field on microbes and the conditions for stability of TOX-biosensor were explored.

2. Experimental section

Chemicals. NaCl, $K_2Cr_2O_7$, $Cd(NO_3)_2$, $Pb(NO_3)_2$, $HgCl_2$, $ZnCl_2$, $CuCl_2$, glucose and glutamic acid were obtained from Beijing Chemical Factory (Beijing, China). 3,5-dichlorophenol (DCP), 5-sulfosalicylic acid dehydrate, p-chlorophenol, diphenylamine, 9,10-diphenylanthracene and 2-chloroanthraquinone were purchased from Aldrich. Stock solutions used in the present work were shown in Table S1. The stock respiration substrate (RS) solution was prepared by mixing 1.5 g L^{-1} glucose and 1.5 g L^{-1} glutamic acid, which was described by APHA methods (APHA, 1997) and originally used as biochemical oxygen demand (BOD) standard solution for that both of the carbon source and nitrox source were contained. Here, this solution was also used as the standard RS solution due to its BOD value of 1980 mg L^{-1} (GGA1980) can be conversed to other certain diluted values, which was benefit for comparing the effect of RS. Unless otherwise stated, all of the reagents used in this work were analytical grade and used as received. All aqueous solutions were prepared with ultrapure water (>18 MΩ) from a Milli-Q Plus system (Millipore).

Biosensor. An oxygen-type biosensor was composed of a biofilm reactor (BFR), a flow system and an electrochemical system. The biofilm was deposited on Gel_{rGONR} that was prepared according to our previous report [35], and put into a plexiglass tube with 1 cm diameter and 2 cm high (Scheme S1). For biofilm culture, BOD seed (E-05466-00, Cole-Parmer) was seeded in LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl, pH 7) and continually circulated by a flow system at 37 °C. The temperature was kept by a thermostat (Zhicheng, China). The flow system was driven by a pump (BT100-1 J, Baoding Longer, China), three solenoid valves (RSD less than 4.95%, Fig. S1) and a dispensing controller (TPC 16-16TD, Duowei, China) to control the sampling sequence and run time of the sensor. The dissolved oxygen (DO) was used as the electrochemical probe due to its concentration will be changed by the microbial respirations. An electrochemical analyzer (CHI832, CHI Co., Shanghai, China) was used as the signal collector. For electroanalysis measurements of DO, a conventional three-electrode system was used, the potential of the gold electrode (2 mm) versus Ag/AgCl (saturated KCl) reference electrode was set at −700 mV, and Pt was used as the counter electrode [36,37]. The biosensor was kept in the flow system by continually pumping PBS (0.08 M KH_2PO_4 and 0.12 M Na_2HPO_4, pH 7) when it was not in use. All solutions were prepared with deionized water being sterilized.

WMF. A weak magnetic field (WMF) was obtained by installing a pair of permanent magnets (cuboids, 0.3 cm × 1 cm × 2 cm for each). The permanent magnets were attached to a pair of removable plates along the axis of the reactor, which can be used for adjusting the intensity of WMF (Scheme S1). Intensities of WMF were measured by a handheld gauss meter (Coliy G92, Germany).

Morphology studies. Ultrathin sections of microbial cells were prepared for transmission electron microscope (TEM) imaging. Firstly, biofilm samples were fixed by using 2.5% glutaraldehyde for 12 h and then 1% osmium tetroxide for 2 h at room temperature, and then dehydrated by a graded ethanol series (50%, 70% and 90%) for 15 min each, and then dehydrated by 100% ethanol for 3 times and 10 min each time. Samples were embedded into Epon 812 resin and polymerized for 8 h to obtain solid blocks. Ultrathin sections (50–70 nm) of the sample blocks were cut by a Leica UC 6 microtome (Leica, Vienna, Austria) with a diamond knife (Diatome, Switzerland). TEM images were obtained using an H-7650 TEM (Hitachi, Ibaraki, Japan) at 80 kV of accelerating voltage. For AFM images, the samples were placed on freshly treated sample stage with sterilized tweezer. All images were obtained from a tapping mode atomic force microscope MultiMode 8-HR (Bruker) with

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Probe Model: SCANASYST-FLUID+ was employed.

3. Results and discussions

3.1. Stress response in acute toxicity assays

An oxygen-type electrochemical biosensor for acute toxicity assay was established based on the biofilm deposed on Gel_{rGONR}, a gel prepared via hydro-thermal synthesis by using graphene oxide and neutral red as the initial materials [35]. The activity of microbes in the biofilm can be evaluated by the strength of microbial respiratory, and which will be affected by toxicants. The strength of respiratory can be reflected by measuring the concentration of DO in the test solution. An electrode system was connected into the sensor for recording the change of DO concentration. Thus, the acute toxicity of toxicants can be measured. As shown in Fig. S2a, the biosensor was kept by continually pumping PBS, and its steady electrochemical signal was recorded as blank. After adding GGA, a respiratory substrate, the electrochemical curve was declined due to the consumption of DO. And then, the signal will be recovered by adding toxicant DCP due to the inhibited effect of toxicant toward respiratory. But in some cases of toxicity assay, a real result was shown in Fig. S2b, the electrochemical signal was continually declined after adding toxicant, which means that the concentration of DO at electrode's surface was reduced continually. This result indicates that more electrons were produced via microbial respiratory caused by adding toxicant. Two reasons might be used to explain the enhancement of microbial respiration. One is that the DCP has acted as the respiratory substance and taken a part of microbial metabolism. The other one is that the DCP has played as an inducted factor to trigger the stress response of microbes. Thus, a large number of microbial self-repair were carried out by taking their autolysis chemicals as the substances of endogenous respiration, and producing a stronger respiratory consumption of DO than that in equilibrium. The aim of present work is to investigate the conditions and reasons for that the signal was continually declined after adding toxicants in the present testing system, and to explore a workable plan for resolving this problem.

Herein, the reason for the enhancement of microbial respiration caused by toxicant was explored by only adding DCP as the exogenous substance. The net currents (ΔIs) of adding substance was used to represent the change of the intensity of microbial respiration. As shown in Fig. 1a, ΔIs of DCP were decreased with the enhancing of DCP concentration. This result indicates that the intensity of microbial respiration was reduced by enhancing the concentration of DCP, so it has not acted as the respiratory substance, and the ΔIs were actually contributed by the endogenous respiration of microbes. Furthermore, six consecutive cycles in each day were operated by alternately adding GGA20 and DCP + GGA20. The ΔIs by only adding GGA20, a fixed concentration of respiration substance for judging the respiratory activity of biofilm, were recorded in Fig. 1b, and they were increased greatly with the expanding of experimental time. This result indicates that, with the stimulated action of DCP under a certain concentration and frequency, the respiratory activity of biofilm was increased distinctly. Thus, a stress response was happened by the present biofilm for its self-repair. The impact of DCP in toxicity assay can be further explored by TEM and AFM characterizations. As shown in Fig. 1c, the suspended microbe was used in toxicity assay, and its cells had been severely deformed even broken, indicating that the microbes were really damaged by DCP. Comparing the results by electrochemical methods and morphological characterization, it can be confirmed that toxic substance had harmed the structure of microbial cells and promoted its metabolic activity, synchronously. The reason for this phenomenon is that a large amount of endogenous respiration was carried out for repairing the microbial cells, meaning the stress response was happened to the microbes in the toxicity assay.

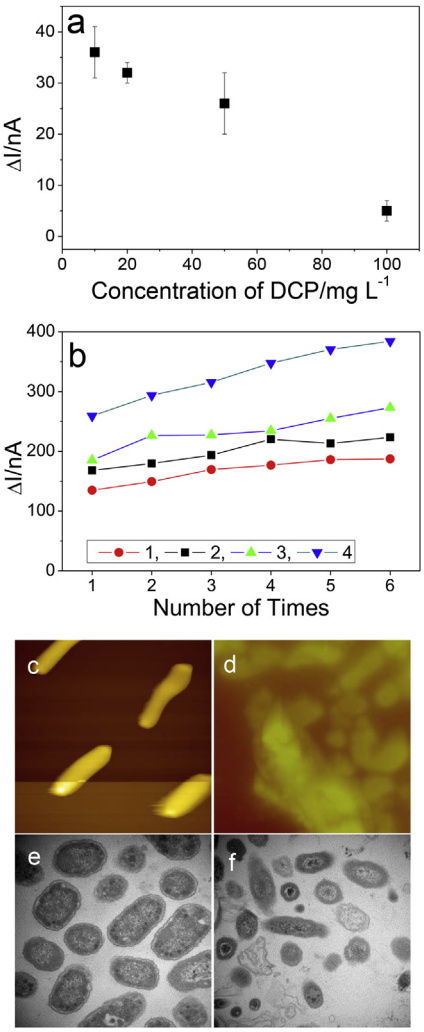

Fig. 1. (a) ΔI by only adding DCP as the exogenous substance. (b) Six consecutive cycles of toxicity test by adding GGA and GGA + DCP alternately, 1 to 4 indicate the signals of GGA in consecutive four days. AFM and TEM images of fresh microbes (c and e), and used for toxicity assay (d and f).

Furthermore, the performances of other toxicants, including heavy metal and organic compounds, were also investigated. As shown in Fig. S3, an overall trend of electrochemical results for all of the toxicants was that the stress response of microbes can caused by low-concentration toxicants. For the real detection of toxic substances, the performance of immobilized microbes is needed to be further experimented and adjusted.

3.2. Characterization of stress response

The conditions that will induce the stress response of microbes were investigated. As shown in Fig. 2, the catalytic ability of biofilm was evaluated by the signals of GGA20 that was added before and after each group of toxic test. As shown in Fig. 2a, four cycles of DCP were orderly pumped into the system with the concentration gradient of 10, 20, 50, 100 mg L^{-1}, and a single testing period with 60 min for continuously pumping 20 mg L^{-1} DCP was shown in Fig. 2b. There were almost no changes for the signals of GGA in the two cases. While the ΔI of GGA was increased from 104 nA to

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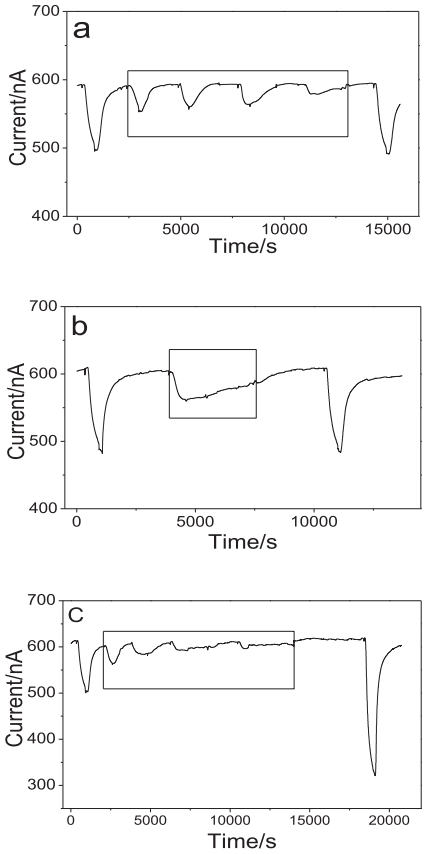

Fig. 2. Toxicity assays, in squares, (a) concentration gradient of DCP (10, 20, 50, 100 mg L^{-1}), 10 min for every concentration, (b) 20 mg L^{-1} DCP for 1 h and (c) 20 mg L^{-1} DCP for 10, 20, 40, 60 min. Before and after each toxicity test, GGA20 was measured for the three experiments.

297 nA for the test shown in Fig. 2c, that was carried out by orderly pumping 20 mg L^{-1} DCP for 10, 20, 40, 60 min. This result indicates that the stress response was stimulated by an accumulated dose with both of measuring time and frequencies. Which means that the repairing processes were performed with the accumulation of time and frequencies due to the persistent and continual destruction of microbial cells, thus, leading to a sharp increase of microbial respiration.

To consider that the stress response of microbes in the present system will also be influenced by the testing procedure, different sampling sequences were tested, and the diagrams and according electrochemical signals for two orders of sampling sequences were shown in Fig. S4. For these two procedures, the values of ΔI_{GGA} by procedure a were big than that by procedure b (Table S1), and the values of ΔI_{inPBS} of toxicant were obviously big than that of ΔI_{inGGA} (Fig. 3), suggesting the strength of microbial stress response obtained under present organic substance was lower than that absent. The ratio of $\Delta I_{inGGA}/\Delta I_{inPBS}$ was lower than 31%, and the ratio was reduced with the increasing of toxicant concentrations, which was induced by the opposite trend of ΔI's changes by using PBS and GGA as the blank control, separately. When PBS used as the blank control, the ΔIs were enhanced by increasing the concentration of toxicant, indicating repair of microbes being increased in this process. But for GGA used as the blank control, the ΔIs were reduced by increasing the concentration of toxicant, indicating that the stress response of microbes was gradually declined under the condition of present organic substance. The reason was that GGA has acted as the substance for microbial repair and the

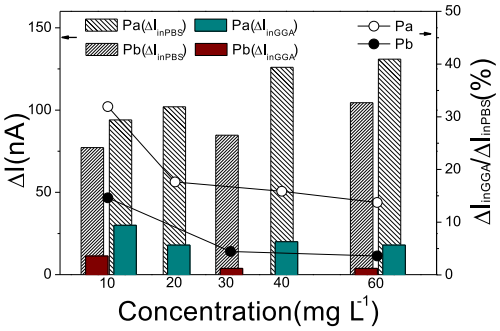

Fig. 3. Procedure a and b were abbreviated as Pa and Pb, respectively. ΔIs obtained by subtracting the signals of GGA + TOX from that of GGA were marked as ΔI_{inGGA}; by subtracting the signals of PBS + TOX from that of PBS were marked as ΔI_{inPBS}. The percent of $\Delta I_{inGGA}/\Delta I_{inPBS}$ for procedure a and b were shown by black circle and black dot, respectively.

consumption of endogenous substance was decreased, leading to a weak stress response. Beyond that, a general trend that the ΔIs obtained by procedure b were small than that obtained by procedure a. It can be seen from Fig. S4, a baseline recovery operation was inserted between the steps for adding of GGA and GGA + TOX in the procedure b. And in procedure a, GGA + TOX was directly added at the condition that the microbes with a high activity, so the stress response was more strong. All of the present results indicate that the stress response of immobilized microbes in toxicity assay has been affected by the conditions that will affect the microbial metabolic process.

3.3. *Effect of WMF on TOX-biosensor*

The monitoring signal of the present TOX-biosensor is based on the transmission of electron in microbial metabolic process. On the other hand, magnetic field can directly affect the movements of electron. Thus, a magnetic field has been adopted for regulating the stability of the present TOX-biosensor. Here, two biosensors were operated at the same conditions, but one of them was placed in WMF. As shown in Fig. 4a, the signal obtained by the biosensor in WMF was more stable. Furthermore, intensities of WMF were optimized for further applications. As shown in Fig. 4b, a low intensity of WMF (10 GS) has shown a beneficial effect for keeping the stability of biosensor, while higher intensities of WMF (50 GS and 100 GS), have promoted stress response of biofilm. The present result was some different from previous reports that high intensity of WMF can inhibit the physiological process of microbes [38]. This difference may be mainly caused by the different conditions that the toxicants were introduced in the present work. On the other hand, the different experiments, such as the place of biosensor in WMF, the volume of biosensor, etc. also be the elements that can effect on biofilm. For the present work, a low intensity magnetic field was adopted to conduct the balance of the microbial activity.

The action of WMF was further investigated by comparing the recovery ability of these two biosensors. Firstly, these biosensors were stored by only pumping PBS for two weeks and not been fed by any exogenous organic substrates. Then, GGA20 was added for activating the biofilm and restarting the biosensors. It can be seen in Fig. 5, for the biosensor in WMF, the signal was recovered within 10 h, but over 50 h were needed for the one not in WMF. This result indicates the activity of biofilm that in WMF is more stable. The reason for that WMF can keep the metabolic activity of microbes at a stable level has not been explored clearly yet. It is known that WMF is a weak physical element and its target site on microbes has not been clear, which can be affected by so many factors. The

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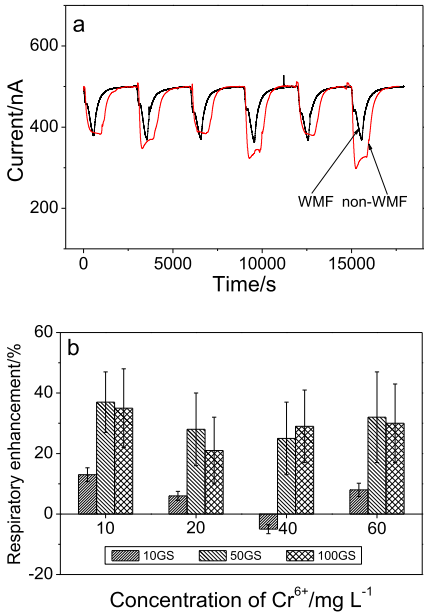

Fig. 4. (a) the electrochemical signals for alternatively injecting GGA20 and toxicant by the biosensors in WMF (black curve) and not in WMF (red curve). (b) biosensor in different magnetic intensities for toxicity assay by measuring Cr^{6+}. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

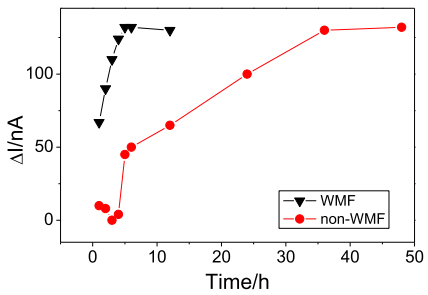

Fig. 5. The recovery process for the biosensors after kept in PBS for 2 weeks, in WMF (black triangles) and not in WMF (red dots). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

present phenomenon was associated with the special magnetic field inside the organisms and the charge transfer happened in the life activity process.

Herein, few relative theories were employed to discuss and explain the phenomenon of the present work. On the one hand, based on the above analysis, the TOX-biosensor has shown a stress response with the accumulation of reacted time and toxicants' dose, but it will be stable when placed it into a WMF. So the WMF has the ability to control the over-high metabolic activity of microbes and avoid the over growth of the biofilm in the sensor. It can be explained by the some conserved mechanisms of microbes to avoid stress response by magnetic field. For example, magnetic field can introduce microbe to avoid oxidative damages by over expressing of associated proteins. This mechanism will be beneficial for microbes to resist the over-high metabolic activities for repairing themselves [39]. And also, the destructive effects of magnetic field on organisms were mentioned by some reports that may be have the relationship for inhibiting the over growth of microbes [40]. On the other hand, the WMF also can enhance the renewable ability of biofilm in the restarting biosensor experiment. There are some reports about enhancement of substrate

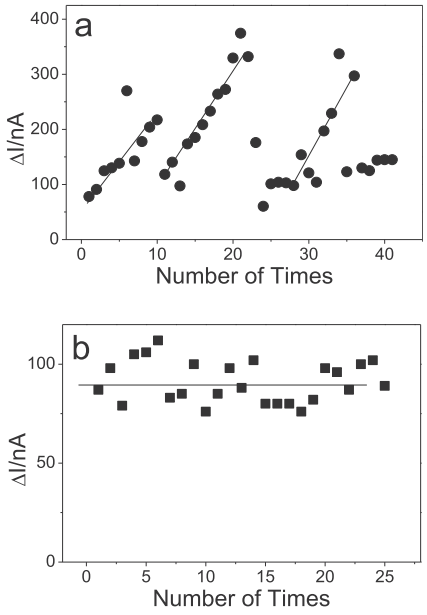

Fig. 6. ΔIs for measuring GGA20 by the biosensors (a) not in and (b) in WMF.

degradation and promotion of microbial growth by magnetic field [41,42]. And these functions were performed by the effect of magnetic field on both of the enzyme's activity and the replication of genetic material of microbes [43,44]. Beyond that, the resistance genes induced by toxicants were another element for inducing stress response of microbes in the present experiment conditions [45], and the microbial extracellular electron transfer can also be affected by the WMF [46].

For the application of TOX-biosensor, its stability was tested by employing GGA20 as the measure index. As shown in Fig. 6, ΔI of GGA20 was about 100 ± 20 nA at the first measurements. But for the biosensor that was not in WMF shown in Fig. 6a, ΔIs were raised gradually and even reached to an extreme value over 300 nA with the increasing of numbers of measuring times. And the signals were not rising continually, after touched an extreme value, it would fall back. This process will be repeated in the following period, indicating that the catalytic ability of the biofilm was fluctuated for the biosensor not in WMF. For the biosensor in WMF, the stable signals were shown in Fig. 6b, indicating the WMF was an effective way for keeping microbial activity. This result indicates that, when the immobilized microbes were used in the toxicity assay, controlling the stimulated elements, such as frequency of measurement, concentrations of toxicants and the time of exposure to toxic substances, was a promising way to reduce the microbial stress response.

4. Conclusions

Here, a stress response of immobilized microbes in acute toxicity assay was proved as a common phenomenon, which was mainly stimulated by the accumulation of both exposure time and dose that reacted with toxicants. WMF was employed to offer a balanced action of the activity of biofilm, and the lower density of WMF was demonstrated to be most effective. The effects of WMF may be associated with the special magnetic field inside the organisms as well as the charge transfer happened in the life activity process.

Declaration of competing interest

The authors declare that they have no known competing

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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.aca.2019.11.036.

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