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# A small microbial three-electrode cell based biosensor for on-line detection of acute water toxicity

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**ABSTRACT:**

The monitoring of toxicity of water is very important to estimate the safety of drinking water and the level of water pollution. Herein, a small microbial three-electrode cell (M3C) biosensor filled with polystyrene particles was proposed for on-line monitoring the acute water toxicity. The peak current of the biosensor related with the performance of bioanode was regarded as the toxicity indicator, and thus the acute water toxicity could be determined in terms of inhibition ratio through comparing the peak current obtained with water sample to that obtained with non-toxic standard water. The incorporation of polystyrene particles in the electrochemical cell not only reduced the volume of the samples used, but also improved the sensitivity of the biosensor. Experimental conditions including washing time with PBS and the concentration of sodium acetate solution were optimized. The stability of the M3C biosensor under optimal conditions was also investigated. The M3C biosensor was further examined by

formaldehyde at the concentration of 0.01%, 0.03% and 0.05% (v/v), and the corresponding inhibition ratios were 14.6%, 21.6% and 36.4%, respectively. This work provides a new insight into the development of an on-line toxicity detector based on M3C biosensor.

**KEYWORDS**: *microbial three-electrode cell (M3C); biosensor; formaldehyde; toxicity; inhibition ratio*

With the rapid development of modern industry and agriculture, monitoring pollution in water is of great importance, whose harmfulness and toxicity are becoming more and more extensive and deep to both plants and animals[1-3]. Hence, it is necessary to develop a reliable, sensitive, less maintained and inexpensive biotoxicity sensor for monitoring water pollution[4-8]. A large number of microbial populations offer good alternatives for responding to the challenge owing to their short life cycle, tenacious vitality, rapid response to toxins and low cost[8-10]. Up to now, there are various types of microbial toxicity testing methods, such as bioluminescent[11-12], colorimetric[8, 10] and bioelectrochemical[5, 13-17] methods, etc.

Since the external mediator-less microbial fuel cell (MFC) was proposed as a lactate biosensor by Kim et al. in 1999[18], many MFCs have been designed and investigated as the biosensors for monitoring the water quality effectively[19-23]. Generally, the decreased current, voltage or output power were recognized as the toxicity response[24-26]. For example, the current output of MFCs could be inhibited by 28-61% in the presence of 1 mg/L of artificial toxicants, such as organophosphorus compound, $Pb^{2+}$, $Hg^{2+}$ and polychlorinated biphenyls (PCBs)[27]. Shen et al. demonstrated that the MFC-biosensor detected toxicity immediately when 5 or 7 ppm of Cu(II) was spiked into the feed of a real wastewater in the continuous operation model[28].

Xu et al. developed a novel flat membrane-based microbial fuel cell (MMFC) sensor through compacting two filter membranes coated with carbon ink. The high micro-porosity and

hydrophilicity of membranes resulted in short acclimation period, simple compact configuration with microliter size, and high sensitivity and stability. The batch-mode tests demonstrated that the MMFC sensors possessed high sensitivity under the shocks of toxic metals ($Cr^{6+}$ and $Ni^{2+}$), and exhibited good reusability and high stability of voltage signals[29]. However, the performance of this biosensor can be disturbed by the cathode, which is similar to the traditional MFC biosensor, which needs further improvement. Microbial three-electrode cell (M3C) is one of the typical bioelectrochemical systems (BESs), which can maintain a stable and explicit electrochemical environment for electrochemical active bacteria (EAB) by accurately poising its working electrode at a constant potential level with the help of a three-electrode system[30]. Compared to conventional MFCs, the M3C requires no cathode chamber, and thus is very simple.

Wang et al. studied the current changes of the M3C biosensor with different concentrations of formaldehyde, and found a linear correlation until 0.08% formaldehyde[20]. Apart from the good stability and reproducibility, the fast response and recovery abilities are also important for a perfect BES-based biosensor. To large scale BESs, the low mass transfer efficiency results in the long response time and recovery time. Therefore, miniaturizing the M3C biosensor is the key point for overcoming the inherent defects of the large size reactors[31].

Ahn et al. developed a bioelectrochemical microfluidic device based on a miniaturized membrane-less M3C biosensor with continuous-flow mode, which was able to detect poisonous substances in wastewater by using only a small amount of reagents in real time[32]. Li et al. fabricated a 3 μL microfluidic M3C biosensor, in which laminar flow was used. Taking advantage of the microliter scale and a short hydraulic retention time (HRT), the fast and reproducible toxicity responses to ferric citrate and formaldehyde were achieved[30]. However,

owing to its inherent defects, the microfluidic chip based M3C biosensor is not suitable in the case of real water with complex ingredient, for example, the number of microorganisms is limited in the microfluidic M3C biosensor, and the microbial membrane is not firm on the electrode, as well as the pipeline is easily to be blocked.

In this work, a small M3C toxicity biosensor filled with polystyrene particles is fabricated with conventional three-electrode system in a single chamber with the volume of 5.3 mL. The inhibition of the bioactivity of electricigens is regarded as the toxicity response according to previous works, and can be determined by measuring the changes in the peak current on the M3C biosensor. Since the soluble formaldehyde has a fast biological toxicity in M3C biosensor, it was selected here as the tested toxin[20, 33-34]. Experimental conditions including injection time of sodium acetate solution, washing time with PBS, pH, temperature, flow rate and concentration of sodium acetate solution are optimized, and the stability of the M3C biosensor under optimal conditions is also investigated. Finally, toxicity responses to formaldehyde are analyzed. The aim of this work is to accumulate some useful data for developing an on-line toxicity detector based on M3C biosensor in water contamination in the future.

## EXPERIMENTAL SECTION

### Chemicals

Formaldehyde was purchased from Sinopharm Chemical Reagent Beijing Co., Ltd. Sodium acetate was purchased from Shenyang the third reagent factory. Graphite felt (thickness: 3 mm, 10 mm×10 mm, volume density: 0.08~0.16 g/cm$^3$, resistance < 0.1 Ω·cm, carbon content: 98%) was obtained from Beijing Sanye Carbon Co., Ltd. The bacterial growth medium contained (per liter deionized water) 1 g sodium acetate dissolved in 100 mM phosphate buffer solution (PBS) amended with 12.5 mL/L minerals and 5 mL/L vitamins[35]. The preparation procedure of PBS,

Minerals and Vitamins are described in previous literature[36]. Unless otherwise stated, all of the chemicals were of analytical or biochemical grade and used as received without further purification. Samples were preserved in car refrigerator (P24, US-Electronics (Shenzhen) Co., Ltd.). All of the solutions were prepared by using 18.25 MΩ/cm deionized water purified with a Milli-Q Gradient System (Millipore).

**Instruments**

The morphology and structure characterizations were obtained with an XL30E SEM field-emission scanning electron microscope (SEM) at an accelerating voltage of 15 kV. The electrochemical measurements were performed using an eight-channel potentiostat (CHI 1030C, Chenhua Instruments Co., Ltd., Shanghai, China) at 30±1℃ in an incubator (Tianjin Taisite Instrument Co., Ltd.). A conventional three-electrode system was used, which consisted of a self-made Ag/AgCl (saturated KCl) electrode as the reference electrode, a titanium wire (diameter 2 mm) as the counter electrode and a 1 $cm^2$ graphite felt as the working electrode. All potentials given here were versus the Ag/AgCl reference electrode.

The flow of the solution was controlled by using a peristaltic pump (model: YZ1515x) in conjunction with silicone tube (14#, ID 1.6 mm and OD 4.8 mm, Baoding Longe Precision Pump Co., Ltd. China). The control section was powered by a DC power converter (NES-100-24, Ming Wei Enterprise Co., Ltd., Taiwan), electromagnetic valves (P20T24-02#, Beion Fluid Systems (Shanghai) Inc.) were controlled by a table program controller (TPC12-12TD, Beijing Table Control Technology Co., Ltd.).

**Sludge acclimation and medium preparation**

The anaerobic sludge was collected from the Changchun second sewage treatment plant. The raw sludge was filtered with a G2 frit funnel and acclimatized at pH 7.0 in the preparatory

stage. The process of sludge acclimation was batch mode, and two-thirds of the supernatant was replaced by fresh medium every two weeks[37].

Medium contained sodium acetate dissolved in PBS was used as nutrients in the startup stage, and it was used as the water sample in toxicity testing stage. Medium free of sodium acetate was used as the cleaning solution, and mediums in the presence of toxic substances were used as the sample solutions. To ensure anaerobic environment, all of the solutions were drunk up with nitrogen for at least 30 min to remove dissolved oxygen before use, and nitrogen was purged continuously during the operation.

**Startup of the M3C biosensor**

The graphite felt electrode was pretreated with 2.6 M $HNO_3$ overnight and washed by deionized water for at least three times. A constant potential of 0.2 V was applied to the working electrode of the M3C biosensor, and the bioelectrocatalytic oxidation of substrate was monitored at a constant temperature (30 ℃) in the startup period[32]. When the output current decreased to 5% of its peak value, the bacterial culture was aspirated with a syringe, then the biosensor was refilled with the fresh medium solution include 30% of the acclimated activated sludge. The cell was launched successfully when peak currents increase of two consecutive cycles could be neglected. The photograph of the M3C biosensor filled with polystyrene particles was shown in Figure S1 in the Supporting Information.

**Principle of on-line toxicity monitoring with the M3C biosensor**

The illustration of current responses of the M3C-based toxicity biosensor filled with polystyrene particles to different solutions is shown in Figure 1. Respiratory activities of electricigens were normal when a sodium acetate solution flowed into the M3C biosensor, where sodium acetate was biodegraded to $CO_2$ and $H_2O$, and a great deal of electrons (x $e^-$) were

transferred from the biofilm to the electrode (Figure 1A). The sodium acetate in the M3C biosensor was gradually replaced by PBS in the cleaning stage, then less and less electrons (y $e^-$) were transferred to the electrode, as a result, the current decreased to almost 0 A (Figure 1B). Respiratory activities of electricigens were inhibited when the same concentration of sodium acetate in the presence of toxicant flowed into the M3C biosensor, then less electrons (z $e^-$) were released (Figure 1C). The current signals referred to the successive processes depicted by Figure 1A, 1B and 1C were shown in Figure 1D.

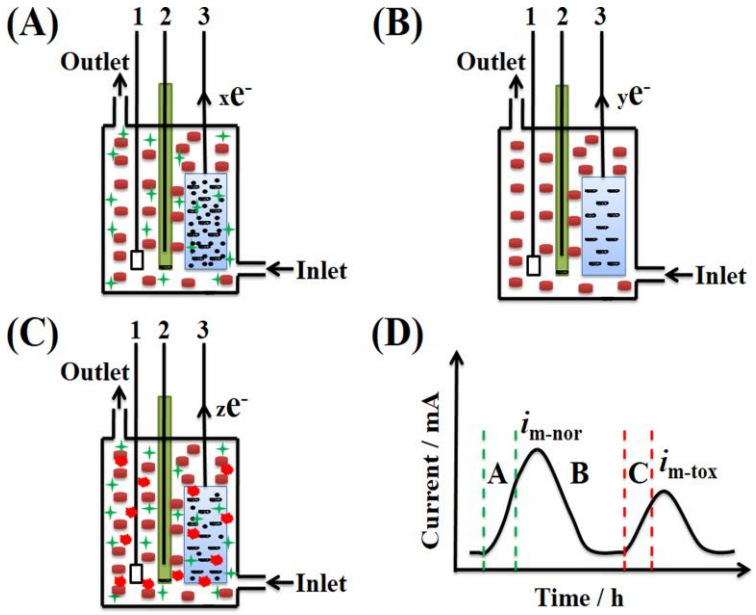


Figure 1. Schematic illustration of the response of the M3C biosensor at 0.2 V and 30℃ when a sodium acetate solution (A), a PBS (B) and the same sodium acetate solution in the presence of toxicant (C) flowing throng the biosensor in sequence, (D) current output of (A), (B) and (C).

According to previous works[27, 33, 36], the inhibition in the bioactivity of electricigens is regarded as the toxicity response of the M3C biosensor to toxicant, and the toxicity can be determined by measuring the changes of the peak currents. The inhibition ratio is calculated by equation (1):

$$\text{Inhibition (\%)} = 100 \times (i_{m\text{-}nor} - i_{m\text{-}tox})/i_{m\text{-}nor} \quad (1)$$

Here, $i_{m\text{-}nor}$ is the maximum current resulted from the M3C biosensor in sodium acetate solution in the absence of toxicant; $i_{m\text{-}tox}$ is the maximum current resulted from the M3C biosensor in sodium acetate solution in the presence of toxicant.

**Schematic of on-line toxicity monitoring with the M3C biosensor**

The single-chamber M3C was constructed by using plexiglass (purchased from Phychemi (Hong Kong) Company Limited), sealed with silicone gaskets and screwed to prevent leakage. The chamber size of the M3C was π × [1.5 cm (*r*)]$^2$ × 2 cm (*h*) with a working volume of 14.13 mL. In order to increase the degradation efficiency of sodium acetate in M3C, polystyrene particles were added into the M3C chamber. As a result, the actual volume of the M3C chamber was reduced to 5.3 mL.

Figure 2 was the flow chart of toxicity monitoring in water with a flow system. The water sample and the washing sample (PBS) were loaded into the sample bottles (1 and 2), respectively. Electromagnetic valves (3 and 10) were controlled by TPC (4), then samples and PBS were drawn from the sample bottles and pushed into the M3C chamber (5) by peristaltic pump, waste water was discharged to waste liquid tank (9), the oxidized current of sodium acetate in the M3C (5) was collected by constant potential instrument (6), then the current signals were displayed and stored in the computer (7).

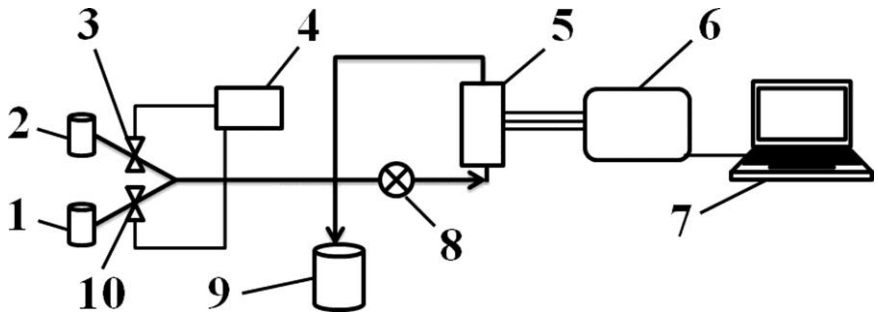

Figure 2. Schematic illustration of the M3C-based biosensor for on-line acute water toxicity monitoring, 1-sample; 2-PBS; 3,10-electromagnetic valve; 4-TPC; 5-M3C; 6-constant potential instrument; 7-computer; 8-peristaltic pump; 9-waste liquid tank.

## RESULTS AND DISCUSSION

### SEM images

SEM images of the graphite felt and the microbial films on graphite felt were shown in Figure 3. We could clearly see the mesh structure of the graphite felt (Figure 3A) and the rod-shaped bacteria growing both inside and outside the graphite felt mesh (Figure 3B). The net structure of graphite felt together with some unique features including high carbon content of 98%, corrosion resistance, good biocompatibility, large bulk density and good electrical conductivity, is beneficial for the enrichment of bacteria and electron conduction.

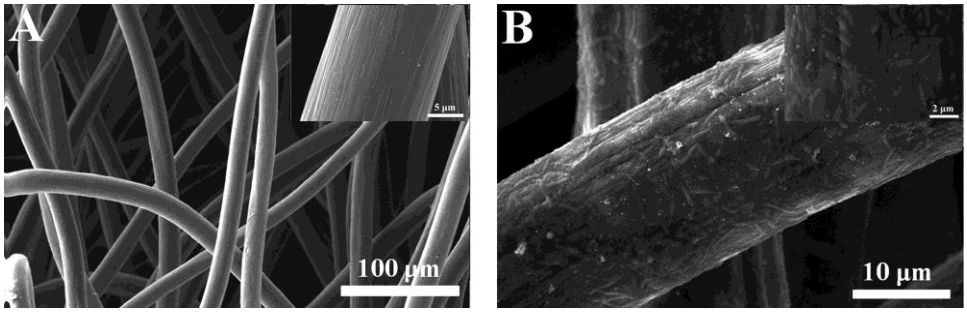


Figure 3. SEM images of graphite felt (A) and electrochemical activity bacteria growing on graphite felt (B).

### The catalytic effect of electrochemical active bacteria

Cyclic voltammetry was employed to investigate the catalytic effect of electricigens on sodium acetate. As shown in Figure S2, the graphite felt shows no catalytic activity to the oxidation of sodium acetate in the potential range from -0.7 to 0.1 V. The electricigens growing on the graphite felt exhibit obvious redox activity at nearby -0.25V in PBS, and the anodic peak intensity increases dramatically when meeting with sodium acetate, demonstrating the catalytic

activity of the electricigens towards the oxidation of sodium acetate. The anodic peak intensity increases obviously.

**Influence of flushing time with PBS**

The quantization of toxicity depends on the difference in peak currents, therefore, it is necessary that the current can return to the baseline at the end of each test cycle. For this purpose, the flushing time of PBS was optimized. As shown in Figure 4A, PBS flows through the M3C biosensor without polystyrene pellets for 5, 10, 20, 30, 60 and 120 min. Currents can not return to the baseline for flushing time of 5, 10, 20, 30 and 60 min, and stable current can not also be obtained for flushing time of 120 min even if. This can be ascribed to the following two reasons. On the one hand, the volume is too large that organic matter cannot degrade completely in a short time; on the other hand, injected organic matter is not spread in time.

We put forward a solution that filling particles were introduced into the M3C biosensor to reduce the volume of the electrochemical cell, and filling particles can change the direction of flow and have indirect effect of stirring at the same time, thus, organic matter can be spread timely and sufficiently. Exciting results appear in Figure 4B at the same program clearly. The currents could not return to the baseline after PBS flowed through the M3C biosensor for 5 and 10 min. The currents could return to the baseline after PBS flowed through the M3C biosensor for 20, 30, 60 and 120 min. But, flushing time of 20 min was too short to ensure organic matter discharging or degrading entirely, and microorganism could also not got enough rest. The purpose of the rapid monitoring could not be achieved for 60 and 120 min. Therefore, 30 min was used as the optimal washing time for a M3C with filler. This can be ascribed to the following reason. Filling polystyrene pellets to the M3C biosensor, the volume of the cell

reduced and the shear stress force increased, which made sodium acetate was degraded adequately by microbes and improved the current stability.

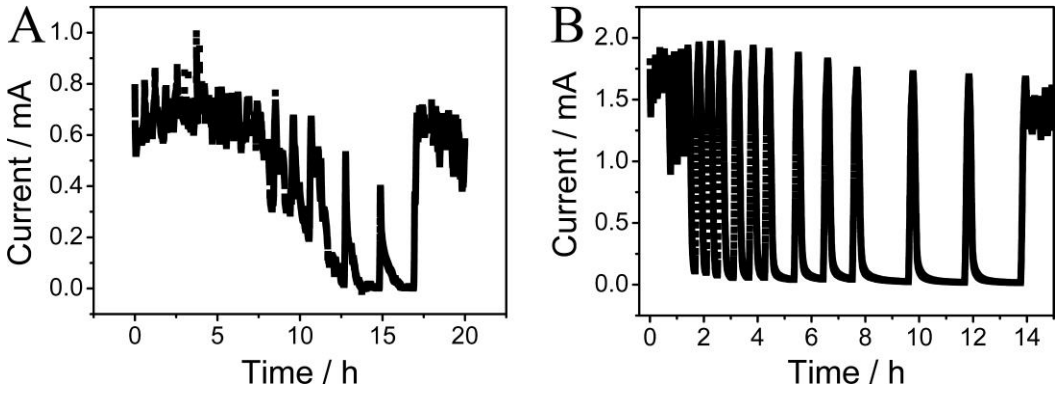


Figure 4. Current response of 1g/L sodium acetate solution injected for 5 min at different flushing times (5, 10, 20, 30, 60 and 120 min) with PBS pumped in the flow rate of 0.72 mL/min in a 30 $^{o}$C incubator by the M3C biosensor (A) without and (B) with filler. Each flushing time ran successively three consecutive cycles.

**Effect of injection time for sodium acetate**

The biodegradation efficiency of the biofilm plays an important role in improving the sensitivity of the toxicity biosensor, therefore, the concentration of sodium acetate used were optimized in terms of injection time interval. It could be clearly seen from Figure S3 that the peak current increases significantly with the increase in the injection time of the sodium acetate solution, and a maximum value is obtained at the injection time of 4 min. However, the larger one current could be obtained by prolonging the injection time (8, 9, 10, 15, 20 and 30 min). Each peak of more than 8 min injection time is divided into two peaks, which is ascribed to the following reason. The reaction ratio was determined primarily by the activity of the biofilms, and high concentration sodium acetate can't be degraded rapidly once it's out of microbial degradation. The duplicate test followed the same trend. Therefore, a period of 4 min is adopted for sodium acetate injection.

**Effect of pH, temperature and flow rate on the current response**

The effect of pH, temperature and flow rate on the peak currents of M3C biosensor were studied and the results are displayed in Figure S4. It can be clearly seen from Figure S4A that the peak current increases with the increasing of pH value in the range of 5.8~7, while the peak current decreases significantly with the further of pH value in the range of pH 7~8, and the maximum peak current is obtained at pH 7. Therefore, pH 7 is used as the optimal pH value

The peak current of M3C biosensor increases along with the temperature, as shown in Figure S4B. In view of maintaining relative high bioactivity and saving energy, 30 $^{o}$C was chosen as the optimal temperature since the peak current increases slowly at more than 30 $^{o}$C.

Effect of sampling rate of sodium acetate on the output peak currents was further investigated in the range of 0.24, 0.48, 0.72, 1.20, 1.92 and 2.40 mL/min. It is obvious that the peak current increased rapidly with the flow rate in 0.24~0.72 mL/min, however, the peak current rises slowly at higher flow rate, and reaches a quasi-stable state after 1.92 mL/min (Figure S4C). Therefore, 0.72 mL/min is chosen as the optimal flow rate. This can be ascribed to the following reason. Microbes need to absorb nutrients from the outside environment and maintain normal growth and reproduction through degrading nutrients, but it has a limit for microbes to degrade the nutrients. Therefore, microbes show strong ability of metabolism for a small number of nutrients, the desire of microorganisms weakens with the increasing of nutrients, and the metabolism ability of microbes reaches saturation for the excess nutrients.

**Effect of the concentration of sodium acetate**

Effect of the concentration of sodium acetate on the output peak currents is also evaluated. It can be seen clearly from Figure 5A that the peak currents basically unchanged with the concentration in the range of 1~160 mg/L sodium acetate. The peak current linearly increased in the range of 200~440 mg/L sodium acetate concentration, a linear equation,

y=0.00712x-1.47509, was obtained by fitting one linear model. It can be seen from figure 5B, the linear correlation coefficient $R^2$ was 0.99734 in the concentration range of 240~440 mg/L. However, the peak currents changed slowly after the concentration of 500 mg/L sodium acetate. Therefore, 500 mg/L was chosen as the optimal concentration of sodium acetate. This can be ascribed to the same reason. Desire and ability of microbial metabolism increased with increasing concentration of sodium acetate. But, excess nutrients were not degraded by microbial as out of the metabolic demands of microbes for nutrients.

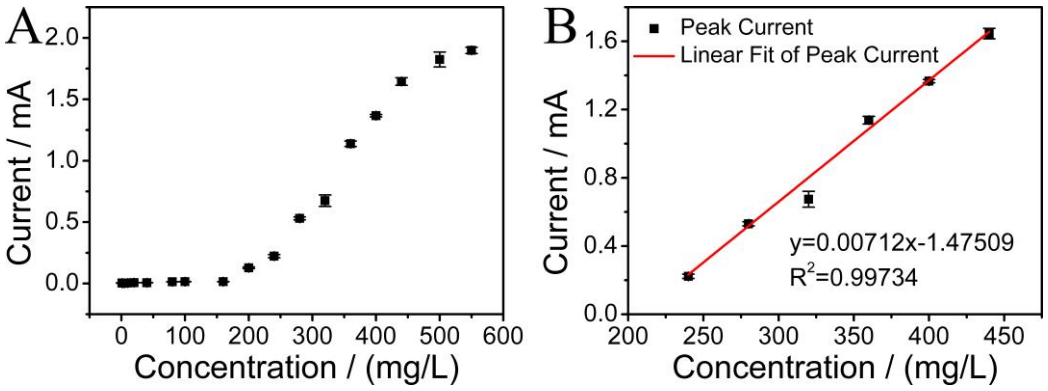


Figure 5. (A) The current response curve of different concentrations of sodium acetate pumped for 4 min at the flow rate of 0.72 mL/min in a 30 $^{o}$C incubator; (B) the linear relationship between the peak currents and the concentration of sodium acetate. Each point plotted was the average of three samples.

**The response time and stability of the M3C biosensor under optimal conditions**

The single output current was shown in Figure 6A. Response time of t=5.7 min was obtained under the above optimal conditions, therefore, the M3C biosensor can quick response to sodium acetate. The stability of output current signals of the M3C biosensor was investigated under the optimal conditions. It was obvious from the Figure 6B that the M3C biosensor could stably output current signals for a long time, and the peak currents were basically unchanged. The catalytic activities of microbial enzyme were stable when the temperature and pH were kept stable, at present, the degradation ratio was kept constant when the appropriate concentration of

sodium acetate solution was fed at a constant flow rate, resulting in number of electrons generated in the M3C biosensor remained stable, accordingly, the peak currents were also basically unchanged.

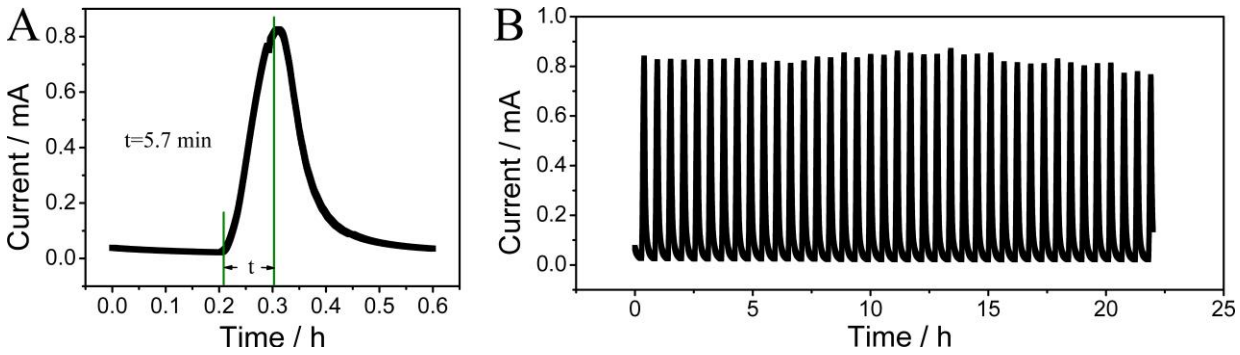


Figure 6. (A) Response time of the M3C biosensor in a single monitoring, (B) Stability of the current response curves under the optimal conditions, i.e. 500 mg/L sodium acetate pumped for 4 minutes at the flow rate of 0.72 mL/min in a 30 $^{o}$C incubator after flushed for 30 min with pH=7 PBS.

**Toxicity tests**

The toxic responses of the M3C biosensor to the artificial wastewaters containing different concentrations of formaldehyde were shown in Figure 7. Firstly, when the output peak current of the M3C biosensor was stable over 3 cycles under the optimal conditions, the substrate containing 500 mg/L sodium acetate was replaced by the substrate containing 500 mg/L sodium acetate and different concentrations of formaldehyde under the same operating conditions, it was clear that the output peak currents of the M3C biosensor decreased significantly.

Figure 7A displayed the toxicity monitoring of the artificial wastewater containing 0.01% formaldehyde, the inhibition ratio of 14.6% was obtained. The currents "a"~"i" correspond to the substrate only containing 500 mg/L sodium acetate, the current "j"~"r" correspond to the substrate containing 500 mg/L sodium acetate and 0.01% formaldehyde solution.

The toxicities of the artificial wastewater containing 0.03% and 0.05% formaldehyde were also detected and shown in Figure 7B and 7C, respectively. When the concentration of

formaldehyde increased to 0.03%, the inhibition ratio significantly increased to 21.6%, and the baseline became unstable. The currents “a”~“e” correspond to the substrate only containing 500 mg/L sodium acetate, while the currents “f”~“l” correspond to the substrate containing 500 mg/L sodium acetate and 0.03% formaldehyde.

When the concentration of formaldehyde increased to 0.05%, the inhibition ratio increased to 36.4%. The peak currents of each cycle decreased significantly, and the peak currents became more and more small, which showed that the toxic formaldehyde needs time to diffuse into the biofilm towards cells and inhibit the activity of bacteria. The baseline became also more unstable (“i”~“p”). The peak currents increased when 500 mg/L sodium acetate was fed into the M3C biosensor again, but it could not be completely recovered to the previous level (“q”~“w”). Figure 7D showed the histogram of the relationship between average inhibition ratio and the concentration of formaldehyde. It could be clearly seen that the inhibition ratios increased significantly with the increasing of the concentration of formaldehyde. The duplicate test followed the same trend.

In a word, formaldehyde has an inhibitory effect on the respiration of microbes when the microbes are in 0.01% formaldehyde solution. The microbes become shallow in respiration in formaldehyde solution, and the inhibition ratio increased with increasing the concentration of formaldehyde in solution. Toxic effects of heavy metal ions on microbes are well known[4, 17], therefore, it is also important to direct toxic sensing of single or mixed heavy metals using the M3C biosensor in future studies.

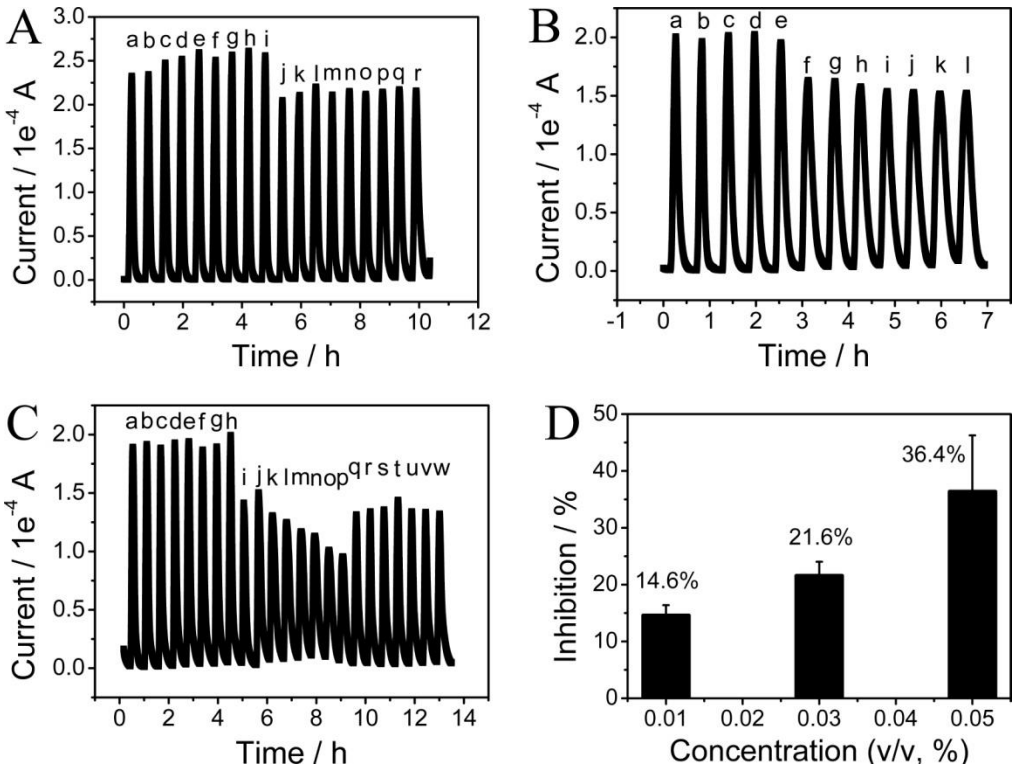


Figure 7. Current response curves of 500 mg/L sodium acetate solution containing formaldehyde with the concentration of 0.01 % (A); 0.03 % (B) and 0.05 % (C); (D) the bar graphs of the inhibition ratios of microbial membranes of the M3C biosensor on different concentrations of formaldehyde.

## CONCLUSION

Herein, a small M3C biosensor is proposed and the key parameters responsible for the performance were optimized. Polystyrene particles are introduced to reduce the volume of the electrochemical cell and improve the sensitivity of the M3C biosensor to toxin. The present results successfully demonstrate the good stability of the M3C biosensor. A typical dose-dependent toxic response to formaldehyde was observed, indicating the great potential of M3C biosensor for on-line monitoring the acute water toxicity. Moreover, compared with traditional fuel cells, there is no interference from cathode in the proposed M3C biosensor. The proposed M3C biosensor has good promise in on-line monitoring the acute water toxicity. The

structure of the M3C biosensor will be optimized, as well as the long-term stability will be explored in future researches.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: ××××.

Photograph of the M3C biosensor; Cyclic voltammetry curves of comparative experiments; Effects of pH, temperature and the flow rate on the peak currents; optimization of injection time of sodium acetate.

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### Notes

The authors declare no competing financial interest.

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