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The biofilm characteristics and enhanced performance of a marine microbial-electrolysis-cell-based biosensor under positive anodic potential

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Abstract: Microbial fuel cells have already been used as biosensors to monitor assimilable organic carbon (AOC). However, their signal production from AOC is known to be completely suppressed by dissoved oxygen (DO). In this study, two identical microbial electrolysis cell (MEC) based biosensors were inoculated with marine sediment and operated at two different anodic potentials, namely −300 mV and +250 mV relative to Ag/AgCl. The MEC biosensor operated under positive anodic potential conditions had electrochemically active microbial communities on the anode, including members of the *Shewanellaceae*, *Pseudoalteromonadaceae*, and *Clostridiaceae* families. However, the strictly anaerobic members of the *Desulfuromonadaceae*, *Desulfobulbaceae* and *Desulfobacteraceae* families were found only in the negative anodic potential MEC biosensor. The positive anodic potential MEC biosensor showed several other advantages as well, such as faster start-up, significantly higher maximum current production, fivefold improvement in the AOC detection limit, and tolerance of low dissolved oxygen, compared to those obtained from the negative anodic potential MEC biosensor. The developed positive anodic potential MEC biosensor can thus be used as a real-time and inexpensive detector of AOC concentrations in high saline and low DO seawater.

Keywords: biosensor; microbial fuel cell; anodic potential; marine biofilm; assimilable organic carbon

正阳极电位下基于海洋微生物电解池的高性能生物传感器及其生物膜特性

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摘　要:作为生物传感器,微生物燃料电池已被广泛用于可同化有机碳(AOC)的检测,但溶液中溶解氧(DO)会抑制AOC信号的产生。构建两个相同的基于微生物电解池(MEC)的生物传感器,传感器以海洋沉积物为接种源,并在不同的阳极电位下运行,其阳极电位相对于Ag/AgCl标准电极分别为−300、+250 mV。在+250 mV正阳极电位条件下运行的MEC生物传感器阳极上电活性

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微生物群落主要包括:*Shewanellaceae*、*Pseudoalteromonadaceae*和*Clostridiaceae*,仅在负阳极电位MEC生物传感器中发现严格厌氧的*Desulfuromonadaceae*、*Desulfobulbaceae*和*Desulfobacteraceae*菌群。与负阳极电位MEC生物传感器相比,正阳极电位MEC生物传感器表现出明显优势,如启动更快、最大电流产量显著提高、AOC检测限提高5倍以及对低溶解氧的高耐受性。提出的实时且经济的正阳极电位MEC生物传感器可以用于高盐度、低DO海水中AOC的检测。

关键词:生物传感器;微生物燃料电池;阳极电位;海洋生物膜;可同化有机碳
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1 Introduction

Membrane biofouling in seawater desalination reverse osmosis (SWRO) plants is a major problem for such operations and is caused by the growth of marine bacteria on the RO membrane in the presence of assimilable organic carbon (AOC) [1-2]. Due to the frequent occurrence of membrane biofouling, an accurate and sensitive AOC monitoring system is needed for biofouling management. In recent years, microbial fuel cell (MFC) based biosensors have demonstrated the potential to monitor trace concentrations of AOC in seawater [3-4]. The current generated represents the rate of oxidation of organic matter by the anodophilic bacteria attached on the anode [5], and the correlation between the current and organic concentration is approximately linear within a range of low AOC concentrations [3,6-8] for a fixed population of bacteria. MFCs generally consist of two chambers, an anodic chamber and a cathodic chamber, separated by an ion exchange membrane. In the anodic chamber, bacteria oxidize organic matter under anaerobic conditions and transfer the electrons towards a cathode through an external circuit, which produces a current [9]. Thus, MFC-based biosensor offers a sensitive, sustainable, and cost-effective method for AOC monitoring. Their capability for real-time detection allows for immediate assessment and intervention, making them a valuable tool for ensuring water quality and biological stability, and their low operational costs and compatibility with other monitoring systems further enhance their utility as an efficient early warning system against potential microbial contamination.

The primary performance criteria of biosensors are sensitivity, reproducibility, detection limit, correlation between signals and organic concentrations, and recovery time [10], and all of these criteria may be influenced by the variation in anodic potential (AP) and the microbial populations in the anode of an MFC based-biosensor [11-12]. Additionally, among the various interfering factors in analyzed samples, the presence of nitrate and oxygen in the anode compartment decreases the current output of the MFC since they compete with the anode for electrons from the biofilm, resulting in an internal circuit. Nevertheless, azide and cyanide, which act as terminal oxidase and nitrate reductase inhibitors, can significantly mitigate this effect[13]. Furthermore, MFC-based biosensors that operate under dissolved oxygen (DO) conditions are not readily implementable because electron flow in conventional MFCs is suppressed by DO[14]. However, the effects of different APs on the interference of oxygen inhibitors with MFC biosensor signal production are not well known.

The bacterial diversity in MFCs has been found to range from containing only a few dominant microorganisms [15-16] to very diverse populations [17] due to differences in operational parameters, including AP. Long-term operation of MFCs under different APs can affect MFC microbial diversity, which in turn affects their performance via their signal output, internal resistance, and mass transfer limitations [18-22].

To date, only a few studies have explored how changes in APs in a marine MFC biosensor affect their microbial communities and thus their performance [23]. Therefore, this study aimed to investigate the characteristics of marine potentiostat-controlled MFC (termed microbial electrolysis cell) biosensors (MEC biosensor) operated under positive and negative APs as tested in previous studies, at both +250 and −300 mV (vs. Ag/AgCl), respectively[3-4]. The positive +250 mV (vs. Ag/AgCl) was selected to overcome the competition for oxygen over the anode by the bacteria since it is higher than the redox potential of oxygen [about +80 mV (vs. Ag/AgCl) considering the effect of overpotential] [4, 23]. In addition, the main focus of this study was on investigat-

ing the role of oxygen as an interfering factor in biosensors that operate with both positive and negative potentials, and the sensitivity, long-term stability, and bio-communities of the MEC biosensors were investigated as well. Finally, continuous flow mode was tested to evaluate the effect of hydraulic retention time on the biosensor's signal production.

2 Materials and methods

2.1 Microbial electrolysis cell (MEC) biosensor setup

Two identical MEC biosensors were created by separating the cathodic and anodic chambers using a cation exchange membrane (with a cross-sectional area of 54 cm^2, Membrane International Inc.) with dimensions of 9 cm×6 cm×1 cm. As per the procedure described[3-4], each chamber was filled with conductive graphite granules that were 2-4 mm in diameter, with anolyte and catholyte volumes of about 30 mL each. The electrodes of the MEC biosensors were connected to a potentiostat to maintain the working electrodes (referred to as anodes) at working potentials (referred to as AP) of −300 mV or +250 mV (vs. Ag/AgCl) throughout the entire experiment. The pH of the analyte, potential of the counter electrode (referred to as cathodic potential (CP)), cell potential, and current were monitored continuously using LabView™ 7.1 software interfaced with a National Instrument™ data acquisition card (DAQ).

2.2 Inoculum

The biosensor was inoculated with marine sediment obtained from Weifang, Shandong province, China, and filtered seawater from the same location was utilized as anolyte and catholyte. The MEC biosensor setup was operated and monitored as per the methodology described in a previously published paper [3].

2.3 MEC biosensor operation

2.3.1 Operational procedure and assessment

To inoculate the anodic chamber (working electrode) of the MEC biosensor, 30 mL of the prepared inoculum, as described above [4], was added along with 30 mL of seawater that contained 0.05 g/L yeast extract and 10 mmol/L sodium acetate. Yeast extract was supplemented every two days, and the cathodic chamber was filled with 30 mL of seawater that was replaced every 4 to 6 weeks. The MEC was operated in fed-batch mode with both catholyte and anolyte continuously re-circulating via their respective compartments. Once the anodophilic biofilm was established (indicated by a stable current generation in the presence of excess organic food), the anolyte was drained, and the anodic chamber was refilled with fresh seawater.

2.3.2 Signal monitoring

The rate of electron flow from anode (working electrode) to cathode (counter electrode) in an MEC is proportional to the oxidation rate of the AOC added by the anodophilic bacterial biofilm, and the electrons generated from the AOC oxidation can be measured as a current using a potentiostat. In this experiment cumulative charges were obtained by integrating the electrons transferred by the biofilm as current throughout the detection period [24], and the detection limit was established using a signal-to-noise ratio between 2∶1 and 3∶1, which is generally considered to be acceptable [24]. Furthermore, the response time was determined by the time needed to reach the peak current achieved at concentrations at the limit of detection.

The established positive MFC biosensor was operated under both batch and continuous flow modes to detect AOC (such as acetate) pollutants. Continuous mode consisted of passing a series of prepared seawater feed solutions, each containing different concentrations of acetate (ranging from 0 to 100 μmol/L), through the anodic compartment only once (without recirculation). Hydrolytic retention times were set at 10 and 20 minutes. The continuous flow system operated under anaerobic conditions with a DO level of 0 mg/L, which was achieved by flushing with nitrogen for at least 30 min beforehand.

2.3.3 Microbial composition analysis

Total DNA was extracted from the biofilm associated with the carbon cloth anodes using a Power Soil DNA analysis extraction kit (MO-Bio, California, USA) according to the manufacturer's instructions. For each extraction, extraction blanks were included to check for naturally occurring background microbial populations introduced by reagents, operators, or consumables, and for pyrosequencing, partial fragments of the 16S rRNA gene were amplified

using universal bacterial fusion primers[25] as previously described[26]. After amplification, the amplicons were purified using the Agencourt AMPure XP system (Beckman Coulter), and DNA concentration was estimated by ethidium bromide gel electrophoresis to obtain approximately equimolar concentrations of each DNA sample[27]. BLAST searches for related 16S rRNA gene sequences from the database were then conducted through Yabi [28], and the files were imported into the program-MEtaGenome ANalyzer (MEGAN version 4.62.1) [29] for taxonomic assignment.

3 Results and discussion

3.1 Positive potential enables faster establishment

Two identical MEC biosensors with same amount of marine inoculum were operated under anaerobic conditions at APs of −300 and +250 mV (vs. Ag/AgCl). Over a period of two weeks in the presence of excess organic food of acetate (5 mmol/L) the current generated by the attached biofilm on the electrode increased steadily until it reached its maximum level (Fig1), suggesting that the establishment of the MEC biosensor was complete. The maximum current achieved in the positive MEC biosensor (refer to +250 mV AP) was roughly tice as much as that of the negative MEC biosensor (refer to −300 mV AP). Moreover, the negative sensor required a 3-4 times longer start-up period to reach steady peak current production (4 days vs. 16 days), indicating that the positive AP may have enabled a faster enrichment and establishment of an anode-respiring-bacteria (ARB) biofilm. This finding is in line with previous studies that indicated that faster establishment of freshwater MECs was achieved when operating at higher APs [17, 30-32]. Reducing the time required for the establishment of an efficient electroactive biofilm on the electrode is a significant challenge in the practical application of both MECs and MEC biosensors [16, 33], and the faster start-up and higher maximum current production observed in the positive MEC biosensor in this study may be attributable to the positive anode supplying a larger amount of energy that is then available for bacterial growth and maintenance[31-32].

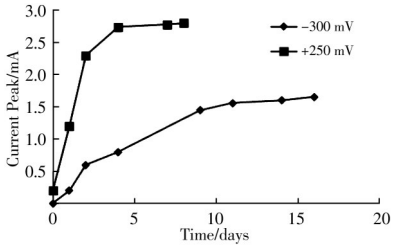


Fig. 1 Start-up of MEC biosensors at −300 and +250 mV (vs. Ag/AgCl) anodic potentials

3.2 Influence of AP on performance

3.2.1 Comparison of current production

To determine the peak current, the maximum current value after adding AOC (acetate) was subtracted from the steady state value, which was defined as no change in current (±0.05 mA) over a 10-minute period. We found that the MEC biosensor, operated at +250 mV anodic potential, generated a current production that was 1.5 times higher than that obtained at −300 mV anodic potential, as demonstrated by the results of acetate spiking experiments with the addition of 25 and 50 μmol/L of acetate (Fig. 2). This higher current production is likely attributable to the faster substrate oxidation rate of the anodophilic bacteria at higher APs [34].

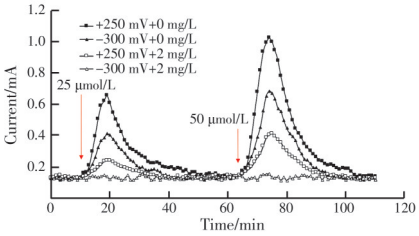


Fig. 2 The effect of AP [−300 and +250 mV (vs. Ag/AgCl)] on the response of the MEC biosensor to the addition of acetate (25 and 50 μmol/L) with and without DO (0 and 2 mg/L)

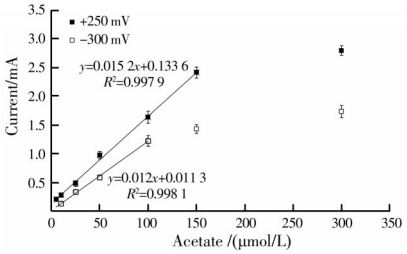
3.2.2 The effects of DO

The presence of oxygen can impede electron transfer from bacteria to electrodes, leading to suppression of current production, and this poses a potential challenge for the application of MEC-based biosensors in real-world desalination plants where these biosensors need to function in environments with DO. To counteract the negative impact of oxygen on electron transfer to the anode, the AP was raised to a level surpassing the oxygen's redox potential [35], and the biosensor's performance was assessed by monitoring its response (current genera-

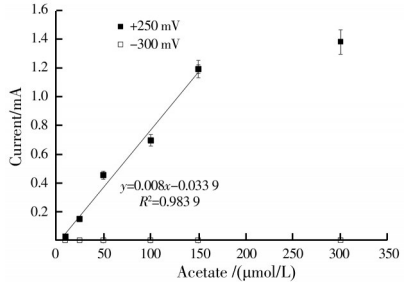
tion) to the introduction of low acetate concentrations (25 and 50 μmol/L) under low DO conditions (2 mg/L) (Fig2). When the DO concentration was low (2 mg/L), the anodic biofilm generated current at a high AP of 250 mV (vs. Ag/AgCl). Nonetheless, a diminished current peak (approximately 3 times lower than the current peak produced in the absence of oxygen) correlated with oxygen consumption (data not presented) indicated that some of the acetate was utilized for microbial aerobic respiration. These findings imply that an anode with a higher positive potential is more effective in capturing electrons from microbial organic oxidation in the presence of DO compared to an anode with a negative potential.

3.2.3　The effects of acetate and oxygen concentrations on current signal production

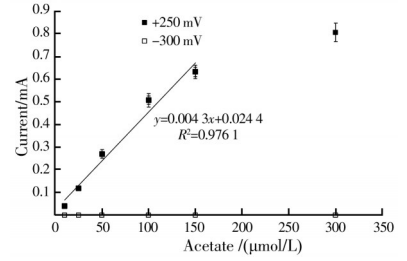
The two identical MEC biosensors were also tested for current signal production (peak current) in the presence of various acetate and DO concentrations. These results showed that the correlation between the added acetate concentrations and generated signals was high for both positive and negative MEC biosensors, indicating that these sensors can be highly precise regardless of the applied AP. However, the positive AP resulted in a fivefold lowering of the detection limit (signal-to-noise ratio of 3) and a twofold shortening of response time compared to the negative AP (Fig. 3 and Table 1). This suggests that the positive AP enables the attached anodophilic bacteria to utilize the anode as an electron acceptor with which to oxidize organics at a higher rate [9, 36].



(a) 0 mg/L



(b) 1 mg/L



(c) 2 mg/L

Fig. 3　The correlation between acetate concentration and current signal at APs of −300 mV and +250 mV (vs. Ag/AgCl) in the presence of 0, 1, 2 mg/L of DO

Table 1　Summary of the performances of the two MEC biosensors operated at −300 and +250 mV (vs. Ag/AgCl)

Anodic Potentials (vs. Ag/AgCl)/mV	Maximum Current (DO=0 mg/L)/mA	Measuring Range (μmol/L of Acetate)	Correlation (R^2, Current Peaks)	Detection Limit (μmol/L of Acetate)	Response Time*/min	
					Current Peak as signal	Total Coulomb as signal
−300	1.732	10-170	0.990	10	10	20
+250	2.797	5-100	0.994	5	5	10

Note: *Response time for detection of acetate at concentrations at the limit of detection.

The positive MEC biosensor also exhibited a more sensitive response to acetate in the presence of low DO concentrations. With low DO levels of 1 and 2 mg/L, no current signal was detected using the negative MEC biosensor. However, a lower, linear response to the acetate addition was observed for the +250 mV positive MEC biosensor (Fig. 3). This finding suggests that the positive MEC biosensor could be employed for substrate detection in the presence of low DO, though we note that DO concentrations higher than 2 mg/L could also completely suppress the signal production of the positive BEC-biosensor (data not shown). Furthermore, although, there are numerous advantages in operating MEC biosensors at positive AP, if the AP is too high, other reactions such as water electrolysis could occur, leading to additional current production that would interfere with the AOC detection [37].

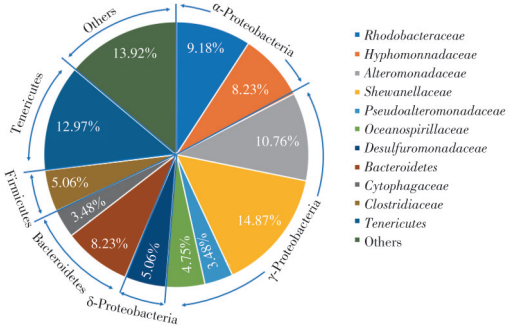
In comparison to previous work, in which a hexacyanoferrate mediator was used to overcome the

toxicity of oxygen[24], the current study demonstrates the feasibility of eliminating the negative effect of DO on the current production of biosensors by simply applying positive AP and avoiding chemical addition.

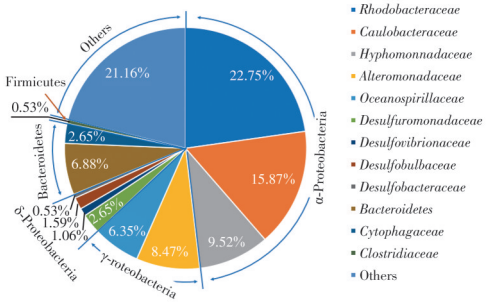
3.3 Comparison of the microbial community composition of anodophilic marine biofilms

Operating the sensors at varying APs necessarily impacts microbial community, which in turn influences the system's startup time needed to generate maximum and stable current and thus its effectiveness in detecting AOC, as previously mentioned. To understand the microbial composition of the anodophilic biofilm enriched at different APs, the mature anodophilic biofilms from the anodes of the two MEC biosensors were assessed using 16S rRNA gene sequence analysis. Even though the two MEC biosensors were only fed with a simple organic substrate (acetate), DNA analysis revealed that both microbial communities were diverse. This was likely due to the raw marine sediment used as inoculum [10, 12, 33]. There was an unexpected dominance of microbes in the positive MEC biosensor such as *Tenericutes* (12.97%), however, that are not known to have exoelectrogenic activity (Fig. 4).

The different APs did not appear to select Gram-positive/negative bacteria differentially as both MEC biosensors were predominated by Gram-negative bacteria. This finding contradicts previous studies that suggested Gram-positive bacteria (negatively charged bacteria) were dominant when positive potential was applied to the electrode due to the increased positive surface charge [15, 38].



(a) +250 mV



(b) −300 mV

Fig. 4 Anodic bacterial communities for the two MEC biosensors operated at APs of +250 mV and −300 mV (vs. Ag/AgCl) characterized by different phyla

Electrochemically active bacteria such as members of the *Shewanellaceae*, *Pseudoalteromonadaceae*, and *Clostridiaceae* families were only found in the positive MEC biosensor (Fig. 4) as well, which might explain the significantly higher maximum current, higher sensitivity, and lower detection limit of the positive sensor. *Shewanellaceae* in particular is known to produce current by forming an electroactive biofilm on anodes [39]. The negative MEC biosensor had an abundance of *Rhodobacteraceae* (22.75%), an exoelectrogenic family usually found in negative anodes of microbial fuel cells [14], and DNA sequences from members of the *Caulobacteraceae*, *Desulfuromonadaceae*, *Desulfobulbaceae*, and *Desulfobacteraceae* families were only obtained from the negative MEC biosensor. Of these, *Desulfuromo-nadaceae* is also a well-known exoelectrogenic family [40].

In a typical MEC biosensor, the presence of trace amounts of DO (DO < 2 mg/L) in the anode compartment completely suppresses current production [24, 39]. However, the presence of low concentrations of DO (up to 2 mg/L) at the anodic compartment of the positive MEC biosensor did not completely suppress the current production, although the signal strength was reduced by a factor of three. In comparison, at this level of DO current production was completely suppressed in the negative MEC biosensor (Fig. 2).

The higher tolerance to DO toxicity of the positive MEC biosensor compared to the negative MEC biosensor could be due to any of the following: 1) the presence of the *Shewanellaceae* family in the positive MEC biosensor, which has been shown to generate current in MECs under both anaerobic and aerobic conditions [41]; 2) the positive AP possibly corresponding to more oxidized environments (for example, micro-aerobic ones) and therefore allowing for more oxygen tolerant bacteria; 3) the presence of 5 times more obligate anaerobic bacteria from the

families of *Desulfovibrionaceae* in the positive MEC biosensor than the negative MEC biosensor; and 4) the presence of strictly anaerobic bacteria from the families of *Desulfuromonadaceae*, *Desulfobulbaceae*, and *Desulfobacteraceae* in the negative MEC biosensor. These last groups could potentially utilize electron acceptors (such as sulfur) that have lower standard potentials (i. e. −470 mV vs. Ag/AgCl).

There was no evidence of *Geobacteraceae* in either of the MEC biosensors' biofilm communities. Although bacteria from the *Geobacteraceae* family, such as *Geobacter*, are usually the most predominate electrochemically active bacteria in the anodophilic biofilms of MECs fed with acetate [14, 30], our results suggest that *Geobacteria* are not essential for the electrochemical activity of MECs, as indicated in a previous study [42].

3.4　Long-term stability

The long-term stability of bio-electrochemical (BEC) sensors is an essential aspect to consider in evaluating the potential success of their implementation in various applications. Ensuring consistent performance over an extended period of time is crucial for maintaining accuracy and reliability in the detection and monitoring of target analytes. Hence, to assess the long-term stability of the MEC biosensors operating at different APs, two biosensors were stored at room temperature [(25±2)℃] and supplied with 100 μmol/L acetate (twice) daily for 15 days, and the corresponding peak current (average of duplicates) was recorded. Between Days 1 to 5 and Days 11 to 15, both sensors were operated under strictly anaerobic conditions. However, from Days 6 to 10, the sensors were maintained under aerobic conditions (DO = 2 mg/L) by periodically introducing air bubbles into the anodic chambers.

As depicted in Fig. 5, both MEC biosensors exhibited relatively stable performance over a 5-day period under anaerobic conditions. It is unsurprising that under aerobic conditions (DO = 2 mg/L), no current was recorded for the sensor operated at −300 mV. After 5 days of operation under aerobic conditions, however, the current signal production of the negative MEC biosensor was significantly reduced, by approximately 60% (from an average of 1.23 mA to an average of 0.515 mA). In contrast, the positive MEC biosensor exhibited only a minor reduction in current production, with a decrease of about 8%. This result indicates that the positive MEC biosensor has relatively long-term stability even under low DO conditions. However, we caution that a high DO in excess of 3 mg/L could also have a detrimental effect on the positive MEC biosensor (data not shown), and this can occur when there is significant growth of heterotrophic nonelectroactive biomass within the anodic chamber.

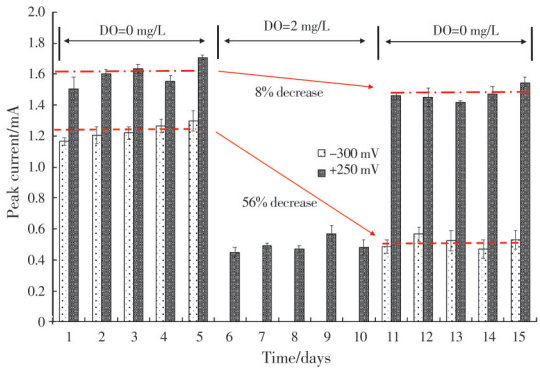
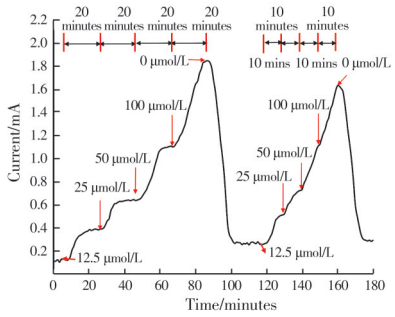


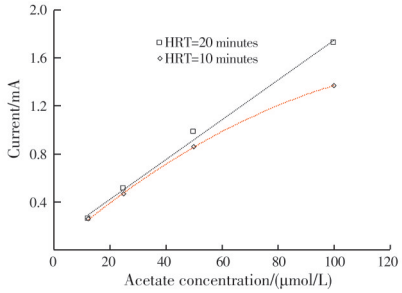
Fig. 5　The long-term stability of MEC biosensors operated at APs of +250 mV and −300 mV. The sensors were supplied with 100 μmol/L of acetate twice daily, and the daily peak current was the average of duplicates. The dashed line represents the average value of peak current over the testing period

3.5　A continuous real-time BOD sensor

A continuous mode MEC biosensor is a type of BEC device that operates continuously to monitor specific target chemicals or environmental parameters. In continuous mode, such a sensor is constantly exposed to a flowing sample, allowing real-time monitoring of an analyte. Among various parameters, hydraulic retention time (HRT), which is calculated as the ratio of the MEC reactor's volume to the flow rate of the influent, is an important factor that can significantly impact the performance of a continuous mode MEC biosensor. Fig. 6 shows that for an HRT of 20 minutes with our sensors, the reproducible responses of current production increased in proportion to the increase in the acetate concentration. However, for a shorter HRT of 10 minutes, the signal for the high concentration of acetate required a longer response time and we did not observe it reaching a steady state. This led to poor correlation between the acetate concentration and current (Fig. 6(b)).



(a) Current responses of the MEC biosensors operated at APs of +250 mV to the step changes in acetate concentration under continuous flow mode and anaerobic conditions



(b) The correlation between the current responses and acetate concentrations

Fig. 6 The acetate concentration in the inflow was changed every 10 or 20 minutes, and the DO concentration was 0 mg/L

HRT is the average time a liquid (in this case, the substrate containing the analyte) spends in biosensor system before being discharged or replaced. As HRT decreases (as the flow rate increases or analyte concentration changes more frequently), the signal generation capacity of the MEC biosensor may decrease due to the reduced contact time between the substrate and the microorganisms. This shorter contact time may not be sufficient for the microbes to oxidize the substrate effectively and transfer the electrons to the anode [43]. HRT can also influence the composition and stability of the microbial community within the MEC biosensor. A longer HRT may favor the growth of slower-growing microorganisms that are better suited for the electrochemical processes needed for the sensors, potentially leading to improved performance [44].

Continuous MEC biosensors are advantageous because they offer real-time, online monitoring capabilities and can provide consistent, reliable results over extended periods. They are often employed in environmental monitoring, wastewater treatment, and bioprocess control, where continuous measurement of specific parameters is crucial for maintaining optimal conditions or assessing the efficiency of a given process.

4 Conclusions

Long-term operational of MEC biosensors at different APs give rise to different anodophilic biofilm communities and resultant current output and performance, and the use of positive AP to develop microbial communities has several key benefits over negative AP.

1) It shortens the startup time by a factor of 3-4.

2) It increases the performance (sensitivity, detection limit, and response time) of the sensor.

3) It allows for a mild DO concentration of up to 2 mg/L.

4) Finally, the positive AP encourages the growth of electrochemically active bacteria.

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