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Electrogenic Bacteria Enhance the Structure and Performance of Nitrite-Reducing Electroactive Biofilms

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Abstract: The reverse polarity biocathode culture (RPBC) is a technology for the rapid preparation of biocathodes, which quickly enrich electroactive bacteria (EAB) in the microbial fuel cell (MFC) anode and then transform the electrode function from bioanode to biocathode by reversing bioelectrode polarity. However, the mechanism of RPBC is still unclear, and methods to regulate performance and ensure the long-term stability of cultured biocathodes have not been established. This study investigated the correlation between electrogenic bacteria and the target reducing EAB, from two aspects: energy supply and the formation of a composite biofilm. The results showed that electrogenic bacteria provided energy for the reducing EAB through interspecies electron transfer. This process could be regulated by changing the electrode potential and substrate concentration to obtain an optimized biocathode. In addition, the RPBC forms a composite biofilm of electrogenic bacteria and reducing EAB, which significantly improves the enrichment efficiency and the amount of reducing EAB (compared with a direct biocathode culture, respectively, shortening the enrichment time by 80%, increasing the electroactivity by 12.4 times, and increasing the nitrate degradation rate by 4.85 times). This study provides insights into regulating the performance and maintaining the long-term stability of RPBC-cultured biocathodes.

Keywords: biocathode; reducing electroactive bacteria; electrogenic bacteria; interspecies electron transfer; composite biofilm structure

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1. Introduction

Bioelectrochemistry, a technology anchored on electroactive bacteria (EAB) and bioelectrochemical systems (BESs), can possibly be used for the detection and degradation of environmental pollutants [1]. Research in this field originated with the exploration of electrogenic bacteria (oxidizing EAB) and bioanodes, which could offer practical solutions for the removal of organic pollutants and the recovery of energy from wastewater [2]. Subsequently, the discovery of reducing EAB, capable of catalyzing reduction reactions on a BES cathode [3], enabled the BES to perform more diversified functions such as hydrogen production [4] and denitrification [5]. However, most biocathode reactions occur at relatively negative reaction potentials, making it difficult to find a coupled anode reaction to form a fuel cell system for energy production (in which the biocathode reaction could occur spontaneously). Therefore, BESs usually require an external voltage to perform or accelerate biocathode reactions. By applying a voltage, the electrode could continuously conduct the biocathode reaction and promote more reducing EAB to colonize the electrode. This technique of directly preparing a biocathode in a BES by providing an external voltage

is known as a direct biocathode culture (DBC). Although DBC is currently the most commonly used biocathode preparation method, it suffers from a quite long preparation time, usually over 90 days [6].

The reducing EAB intrinsically grew slowly and have a long generation time because most of them belong to autotrophic bacteria that cannot use an organic substrate in a solution to provide energy for growth [7]. In DBC, due to the repulsion between the negatively charged bacteria and the cathode [8], the reducing EAB find it difficult to adhere to the cathode surface and to establish an effective connection with the cathode. This weak connection slows down the bioelectrochemical reaction rate and reduces the energy obtaining rate of the reducing EAB, thereby further slowing down the enrichment of reducing EAB on the cathode [8]. In 2010, Cheng et al. discovered that bioanodes cultured under heterotrophic conditions in a microbial fuel cell (MFC) also had reducing electroactivity, and then the bioanodes were successfully used as biocathodes by reversing the polarity [9]. This RPBC technology could shorten the preparation time of biocathodes from more than 90 days of DBC to less than 14 days, and has been proven to be effective in preparing a variety of biocathodes with different functions [10]. However, there is still a lack of research on the enrichment process of reducing EAB in an RPBC, including the main functional bacteria, attachment mechanisms, and the energy sources. Therefore, effective strategies for further regulating and improving RPBC technology have not yet been established.

Generally, the anode of an MFC is easier to enrich with EAB because of the attraction between the positively charged anode and negatively charged microorganisms [8]. However, the electrostatic attraction alone is insufficient to realize the colonization of the reducing EAB on the electrode since the reducing EAB cannot obtain energy through a bioelectrochemical oxidation reaction. In our previous report, the oxidizing and reducing electroactivity of bioelectrodes showed a strong positive correlation ($R^2 > 0.9$) in an RPBC [6]. Therefore, we speculated that the rapid enrichment of reducing EAB in the RPBC was related to the electrogenic bacteria. First, the electrogenic bacteria might metabolize organic matter and provide electrons for reducing EAB to synthesize energy substances [11] through interspecies electron transfer. Similarly, *Geobacter metallireducens* could oxidize formate or ethanol, and transfer electrons to *Geobacter sulfurreducens* [12] or to *Methanosaeta harundinacea* [13] to support them in reducing fumaric acid or in reducing carbon dioxide. Secondly, electrogenic bacteria might change the contact mode between reducing EAB and an electrode to that between reducing EAB and electrogenic bacteria, thereby increasing the enrichment rate and biomass of reducing EAB on an electrode through interspecies interaction [14]. While surface modifications, such as oxygen- and nitrogen-reducing functional groups, could improve the biocompatibility of the electrodes, direct electron transfer between microorganisms via nanowires, pili, or extracellular proteins proved to be more effective [15]. Additionally, electrogenic bacteria might also create a favorable local microenvironment for reducing EAB growth, such as making the pH of the solution around the reducing EAB more suitable for their growth [16]. However, further research is needed to elucidate the complex interplay between electrogenic bacteria and reducing EAB in an RPBC.

In this study, nitrite-reducing EAB enrichment and its biofilm structure in an RPBC were investigated. The effect of electrogenic bacteria in the process was explored regarding the energy issue regulated by adjusting the electric potential and substrate concentration, and regarding the structure issue obtained from different biocathode culture methods. In addition, the strategy of how to maintain the long-term stability of biocathodes cultured in an RPBC was also studied. The results revealed the action mechanism of electrogenic bacteria on the fast enrichment and long-term stabilization of the biocathode.

2. Materials and Methods

2.1. Inoculation and the Culture of the Biocathode

Bioelectrochemical system: the bioelectrochemical system (BES) reactor was constructed as a dual-chamber three-electrode system and was connected to an electrochemical analyzer (Bio-Logic, Claix, France), according to the previous study [6]. The bioelectrode (working electrode) was a graphite sheet electrode with a thickness of 2 mm and a diameter of 20 mm. The counter electrode was an air cathode-type electrode [17]. A cation exchange membrane (CEM) was set between the working electrode chamber and the counter electrode chamber, which held volumes of 30 mL and 10 mL, respectively. Within the working electrode chamber, an Ag/AgCl electrode (+197 mV vs. SHE) was installed as a reference. In this study, all potential measurements were calibrated against the Ag/AgCl.

Inoculation and culture: In this study, EAB were divided into two types: oxidizing EAB and reducing EAB, which have the ability of outward electron transfer and inward electron transfer, respectively. The main oxidizing EAB in this article are electrogenic bacteria, and the main reducing EAB are nitrite-reducing EAB. The tested biocathodes were prepared in both DBC and RPBC. In DBC, the working electrodes acted as the cathode of the BES to directly enrich nitrite-reducing EAB under potential control (of −0.4 V) by using an electrochemical analyzer. A mixture of 50% (v/v) inoculum and 50% (v/v) nitrite medium was used to inoculate the DBC biocathodes. The inoculum was either anoxic sludge or the metronidazole-treated anode sludge. The anoxic sludge was from the anoxic ponds of a local municipal sewage treatment plant. The metronidazole-treated anode sludge was obtained by scraping the anode biofilm of an MFC that operated for at least 30 days, followed by soaking with 15 mL of 120 mg L^{-1} metronidazole solution overnight. The nitrite medium was a 50 mM phosphate-buffer solution (PBS; 11.47 g L^{-1} $Na_2HPO_4{\cdot}12H_2O$, 2.75 g L^{-1} $NaH_2PO_4{\cdot}2H_2O$) containing 12.5 mL L^{-1} trace minerals, 5 mL L^{-1} vitamins [18], and 30 mgN L^{-1} $NaNO_2$. In the inoculation stage, the mixture (50% (v/v) inoculum, 50% (v/v) nitrite medium) was replaced daily. After five days of inoculation, the bioelectrode was cultured with the nitrite medium in fed-batch mode with daily medium changing. The solution of the counter electrode chamber was a 50 mM PBS solution containing 12.5 mL L^{-1} trace mineral solution and 5 mL L^{-1} vitamin solution, which was replaced every 7 days. Three reactors were set up for each inoculum as parallel reactors. The biocathodes cultured with the inoculum of metronidazole-treated anode sludge and anoxic sludge in DBC were abbreviated as MT-DBC and AS-DBC, respectively.

In the RPBC, the working electrode was initially cultured as the anode of the MFC at the external resistance of 1000 Ω during the observation of the repeatable consistent voltage of the MFC over three cycles (demonstrating a stable oxidizing electroactivity cultured on the electrode), and was then switched to a biocathode operated under controlled potential conditions that were same as those in the DBC. The inoculum of the working electrode chamber was a mixture of 50% (v/v) anoxic sludge and 50% (v/v) organic medium. The organic medium was a 50 mM PBS containing 12.5 mL L^{-1} trace minerals, 5 mL L^{-1} vitamins [18], and 1500 mg COD L^{-1} acetate. The mixture (50% (v/v) inoculum and 50% (v/v) organic medium) was replaced daily. After 5 consecutive days of inoculation, the working electrode was continually cultured using only organic medium in fed-batch mode with the organic medium was changed daily. The solution of the counter electrode chamber was a 50 mM PBS solution containing 12.5 mL L^{-1} trace mineral solution and 5 mL L^{-1} vitamin solution, which was replaced every 7 days. Ten MFCs operated with an external resistance of 1000 Ω as experimental groups, and three MFCs operated in open-circuit conditions as control groups. The biocathodes cultured with anoxic sludge the inoculum in the MFC and in the open-circuit condition were abbreviated as AS-RPBC and AS-OC, respectively. The MFC voltage was continuously recorded by a digital data collection

instrument (KEYSIGHT Model 34970A, Loveland, CO, USA). The nitrite removal rate in the working electrode chamber was assessed by employing chronoamperometry at a constant potential of −0.5 V for a period of 12 h in 50 mM PBS containing 30 mgN L^{-1} $NaNO_2$. In all the above processes, the mixture and the medium were flushed with pure N_2 for 20 min to remove dissolved oxygen before being added to the chambers. All the experiments were conducted in a constant-temperature room at 30 °C.

2.2. Electrochemical Characterization

Cyclic voltammetry (CV) was used to assess the electroactivity of different bioelectrodes. CV was performed at a scan rate of 2 mV s^{-1} in the potential range of −0.6~−0.1 V in 50 mM PBS with 1000 mg COD L^{-1} acetate for bioelectrochemical oxidation activity, or in 50 mM PBS with 30 mgN L^{-1} $NaNO_2$ for bioelectrochemical reduction activity. In this paper, oxidizing electroactivity was defined as the oxidation current at −0.15 V of a positive scan where no reduction reaction occurred, while reducing electroactivity was defined as the reduction current at −0.55 V of a negative scan where no oxidation reaction occurred.

2.3. Regulation of RPBC

To investigate the effect of electrogenic bacteria on the growth of nitrite-reducing EAB during RPBC, the working electrode was cultivated at different control potentials for regulating the electronic competitiveness of electrodes or in different substrate concentrations for regulating the energy supplies to grow the electrogenic bacteria. The set electrode potentials were −0.4 V, −0.3 V, and −0.2 V. The varied substrate concentrations were 100 mg COD L^{-1} acetate, 500 mg COD L^{-1} acetate, and 1500 mg COD L^{-1} acetate. CV was conducted every two days during RPBC to monitor the oxidizing electroactivity and reducing electroactivity of the cultivated electrode and to identify the growth status of the electrogenic bacteria and reducing EAB, respectively.

To find out the effects of the electrogenic bacteria in the bioelectrodes cultured in RPBC on the long-term reducing electroactivity of reducing EAB, the cultured bioelectrodes were operated in MFC mode or MEC mode under various operation conditions. In MFC mode, four experiments were carried out to observe the changes in the reducing electroactivity of reducing EAB before/after killing the electrogenic bacteria with metronidazole solution. The cultured bioelectrode, before killing the electrogenic bacteria, was operated in 50 mM PBS with 1500 mg COD L^{-1} acetate as a control group (referred to as MFC-0). Three electrogenic bacteria-killed bioelectrodes were operated in 50 mM PBS with 1500 mg COD L^{-1} acetate (referred to as MFC-1), MFC-2 operated in a mixture of 2/3 (v/v) 50 mM PBS and 1/3 (v/v) the filtered MFC effluent with 1500 mg COD L^{-1} acetate (referred to as MFC-2), and in a mixture of 2/3 (v/v) 50 mM PBS and 1/3 (v/v) MFC effluent with 1500 mg COD L^{-1} acetate (referred to as MFC-3), respectively, in which the MFC effluent was obtained from an MFC reactor that was initially inoculated with anoxic sludge and operated with acetate solutions for over 30 days, and the filtered MFC effluent was obtained by filtering the MFC effluent with a 0.22 μm membrane to remove microorganisms but retain biomass such as extracellular polymeric substances (EPSs). In MEC mode, the cultured bioelecrodes were operated under a constant potential of −0.4 V, separately, in 50 mM PBS with 30 mgN L^{-1} $NaNO_2$ (referred to as MEC-1) and in 50 mM PBS adding 1500 mg COD L^{-1} acetate and 30 mgN L^{-1} $NaNO_2$ (referred to as MEC-2), to understand the influence of organic substrate availability on the long-term stability of reducing electroactivity in bioelectrodes cultured in an RPBC. In the above regulation process and the following electrochemical characterization, the counter electrode chamber was con-

sistently filled with 50 mM PBS solution and this was replaced before every test. And a comprehensive summary of the experimental conditions can be found in Table 1.

Table 1. Summary of experimental conditions and purposes in this study.

Assay	Conditions	Purpose
Biocathode Culture—DBC	Electrodes enriched nitrite-reducing EAB at −0.4 V; inoculum: anaerobic sludge (AS-DBC) or metronidazole-treated anode sludge (MT-DBC) mixed with nitrite medium	To investigate the effects of different inocula on biocathode performance under DBC conditions
Biocathode Culture—RPBC	Electrode initially working as an MFC anode until the voltage is stable and then switched to being a biocathode at −0.4 V; inoculum: anoxic sludge (AS-RPBC) mixed with nitrite medium; ten MFCs as the experimental group (1000 Ω) and three MFCs as the control group (AS-OC)	To study the enrichment process of nitrite-reducing EAB and biocathode performance during RPBC, comparing differences between RPBC and DBC
Electrochemical Characterization	Cyclic voltammetry (CV) at 2 mV s^{-1} scan rate; oxidizing electroactivity evaluated in 50 mM PBS with 1000 mg COD L^{-1} acetate; reducing electroactivity evaluated in 50 mM PBS with 30 m N L^{-1} $NaNO_2$	To evaluate the oxidizing and reducing electroactivity of different bioelectrodes
RPBC Regulation—Electrode Potential Study	Electrode potentials set to −0.4 V, −0.3 V, and −0.2 V; substrate: 1500 mg COD L^{-1} acetate	To explore the effects of electrode potential on the growth and interspecies electron transfer of electrogenic bacteria and nitrite-reducing EAB growth during RPBC
RPBC Regulation—Substrate Concentration Study	Substrate concentrations set to 100, 500, and 1500 mg COD L^{-1}; potential fixed at −0.3 V	To explore the role of electrogenic bacteria in providing energy to nitrite-reducing EAB via interspecies electron transfer during RPBC
RPBC Long-Term Stability—MFC Mode	Control group (MFC-0): 50 mM PBS containing 1500 mg COD L^{-1} acetate; test group (MFC-1): After metronidazole treatment, operated in 50 mM PBS containing 1500 mg COD L^{-1} acetate; test group (MFC-2): After metronidazole treatment, operated in a mixture of 2/3 (v/v) 50 mM PBS and 1/3 (v/v) filtered MFC effluent with 1500 mg COD L^{-1} acetate; test group (MFC-3): After metronidazole treatment, operated in a mixture of 2/3 (v/v) 50 mM PBS and 1/3 (v/v) MFC effluent with 1500 mg COD L^{-1} acetate	To investigate the impact of the existence of electrogenic bacteria on the growth of nitrite-reducing EAB on bioelectrodes cultured in RPBC-, clarifying the role of electrogenic bacteria in maintaining the stability and performance of biocathodes
RPBC Long-Term Stability—MEC Mode	MEC-1: Operated in 50 mM PBS containing 30 mgN L^{-1} $NaNO_2$ at −0.4 V; MEC-2: Operated in 50 mM PBS containing 1500 mg COD L^{-1} acetate and 30 mgN L^{-1} $NaNO_2$ at −0.4 V	To examine the influence of organic substrate presence on the long-term stability of the reducing electroactivity of nitrite-reducing EAB on bioelectrodes cultured on an RPBC

Note: Nitrite medium: 50 mM PBS containing 12.5 mL L^{-1} trace minerals, 5 mL L^{-1} vitamins, and 30 mgN L^{-1} $NaNO_2$;Organic medium: 50 mM PBS containing 12.5 mL L^{-1} trace minerals, 5 mL L^{-1} vitamins, and 1500 mg COD L^{-1} acetate.

2.4. Characterization of Electrode Biofilm

After the regulation of the bioelectrodes cultured on RPBC, the bioelectrodes were divided into three kinds of samples, respectively, for fluorescence in situ hybridization (FISH), scanning electron microscopy (SEM) analysis, and microbial community analysis.

The samples for FISH were stained using a LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, Carlsbad, CA, USA; SYTO9 and PI fluorescent dyes) to evaluate cell viability based on membrane integrity. The staining resulted in a red coloration when the cell membrane was compromised, whereas intact cells stained green [19]. Confocal images were obtained on a TCS SPE system equipped with a DMI 4000B confocal microscope (Leica, Solms, Germany) at 485/535 nm excitation and 498/617 nm emission [20].

The morphology of the bioelectrode was characterized by SEM. The SEM samples were first fixed using the 2.5% glutaraldehyde solution and rinsed with a phosphate buffer (100 mM, pH = 7.0), and then fixed with a 1% osmic acid solution for 1–2 h following being rinsed three times with the phosphate buffer. Finally, the samples were immersed in a series of ethanol solutions with increasing concentrations (30%, 50%, 70%, 80%, 90%, 95%, and 100%). After the final ethanol immersion, the samples were dried with a critical-point drier using liquid CO_2 and sputtered with a layer of gold. The coated samples were analyzed by a scanning electron microscope (ZEISS GEMINI 300, Oberkochen, France) operated at 3.0 kV, and the images were captured digitally with a 1 k~10 k× magnification.

For community analysis samples, the biofilm was scraped from the bioelectrode surface and collected in a 2 mL centrifuge tube. DNA was extracted using a DNA isolation kit (PowerSoil, Carlsbad, CA, USA). The biomass was evaluated based on the total DNA content of the biofilm [21]. For community analysis, DNA extraction was carried out, followed by PCR amplification (the paired primers in the V3–V4 variable regions used for PCR amplification were F: 5′-ACTCCTACGGGAGGCAGCAG-3′ and R: 5′-GGACTACHVGGGT-WTCTAAT-3′). Fragment purification, database construction, and sequencing were subsequently performed, utilizing the Miseq Illumina sequencing platform [19].

3. Results and Discussion

3.1. Oxidizing and Reducing the Electroactivity of Bioelectrodes During the Culture Process

During the cultivation process, under −0.2 V and −0.3 V, the oxidizing and reducing electroactivity of all the bioelectrodes exhibited a similar changing trend over time, displaying three distinct stages: an initial enrichment period, a rapid growth period, and a plateau period (Figure 1A). The electroactivity initially increased from zero to a relatively high value (>0.573 mA cm^{-2}), reaching 90 ± 5% of the electroactivity observed in mature bioelectrodes cultured in traditional MFCs [21], and then tending to be stable. This trend was similar to the oxidizing electroactivity change in the bioanode culture process of common MFC. Notably, the electrodes cultured with the potentials of both −0.2 V and −0.3 V showed that the oxidation/reduction current started to increase rapidly on day 4 and reached a current plateau on day 8. The oxidizing and reducing electroactivity of each bioelectrode entered different stages simultaneously. However, the oxidizing and reducing electroactivities of the bioelectrode cultured at −0.4 V increased very slowly during the 20 days of culture. The synchronous evolution of the oxidizing and reducing electroactivity of bioelectrodes indicated that the enrichment process of nitrite-reducing EAB might be highly correlated with the growth process of electrogenic bacteria on the bioelectrodes cultured in RPBC.

Although the oxidizing and reducing electroactivity of the bioelectrodes changed synchronously, the reducing electroactivity-to-oxidizing electroactivity ratio (−Ire/Iox) of the electrodes showed significant differences for the various cultured potentials. This ratio demonstrated a consistent decline with the increasing cultured potential, with the average value of −Ire/Iox decreasing from 2.717 at −0.4 V to 1.391 at −0.3 V, and further to 0.804 at −0.2 V (Figure 1B). The possible reason for the change in the -Ire/Iox ratio was that the more positive electrode potential enhanced the competition for electrons produced by

electrogenic bacteria, thereby reducing the electron transfer from electrogenic bacteria to nitrite-reducing EAB, which affected the enrichment rate of the reducing EAB in RPBC.

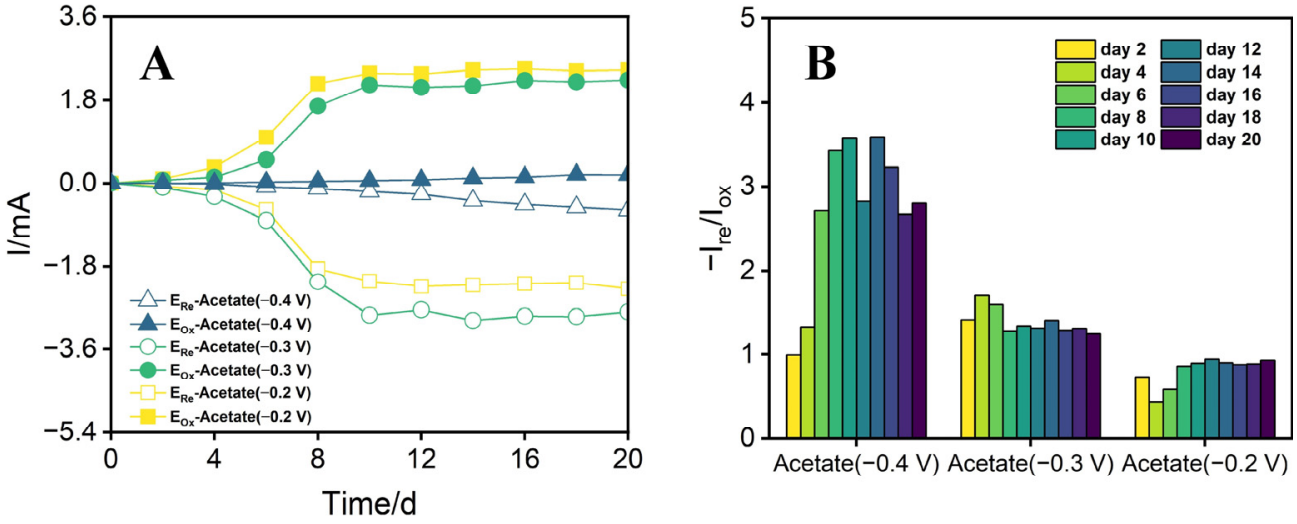


Figure 1. (**A**) The electroactivity of bioelectrodes cultured in RPBC at different electrode potentials (−0.4 V, −0.3 V, and −0.2 V) in 50 mM PBS containing 1500 mg COD L^{-1} acetate; (**B**) the $-I_{re}/I_{ox}$ of the electroactivity of bioelectrodes cultured in RPBC at different electrode potentials (−0.4 V, −0.3 V, and −0.2 V) in 50 mM PBS containing 1500 mg COD L^{-1} acetate.

3.2. Characteristics of Biocathodes Cultured by Different Methods

In this research, different inocula and culture methods were used to culture biocathodes, in order to explore the effect pathways of electrogenic bacteria regarding the enrichment of reducing EAB. The results show that the RPBC had significant advantages over other culture methods in terms of the preparation time and performance of the biocathodes. The biocathode cultured in RPBC achieved stable reducing electroactivity within 6 days (Figure 2A), which was 80% shorter than the biocathode cultured in DBC.

The biocathode cultured in RPBC achieved stable reducing electroactivity within 6 days (Figure 2A), unlike the biocathode cultured in DBC, whose electroactivity did not stabilize within the 30-day culture period (Figure 2B). The reducing electroactivity of the biocathode cultured in RPBC was 1.01 mA (measured at −0.5V), which was 3.26 (MT-DBC), 5.84 (AS-DBC), and 6.60 (AS-OC) times that of biocathodes cultured by other methods, respectively (Figure 2D). The short culture process and high reducing electroactivity of biocathodes cultured in RPBC in this study were consistent with other reports [22]. Under open circuit conditions, the nitrite-reducing electroactivity of AS-OC was minimal, with a reduction current below 0.15 mA, indicating the difficulty of enriching nitrite-reducing EAB on electrodes without an external electron supply. Meanwhile, the MT-DBC and AS-DBC were controlled at a constant negative potential (−0.4 V), so that the reducing EAB could obtain electrons from electrodes to complete enrichment, and both bioelectrodes exhibited reducing electroactivity. However, the MT-DBC exhibited a higher nitrite-reducing electroactivity than AS-DBC. This enhanced performance might be due to the anode biofilm treated with metronidazole (as the inoculum) containing a higher content of nitrate-reducing EAB compared to the anaerobic sludge inoculum, or because of the effects of substances secreted by electrogenic bacteria such as EPS and nanowires. By comparing the reducing electroactivity of MT-DBC and AS-DBC, it could be known that electrogenic bacteria did not have a direct impact on the reducing EAB enrichment process which conducted biocathode-reducing reactions. The biocathode cultured in RPBC exhibited high oxidizing electroactivity different from the other biocathodes cultured in DBC (Figure 2C), suggesting that the promoting

effect of electrogenic bacteria on the enrichment of reducing EAB was through interspecies interactions during co-growth on the electrode surface.

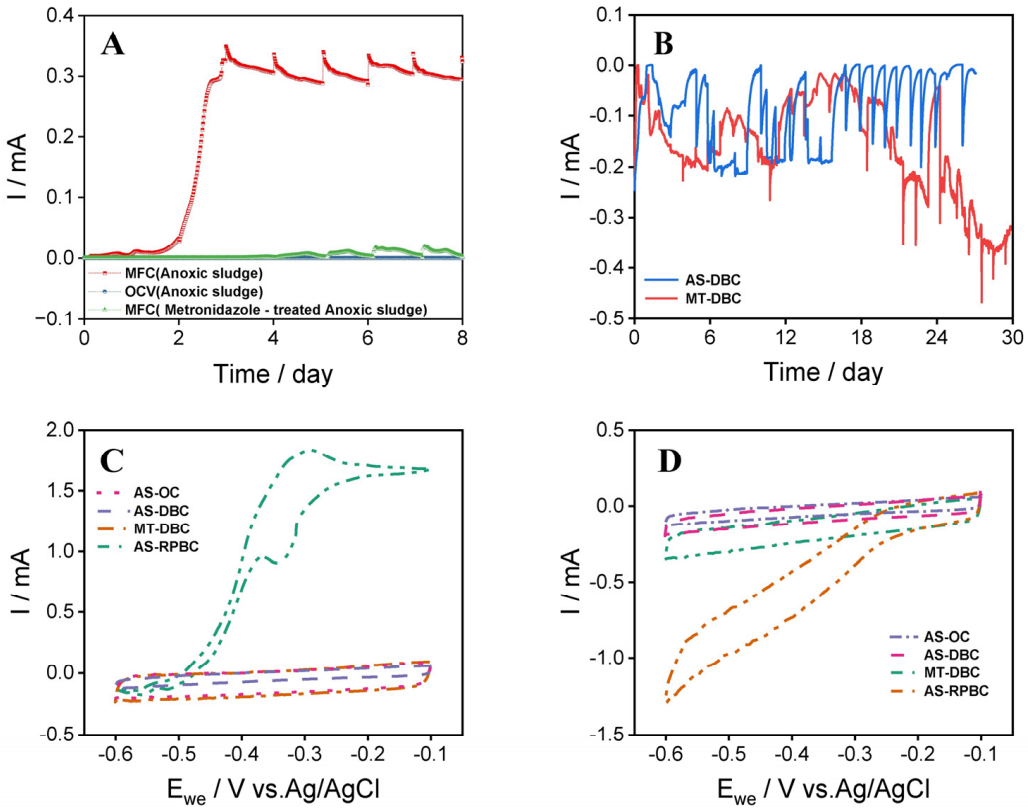


Figure 2. (**A**) The current output of an MFC cultured with anoxic sludge, open circuit condition (OCV), and metronidazole-treated anoxic sludge; (**B**) the current output of AS-DBC and MT-DBC biocathodes operated at a constant potential of −0.4 V; (**C**) the CV of the AS-OC, the AS-DBC, the MT-DBC, and the AS-RPBC recorded in nitrite solutions at a scan rate of 2 mV s^{-1}; (**D**) the CV of the AS-OC, the AS-DBC, the MT-DBC, and the AS-RPBC recorded in acetate solutions at a scan rate of 2 mV s^{-1}.

The average nitrite removal rate of the biocathodes corresponded to their nitrite-reducing electroactivity. The AS-RPBC exhibited the highest rate of 4.4 mgN L^{-1} h^{-1}, significantly outperforming the MT-DBC (1.3 mgN L^{-1} h^{-1}), AS-DBC (0.34 mgN L^{-1} h^{-1}), and AS-OC (0.06 mgN L^{-1} h^{-1}) (Figure 3A). And the biomass (measured by protein content) of the AS-RPBC (29.6 mg) was 9.5 times that of the MT-DBC (3.11 mg) and 32.9 times that of the AS-DBC (0.9 mg) (Figure 3B). Therefore, the high nitrite reduction capacity of the biocathode cultured in RPBC was mainly due to its high biomass. It was observed that the protein content increased more than the removal rate. In fact, proteins that play a role in electron transfer only account for a small part of the total protein content, so changes in the total protein content may not correspond proportionally to changes in nitrite reduction electroactivity and nitrite removal rate. In mixed bacterial systems, there may be other microorganisms whose growth is promoted by electrogenic bacteria, and the proteins they contain may also affect the total protein content of the biofilm, causing the phenomenon. The biofilm cultured in RPBC had a high abundance of *Geobacter* (over 37%) which was a typical electrogenic bacterium [23]. It is speculated that electrogenic bacteria might positively influence the enrichment process of reducing EAB, thereby enabling RPBC to cultivate biofilms with higher reducing EAB biomass.

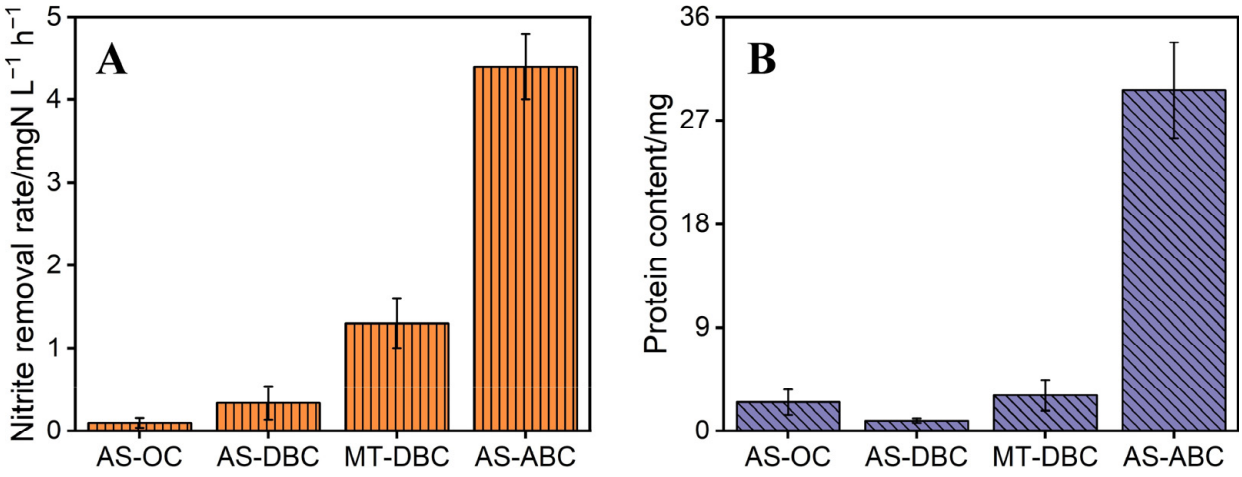


Figure 3. (**A**) The nitrite removal rate of the AS-OC, the AS-DBC, the MT-DBC, and the AS-RPBC in 12 h; (**B**) the protein content of the electrode biofilm of the AS-OC, the AS-DBC, the MT-DBC, and the AS-RPBC.

3.3. Research on the Interspecies Energy Transfer in RPBC

A significant positive correlation between the nitrite-reducing electroactivity of biocathodes cultured in RPBC and substrate concentration was demonstrated at various electrode potentials. Specifically, at −0.3 V, as the substrate concentration increased from 100 mg COD L^{-1} to 1500 mg COD L^{-1}, the reduction current increased from 0.40 mA to 1.65 mA, representing a substantial increase of 313% (Figure 4A). Generally, a higher concentration of acetate could provide electrogenic bacteria with more electron donors and energy, thus promoting their growth and metabolic activity. However, since reducing EAB could not directly metabolize organic matter [24], acetate did not have a direct positive impact on their growth. Therefore, biocathodes in an environment with a high acetate concentration exhibited higher reducing electroactivity, which might be because an increase in the acetate concentration increases the number of electrons transferred to reducing EAB through interspecies electron transfer channels, thereby promoting the growth of nitrite-reducing EAB. The influence of electrode potential on the reducing electroactivity of biocathodes demonstrated a peak performance at a specific potential, with decreased electroactivity at both higher and lower potential. The reducing electroactivity of the biocathodes was highest at a culture potential of −0.3 V, followed by −0.2 V, with the lowest at −0.4 V. For example, under the culture condition with 1500 mg COD L^{-1} acetate, the reducing electroactivity of the biocathodes was 0.104 mA at −0.4 V, 1.652 mA at −0.3 V, and 1.235 mA at −0.2 V, respectively. The variation in reducing electroactivity observed across different culture potentials might be attributed to the interplay between two key factors: the abundance of electrogenic bacteria and the competition for electrons by the electrode. At a more negative potential, the low loading of electrogenic bacteria might lead to limited attachment sites for nitrite-reducing EAB and an insufficient electron supply for energy synthesis, thereby preventing the effective enrichment of nitrite-reducing EAB. Conversely, at higher a potential, the enhanced competition of electrodes towards electrons limited the flow of electrons from electrogenic bacteria to reducing EAB.

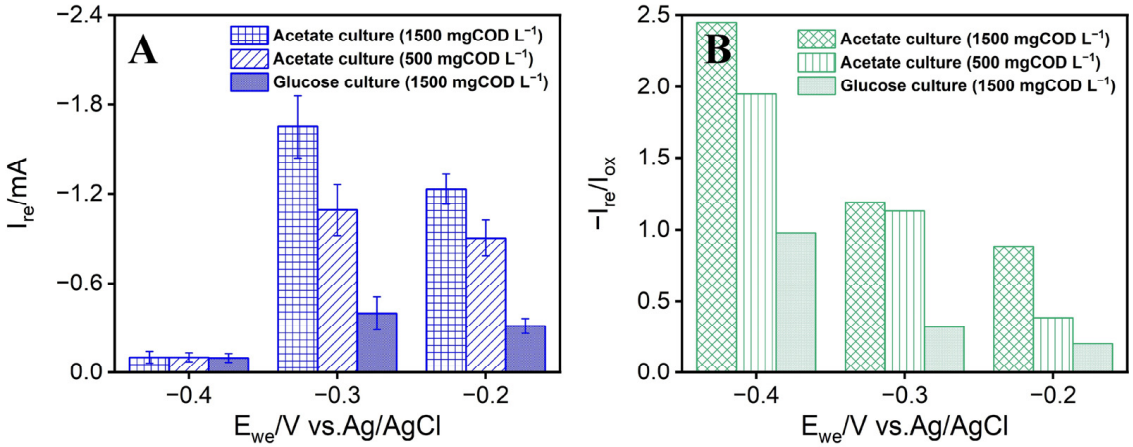


Figure 4. (**A**) The nitrite-reducing electroactivity of a bioelectrode cultured in RPBC under different electrode potentials (−0.4 V, −0.3 V, and −0.2 V) and substrate conditions (100 mg COD L^{-1} acetate, 500 mg COD L^{-1} acetate, and 1500 mg COD L^{-1} acetate); (**B**) the $-I_{re}/I_{ox}$ of a bioelectrode cultured in RPBC under different electrode potentials (−0.4 V, −0.3 V, and −0.2 V) and substrate conditions (100 mg COD L^{-1} acetate, 500 mg COD L^{-1} acetate, and 1500 mg COD L^{-1} acetate).

To further validate the effects of electrode potential and substrate conditions on RPBC, the $-I_{re}/I_{ox}$ ratio of biocathodes cultured under various conditions was compared (Figure 4B). Under these circumstances, a positive correlation was observed between the quantity of directly available electron donors in the culture medium and the $-I_{re}/I_{ox}$ ratio of the biocathode. For instance, at −0.3 V, the $-I_{re}/I_{ox}$ ratio of biocathodes in different solutions decreased from 1.19 (1500 mg COD L^{-1} acetate) to 1.11 (500 mg COD L^{-1} acetate), and further to 0.33 (100 mg COD L^{-1} acetate). The highest $-I_{re}/I_{ox}$ ratio exhibited by biocathodes cultured in 1500 mg COD L^{-1} acetate solution suggested that in medium richer in directly available electron donors, a greater number of nitrite-reducing EAB could be enriched per unit of electrogenic bacteria. With the potential positively shifted from −0.4 V to −0.2 V, the competition of the electrode for electrons gradually strengthened, and the $-I_{re}/I_{ox}$ rate gradually decreased. For instance, under the culture condition with 1500 mg COD L^{-1} acetate, the $-I_{re}/I_{ox}$ ratio of the biocathode decreased from 2.44 to 0.88 as the electrode potential shifted from −0.4 V to −0.2 V. This phenomenon might be attributed to the increased competition of the electrode for electrons, which, by affecting the process of nitrite-reducing EAB obtaining energy from electrogenic bacteria, reduced the number of nitrite-reducing EAB enriching on the electrode and consequently decreased the nitrite-reducing electroactivity of the biocathode cultured in RPBC. The above results indicated that the competition of bioelectrodes for electrons and the quantity of directly available electron donors could significantly influence the performance of biocathodes cultured in RPBC. The optimization of the RPBC to obtain high-performance biocathodes could be achieved through the regulation of electrode potential and substrate conditions.

3.4. Research on the Interspecies Structural Functions in RPBC

The scanning electron microscopy (SEM) image revealed significant differences in biomass accumulation and biofilm structure between biocathodes cultured in DBC and RPBC. After 3 days of culture, the biocathode cultured in DBC exhibited sparse microbial adhesion to the electrode surface (Figure 5A). Although a prolonged culture (30 days) led to an increase in microbial population (Figure 5B), the biocathode cultured in DBC maintained a relatively dispersed distribution without forming distinct connective structures. The biofilms exhibited a complex microbial composition without any apparent dominant species.

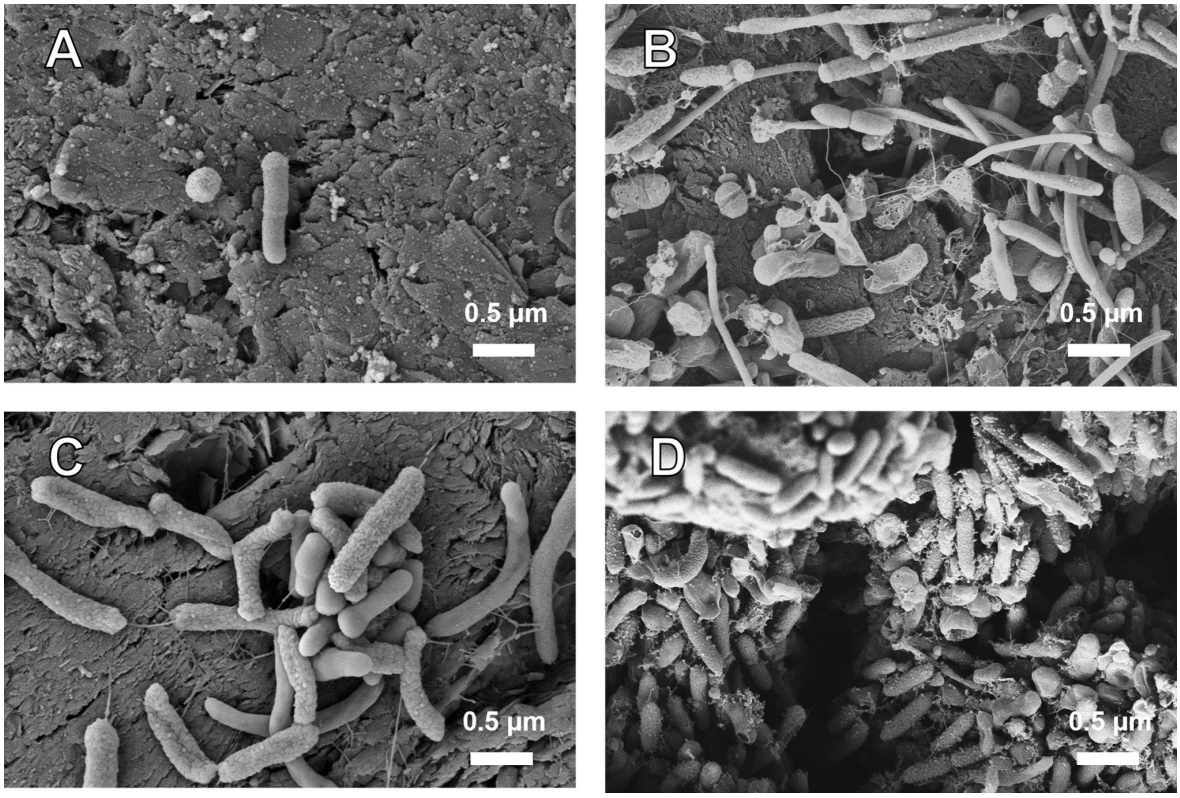


Figure 5. (**A**) The SEM results of the electrode biofilm on the 3rd day of the biocathode cultured in DBC and (**B**) the 30th day of the biocathode cultured in DBC; (**C**) the SEM results of the electrode biofilm on the 3rd day of the biocathode cultured in RPBC and (**D**) the 30th day of the biocathode cultured in RPBC.

In contrast, the biocathode cultured in RPBC demonstrated superior biomass accumulation and interconnected microbial structures within the same initial 3-day period (Figure 5C). There were two dominant bacterial species in the early biofilm cultured in RPBC: rod-shaped bacteria with distinct surface wrinkles (potentially electrogenic bacteria, such as *Geobacter*) and smaller rod-shaped or coccoid bacteria (potentially nitrite-reducing EAB, such as the *Pseudomonas* species). Notably, the biofilm structure on the biocathode cultured in RPBC exhibited a network-like architecture, formed by electrogenic bacteria and nitrite-reducing EAB. This three-dimensional biofilm structure differed from the biofilm cultured in DBC, where bacteria were observed to be more dispersed and merely attached to the electrode surface.

The SEM image (Figure 5D) showed that after 30 days of culture, the biocathode cultured in RPBC developed a multi-layered, stacked biofilm structure with intimate connections between electrogenic and nitrite-reducing bacteria. And the microbial density of biofilm cultured in RPBC was significantly higher than that observed in biofilm cultured in DBC, which aligned with the high protein content measured in the biofilm cultured in RPBC (Figure 3B). This observation suggested that the formation of the composite biofilm structure might provide more attachment sites for nitrite-reducing EAB, which thereby contribute to the enhanced reducing electroactivity of the biocathode cultured in RPBC. Moreover, the nanowire structures (Figure 5C,D) observed in the biofilm cultured in RPBC further supported the hypothesis that electrogenic bacteria might provide energy for nitrite-reducing EAB growth through electron transfer. Due to the uncertainty of biofilm growth and the complexity of the microbial population in mixed biofilms, no significant differences in biofilm structure were observed with different substrate concentrations.

3.5. The Effect of EAB Interspecies Interactions on the Maintenance of a Biocathode Cultured in RPBC

The factors affecting the growth and maintenance of reducing EAB on the electrodes were analyzed by operating the stable biocathodes cultured in RPBC under different conditions, and using metronidazole to selectively remove the electrogenic bacteria on the biocathodes. After metronidazole treatment, the nitrite-reducing electroactivity of the bioelectrodes (MFC-1 and MFC-2) under both solution conditions (acetate solution with and without added EPS) did not change immediately but gradually decreased with the operational time and finally stabilized at 26% and 5% of that before metronidazole treatment, respectively (Figure 6). And after 20 days of operation, the biofilm thickness of the two bioelectrodes decreased by 25.10 μm and 27.42 μm, making them 30.1% and 34.1% lower than before treatment. The viability of the microorganisms on the two bioelectrodes decreased by 45.8% and 35.8%, respectively (Figure 7). The delayed reduction in electroactivity after metronidazole treatment indicated that metronidazole did not directly inhibit the activity of nitrite-reducing EAB but indirectly affected the growth of nitrite-reducing EAB by removing electrogenic bacteria. In addition, this gradual decrease in reducing electroactivity, rather than an immediate drop, suggested the possible existence of alternative electron transfer pathways in biofilms cultured in RPBC. These pathways, which were not immediately interrupted by the death of the electrogenic bacteria, might involve extracellular electron transfer mechanisms such as conductive nanowires and redox-active mediators.

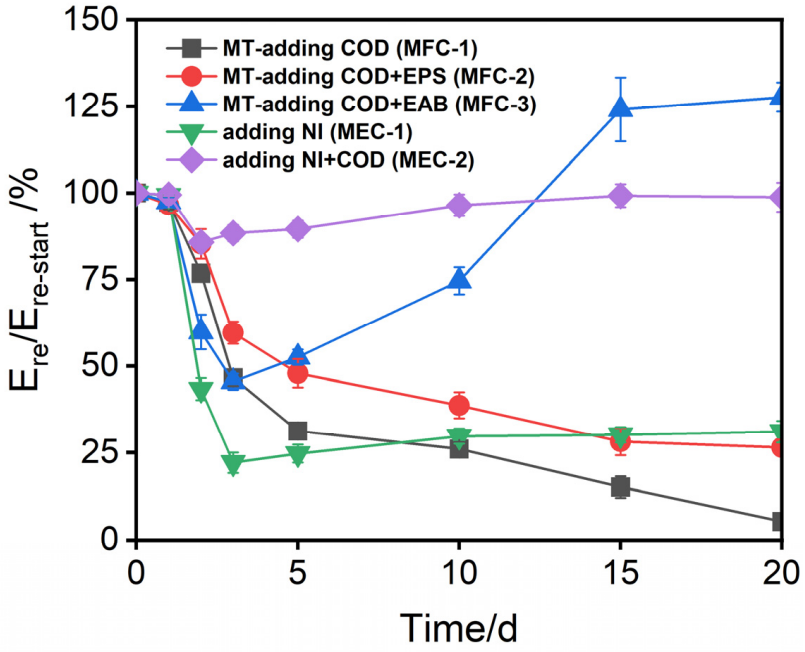
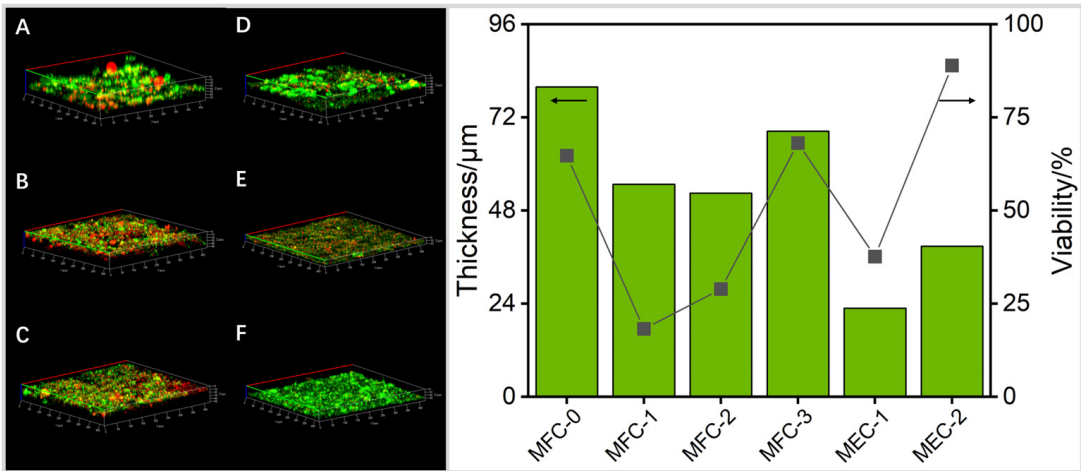


Figure 6. The electroactivity of bioelectrodes, including the bioelectrode cultured before the killing of the electrogenic bacteria, operated in 50 mM PBS with 1500 mg COD L-1 acetate (MFC-0); the bioelectrode with the electrogenic bacteria killed operated in 50 mM PBS with 1500 mg COD L^{-1} acetate (MFC-1); the bioelectrode with the electrogenic bacteria killed operated in a mixture of 2/3 (v/v) 50 mM PBS and 1/3 (v/v) of filtered MFC effluent with 1500 mg COD L-1 acetate (MFC-2); the bioelectrode with the electrogenic bacteria killed operated in a mixture of 2/3 (v/v) 50 mM PBS and 1/3 (v/v) MFC effluent with 1500 mg COD L^{-1} acetate (MFC-3); the cultured bioelectrode operated under a constant potential of −0.4 V separately in 50 mM PBS with 30 mgN L^{-1} $NaNO_2$ (MEC-1); and the cultured bioelectrode operated in 50 mM PBS with 1500 mg COD L^{-1} acetate and 30 mgN L^{-1} $NaNO_2$ (MEC-2) during twenty days of operation under different solution conditions and in various operational modes.



(**a**) (**b**)

Figure 7. (**a**) Confocal laser scanning imaging of bioelectrodes of MFC-0 (**A**), MFC-1 (**B**), MFC-2 (**C**), MFC-3 (**D**), MEC-1 (**E**), and MEC-2 (**F**); (**b**) the thicknesses (corresponding to the left ordinate) and viabilities (corresponding to the right ordinate) of different bioelectrodes.

Comparing the nitrite-reducing electroactivity of MFC-1 and MFC-2 over time, it could be observed that MFC-2 maintained a higher electroactivity within 20 days after metronidazole treatment than MFC-1. This difference showed that the addition of EPS partially alleviated the impact of removing electrogenic bacteria on the bioelectrode cultured in RPBC. However, the overall trends in the electroactivity and biofilm structure of MFC-2 were similar to those observed in MFC-1 operating in acetate solutions, which suggested that relying on EPS and substrate supplementation alone was insufficient to fully maintain the reducing electroactivity of the biocathodes cultured in RPBC. Thereby the promotion of nitrite-reducing EAB growth by electrogenic bacteria was not solely driven by extracellular secretions, but more significantly by the electrogenic bacteria themselves.

On the other hand, the nitrite-reducing electroactivity of MFC-3 in the acetate solution with electrogenic bacteria initially declined, followed by a gradual increase, eventually reaching 127% of the pre-metronidazole treatment level after 20 days of operation. The re-inoculation reactivated the nitrite-reducing EAB and rebuilt the biofilm, with its thickness returning to 85.7% (68.44 μm vs. 79.85 μm) and its activity increasing compared with that before the metronidazole treatment (68.1% vs. 64.7%). Therefore, we thought the existence of electrogenic bacteria was the main reason for the quick accumulation and stable maintenance of the nitrite-reducing EAB on the electrode. In RPBC, electrogenic bacteria likely not only served as the energy source for the growth of nitrite-reducing EAB but also, together with the nitrite-reducing EAB, formed a stable three-dimensional composite biofilm structure, thereby increasing the enrichment quantity of nitrite-reducing EAB.

After switching to potentiostatic culture mode and changing the substrate from a medium containing 1500 mg COD L^{-1} acetate to the one containing 30 mgN L^{-1} $NaNO_2$, the nitrite-reducing electroactivity of MEC-1 declined by 75% within 3 days, eventually stabilizing at about 31% of the value before the polarity was reversed. This result indicated that, despite the decline, nitrite-reducing EAB could partially obtain electrons through the electrode to maintain their metabolic activity. Concurrently with the electroactivity decline, the thickness of the MEC-1 biofilm significantly decreased from 79.85 μm to 22.81 μm (only 28.6% of that before treatment), and the biofilm's viability reduced from 64.7% to 37.6%. This phenomenon might be attributed to the unfavorable conditions without substrate supplementation, which made it difficult to maintain the amount and

activity of electrogenic bacteria within the biofilm. Without electrogenic bacteria, the biocathode failed to form a complex three-dimensional composite structure, instead tending towards a planar configuration. This structural limitation led to a gradual decline in the nitrite-reducing EAB population, consequently causing a decrease in the nitrite-reducing electroactivity of the biocathode.

Conversely, the reactor operating in a solution containing both nitrite and acetate maintained the reducing electroactivity of the bioelectrode at a relatively constant level. The confocal images showed that the viability of the MEC-2 biofilm was higher than that of MEC-1 (88.9% vs. 37.6%), and the thickness of the MEC-2 biofilm was also 1.7 times greater than that of MEC-1. In addition, the bioelectrode biofilms cultured in MEC mode exhibited a reduced thickness compared to that of those cultured in MFC mode. This might be because the potential control accelerated the bioelectrochemical oxidation rate, which led to the increased utilization of EPSs as energy substrates. And the accelerated consumption of EPSs might result in a more compact arrangement of bacteria within the biofilm matrix, which consequently reduced the overall biofilm thickness [25]. The above results indicate that, in addition to electron transfer with the electrode, nitrite-reducing EAB could also exchange electrons with the electrogenic bacteria, explaining the promoted enrichment of nitrite-reducing EAB on the bioelectrode cultured in RPBC. And the composite structure formed by the electrogenic bacteria and nitrite-reducing EAB improved the enrichment quantity, which was the main reason for the high nitrite-reducing electroactivity of the biocathode cultured in RPBC.

The microbial community results showed that the bioelectrode cultured in RPBC significantly enriched both electrogenic bacteria and the reducing EAB from the anoxic sludge inoculum. Specifically, the relative abundance of the main electrogenic bacteria *Geobacter* [26] increased from 1.02% to 37.64%, and the nitrite-reducing EAB increased from 1.13% to 7.19% (Figure 8). *Pseudomonas*, with a relative abundance of 7.19%, was considered to be the main nitrite-reducing EAB enriched in RPBC [27]. The concurrent increase in the relative abundance of electrogenic bacteria and nitrite-reducing EAB corresponded to the progressive enhancement of redox electroactivity in RPBC. After metronidazole treatment, the relative abundance of *Geobacter* in MFC-1 and MFC-2 decreased from 37.64 to 1.15%~2.23%. Moreover, the relative abundance of *Pseudomonas* reduced from 7.19% to 1.66~4.42%. The decrease in the relative abundance of the two main functional bacteria was consistent with the reduction in both oxidizing and reducing electroactivity of the bioelectrodes after metronidazole treatment. The average decrease in Pseudomonas abundance was close to 5%. This obvious abundance change indicated that the abundance of electrogenic bacteria impacted the growth of reducing electroactive microorganisms, rather than slight fluctuations in microbial abundance caused by the operation process. Since *Pseudomonas* is a facultative anaerobic microorganism, its activity was not directly affected by metronidazole [27]. However, the decrease in *Pseudomonas'* relative abundance following the removal of *Geobacter* by the metronidazole treatment suggests that the growth of *Pseudomonas* in RPBC is dependent on the presence of *Geobacter*. On the bioelectrode re-inoculated with electrogenic bacteria, the relative abundance of *Geobacter* and *Pseudomonas* increased and finally reached 57.65% and 9.77%, respectively. Similarly, the MEC-2 also possessed a high relative abundance of *Geobacter* (32.21%) and *Pseudomonas* (10.12%). This result further proved that the existence of electrogenic bacteria was the key factor for the enrichment of nitrite-reducing EAB on the bioelectrode cultured in RPBC. Interestingly, after re-inoculation, the relative abundances of the two primary functional bacteria surpassed their pre-metronidazole treatment levels, possibly due to metronidazole treatment eliminating non-functional anaerobic bacteria in the inner biofilm layer, thereby indirectly increasing the relative abundances of electrogenic bacteria and nitrite-reducing EAB. The

MEC-1 operated in the nitrite solution without organic substrate was the only bioelectrode with a high *Pseudomonas* abundance (46.14%) and a low *Geobacter* abundance (0.11%), which verified that nitrite-reducing EAB could directly use the electrode to obtain electrons to maintain growth.

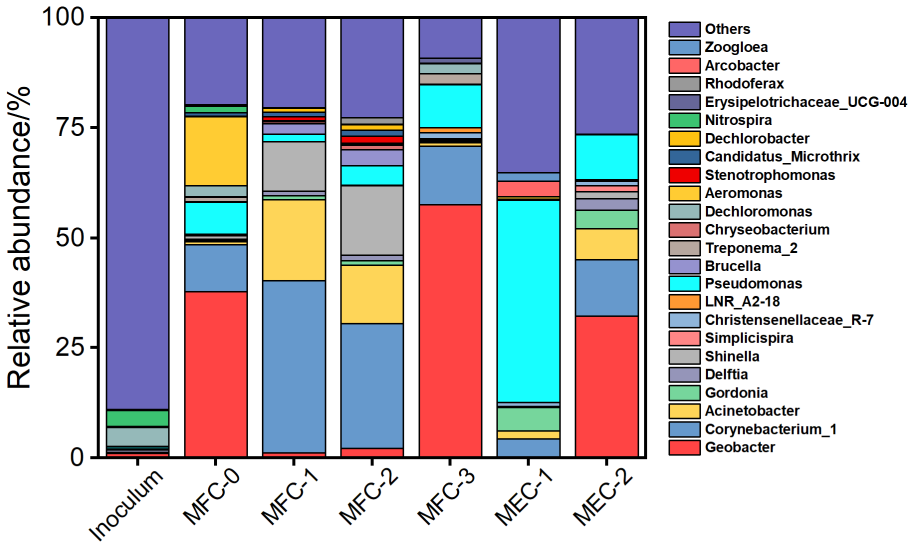


Figure 8. The relative abundance of the microbial community in the inoculum and the bioelectrodes of MFC-0, MFC-1, MFC-2, MFC-3, MEC-1, and MEC-2 at the genus level.

4. Conclusions

This study investigated the mechanism of the rapid culture of high-performance biocathodes using RPBC technology. It was found that the oxidizing and reducing electroactivity of the bioelectrodes always change synchronously in RPBC. The reducing electroactivity of biocathodes cultured in RPBC was influenced by the operational parameters (substrate concentration and electrode potential) of electrogenic bacteria during the culture process. The higher the number of available electron donors and the weaker the electron competition of the electrode, the higher the ratio of the reducing electroactivity to the oxidizing electroactivity of the bioelectrode. This phenomenon revealed the role of electrogenic bacteria in energy supply for promoting the enrichment of reducing EAB in RPBC. On the other hand, the high reducing electroactivity of biocathodes cultured in RPBC showed the high biomass of both electrogenic bacteria and reducing EAB. The morphology of biofilms cultured by RPBC showed that electrogenic bacteria and reducing EAB formed a three-dimensional composite biofilm structure on the electrode surface, increasing the biomass of reducing EAB loaded onto electrodes, resulting in thick, dense, and highly reducing electroactive biocathode biofilms. Our research elucidated the interspecies mechanism between electrogenic bacteria and reducing EAB in RPBC, whereby the enrichment of reducing EAB was promoted through both the energy supply from electrogenic bacteria and the formation of a composite biofilm structure by both electrogenic bacteria and reducing EAB. Based on this mechanism, a regulation and maintenance method for biocathodes cultured in RPBC has been proposed, which should be beneficial for promoting the future application of biocathodes in fields such as sensors and pollutant degradation.

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