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Effects of azide on current generation and microbial community in air-cathode MFCs

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Azide is known to be a respiratory inhibitor, which can disrupt electron transfer in the process of aerobic respiration. It has been proposed for preventing the reduction of oxygen in the anode compartment of MFC-based biosensors, but has also been found to function as an electron acceptor in recent research. However, there are few reports about the effects of azide on the structure and composition of the microbial community in air-cathode MFCs, as well as on their corresponding performance. Therefore, the current generation, electroactivity and community structure of anodic biofilms were investigated using air-cathode MFCs acclimated with (1.5 mM) and without azide. The enrichment process was much slower in the presence of azide compared to the control. Biofilms enriched with and without azide were found to produce similar voltammograms, but the difference lay in the current intensity of the predominant peaks. Pyrosequencing indicated that the distribution of microbes at the genus level was more uniform, with *Geobacter* and *Ignavibacterium* being the dominant genera on both biofilms, although the community of the azide-enriched film was less diverse than that of the control. These results demonstrate that the microbial community enriched with azide was not significantly altered compared to the control and the difference in the maximum current or peak current of cyclic voltammograms (CVs) was thought to be related to the amount of biomass.

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1 Introduction

Organic contaminants in wastewater are potential sources of energy.[1] Microorganisms convert them to useful forms of energy through various biochemical processes, such as methanogenesis. Among these, microbial fuel cells (MFCs) are attractive because they have functional and operational advantages,[2] although much effort is required to enable their practical application. Besides electricity generation, MFCs are used to determine biochemical oxygen demand (BOD),[3] since the amount of coulombs obtained from MFCs is proportional to the concentration of the fuel used.[4] MFC-based biosensors, compared with conventional methods, have shown prolonged operational stability and very high substrate versatility.[5] Therefore, several types of MFC-based biosensor have been developed.[6–8] Generally, single-chamber MFCs with a membrane electrode assembly (MEA) show better performance than two-chamber systems.[9]

During the sensing process, oxygen diffuses through a membrane into the anode chamber and nitrate is contained in the samples to be analyzed for BOD. The presence of these electron acceptors in the samples results in substantial electron losses, because oxygen and nitrate are preferred electron acceptors over the anode.[10] Consequently, their presence in the anode compartment is a major reason for low coulombic efficiency (CE). However, the CE should be improved to achieve better performance in MFC-based biosensors. Respiratory inhibitors, such as azide, can inhibit oxygen and nitrate reduction. Hence, adding azide into anode compartments has been considered for improving CE. A current increase has been observed when azide was added into air-saturated or nitrate-containing anode compartments,[11] because current generation from an MFC was inhibited by NADH dehydrogenase, coenzyme Q and quinol-cytochrome b oxidoreductase inhibitors, but not by terminal oxidase inhibitors.[12] This contrasts with recent reports that anodes enriched with azide from the beginning generated a lower current than those without azide. For the latter, further analyses showed that azide added into the anode compartment was used as an electron acceptor during the enrichment process.[13] Presumably, azide not only inhibited aerobic respiration, but also selected for azide-respiring bacteria when azide was used from the beginning of the enrichment process. Various electron donors and acceptors are used by prokaryotes, employing different electron transport chains.

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Previous researchers have demonstrated that various factors, such as fuel type[14] and external resistance,[15] as well as anode potential,[16] have an impact on the structure and composition of the microbial community, which in turn affects the current generation or power output.

The effect of azide on the composition of the microbial community was observed in two-chamber systems, where electrochemically active bacteria (EAB) were enriched with and without azide from the beginning. Microbial communities on the anodes of MFCs with azide were dominated by *Geobacter sulfurreducens* (>94.5% of all clones) and overall 98% of the clones were *Deltaproteobacteria*.[13] Unfortunately, a community analysis was not carried out for the control, because beforehand a similar test, that used acetate to enrich EAB, had been conducted in the same two-chamber reactors, where the population of the MFCs without azide consisted of *Deltaproteobacteria* (68.8%), *Gammaproteobacteria* (17.3%), *Alphaproteobacteria* (7.0%), and *Bacteroides* (3.4%). Obviously, azide could reduce the diversity of populations in the anode compartment of two-chamber MFCs.[12] Despite the availability of a high percentage of *G. sulfurreducens* on the azide-enriched biofilm, the peak current and CE (4.0 mA, 65.0 ± 5.0%) in the presence of azide (0.2 mM) did not increase relatively, compared to that (4.5 mA, 70.0 ± 5.0%) without azide. The difference in current generation was possibly due to azide, which was believed to consume more electrons than oxygen did in the anode compartment of MFCs without azide.[13]

Differently from two-chamber MFCs, air-cathode single-chamber MFCs have a unique MEA configuration, which enables more oxygen to diffuse from the cathode to the anode and reduces the system resistance by avoiding the use of exchange membranes. It has been reported that a difference in dissolved oxygen[13,17,18] and system resistance[9] can affect the microbial community as well as the system performance. So the effect of azide on single-chamber MFCs cannot be simply explained by results from two-chamber models. In addition, there are few reports about the effect of azide on the structure and composition of the microbial community in air-cathode MFCs, as well as on their corresponding performance. So far, cyclic voltammetry has been thought the preferred method to test the electrochemical behavior of a biofilm and its community characteristics.[19] To investigate whether there exist specific bacterial species able to reduce azide, and how the microbial community or composition obtained from an azide-enriched biofilm influences the performance of MFCs, air-cathode MFCs were acclimated with and without azide from the beginning. Therefore, the electrochemical behavior of a biofilm and its community structure were examined for MFCs acclimated with and without azide.

2 Materials and methods

2.1 Reactor construction and operation

A cube-shaped reactor with a cylindrical chamber (3 cm in diameter, 4 cm in length) was constructed as previously reported.[20] The anode (3 cm in diameter, 3 cm in length) was made of graphite fibers, wound into two twisted titanium wires and heat-treated according to previous procedures.[21] The cathode was carbon cloth (B1B30WP, 30% wet-proofing, E-Tek Division^{SM}), with one side as the catalyst layer (0.5 mg cm^{-2} Pt, Hesen, Shanghai) and the other side as the diffusion layer (4 PTFE layers).[22] Titanium contacts were used to connect the electrodes to an external circuit.

Four reactors (tested in duplicate) were initially inoculated (at intervals of 2 days) with a 20 : 80 mixture of domestic wastewater and acetate medium. Eight days later, these reactors were divided into two groups: one as the control, only fed with acetate medium, the others with acetate medium plus 1.5 mM azide. The acetate medium (1.64 g L^{-1}, pH = 7.0, 7.8 mS cm^{-1}) contained the following (per liter): KCl, 0.13 g; $NaH_2PO_4 \cdot 2H_2O$, 3.32 g; $Na_2HPO_4 \cdot 12H_2O$, 10.32 g; NH_4Cl, 0.31 g; vitamin (5 mL); trace mineral (12.5 mL).[23] During operation, the reactors were placed in a temperature-controlled chamber (30 °C) and were refilled with fresh medium when the voltage dropped below 50 mV.

2.2 Analyses and calculations

The voltage (U) across the external resistance (1000 Ω) was recorded every 30 min using a data acquisition system (PISO-813, ICP DAS Co., Ltd). Current (I) and power (P) were calculated based on Ohm's law and normalized by the cathode surface area (7 cm^2).

Cyclic voltammograms were performed for the anode biofilms and cell-free effluents from the anode compartment using a potentiostat (PGSTAT128N, Utrecht, Netherlands). To prevent interference of excreted redox mediators in the spent medium, the CV test for the biofilm was conducted in 50 mM PBS without acetate or azide. The anode was the working electrode, and the cathode was the counter electrode with an Ag/AgCl reference electrode (0.197 V *vs.* SHE). Scans started from −0.700 V and went up to 0.200 V and back at a scan rate of 5 mV s^{-1}. The spent medium was collected using a test vial and centrifuged (12 000 rpm, 4 min) to remove suspended solids. The CV test for the supernatant was conducted in a three-electrode electrochemical system. A carbon cloth working electrode (B-1 designation B, 30% wet-proofing, E-Tek, USA; 7 cm^2), an Ag/AgCl reference electrode and a platinum counter electrode were used in a 28 mL electrochemical cell. The potential was varied from −0.7 V to 0.2 V at a scan rate of 5 mV s^{-1}. Both PBS and supernatant were flushed (15 min) with nitrogen gas prior to analysis. These tests were performed using samples taken from 6-month-old MFCs.

Samples taken from these reactors were immediately filtered through 0.22 μm pore diameter syringe filters before acetate was analyzed using HPLC equipped with an Aminex HPX 87H column (Bio-Rad Laboratories, Hercules, CA).

2.3 Bacterial community analysis

Bacterial communities were analyzed using samples taken from thirteen-month-old MFCs. Two representative biofilms, one taken from controls (0 mM azide), and the other from azide-acclimated (1.5 mM azide) reactors, were analyzed using a 454/Roche GS-FLX instrument to describe the community

differences. The graphite fibers were cut from the anodes of the azide-acclimated MFCs and those of the controls. The total genomic DNA was extracted using a Bacteria DNA Mini Kit (Watson Biotechnologies, Inc., Shanghai) according to the producer's instructions. The DNA extracts were inspected by electrophoresis in 0.7% agarose gels with ethidium bromide staining. The extracts were subjected to PCR to amplify bacterial 16S rDNA genes. PCR and sequencing were performed using the universal bacterial primers: F (5′-CGTATCGCCTCCCTCGCGC CATCAG-3′) and R (5′-CTATGCGCCTTGCCAGCCCGCTCAG-3′). A mixture of amplicons was pyrosequenced using a Roche 454 FLX instrument with Titanium reagents. Raw sequencing data were processed using the QIIME (Quantitative Insights Into Microbial Ecology) pipeline. Sequences with lengths lower than 200 base pairs (bp) were removed according to standard protocols. Sequences with similarity at or above 97% were clustered and defined as an operational taxonomic unit (OTU) for generating rarefaction curves and calculating the richness (Chao 1 and abundance-based coverage estimator) and diversity indices. Representative sequences from each OTU were phylogenetically assigned to phylum, family and genus taxonomic groups using a RDP naïve Bayesian rDNA classifier with a confidence threshold of 80%.[24]

3 Results

3.1 Effect of azide on MFC performance

The control MFCs generated a stable current of 0.533 ± 0.005 mA in repeated feeding cycles after 5 weeks of operation. However, for the azide-acclimated MFCs the currents were much lower than for the controls and increased during the feeding cycles from 0.115 mA to 0.511 mA (Fig. 1). Correspondingly, the cycle time was almost stable (45.5 ± 1.5 h) for the controls and comparable to that (43.5 ± 1.4 h, except for 83 h obtained at the end of the second month) for the azide-acclimated reactors (Fig. 1A). Upon the end of each cycle, the depleted medium was replaced with acetate medium with the same concentration. Therefore, the gradual increase in (maximum) current with time should be associated with the increase in biofilm mass. The longer cycle time at the end of the second month suggested much slower substrate utilization rates in azide-acclimated MFCs compared to those in controls. In contrast, the times for the following batch cycles of azide-acclimated MFCs were very close to, and slightly shorter than, those of the controls (Fig. 1B–D), and acetate was below the detection limit at the end of each cycle (data not shown). This further demonstrates that the substrate utilization rates in azide-acclimated MFCs were not influenced by azide once these reactors had been run for more than a certain period of time.

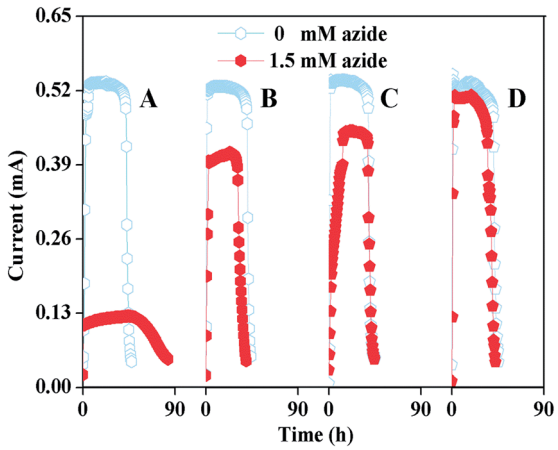

Fig. 1 Current evolution over one batch cycle at the end of the second (A), fourth (B), sixth (C) and eighth (D) months for reactors with and without the addition of azide.

In addition, acetate was not detected at the end of each cycle, which means that CO_2 was the end product and the released electrons (370 C) from acetate oxidation were diverted into other electron sinks. The amount of coulombs recovered per cycle for the controls was constant (79.2 ± 0.5 C), but increased from 32.3 C to 67.5 C for the azide-added reactors. This further indicates that the biomass of EAB increased during the enrichment process. The slow enrichment in EAB in the presence of azide might be due to molecular oxygen diffusing through the cathode that is not consumed by aerobic respiratory organisms, since azide is a respiratory oxidase inhibitor.[8]

3.2 CV analysis of anode biofilms

CVs were performed to study the mechanism of electron transfer and the electroactivity of anode biofilms acclimated with and without azide in PBS (50 mM) containing no acetate. A lack of acetate could prevent a current from acetate oxidation.[25]

For the anode of the control, oxidation (positive) current with a threshold (~−0.53 V *vs.* Ag/AgCl) was observed during the forward scan, reflecting electron transfer to the anode. In the narrow region (between −0.53 and −0.47 V), the current was low and constant. Subsequently, the current increased rapidly until redox species in the reduced state at the electrode surface were substantially oxidized, causing the current to peak (Fig. 2A). Thereafter, the current significantly decayed, which was possibly due to the slow kinetics of electron transfer from adjacent cell layers by cell–cell interactions. In the wake of the first peak, a peak-like shape (−0.26 V *vs.* Ag/AgCl) appeared on the voltammogram (Fig. 2A). This observation may support the idea that electrons at the outer edges of biofilms can also be depleted at sufficiently positive potentials.[26,27] This can provide an explanation of the ensuing limiting current produced in the high-potential region (from ~−0.16 to 2.00 V *vs.* Ag/AgCl). When the scan direction was switched to negative at 0.2 V for the reverse scan (Fig. 2A), a reversible peak (−0.28 V) was observed but was not evident on the voltammogram. This feature can easily be created by a rate-limiting process, where oxidized redox proteins not closely attached to the electrode surface may not have time to accept more electrons from the anode surface. In this study, biofilms enriched with and without azide were found to produce similar voltammograms, but the difference lay in the current intensity of the predominant peaks: 2.80 mA (−0.34 V, 0 mM); 1.22 mA (−0.33 V, 1.5 mM). The biofilm enriched without azide showed the highest electroactivity,

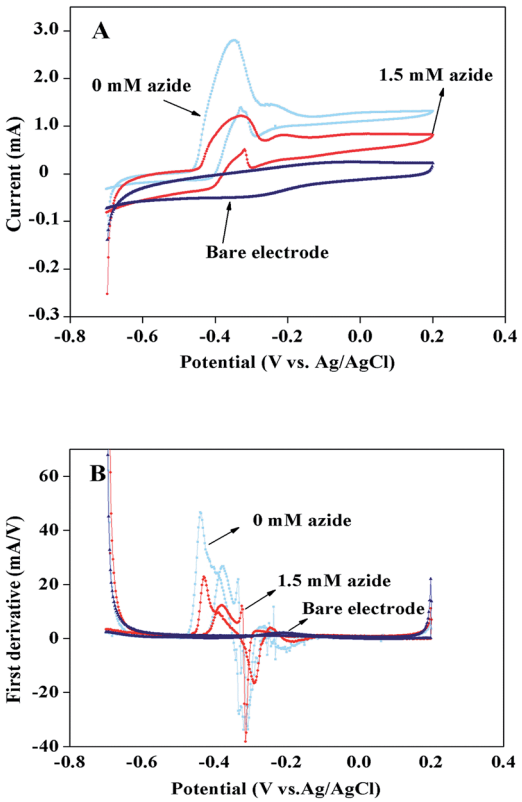

Fig. 2 (A) Cyclic voltammogram of the bacterial biofilms enriched with and without azide; (B) first derivatives of the voltammetric curve over the potential.

which was consistent with the maximum currents being produced in the controls. The decay in peak current of the azide-acclimated MFCs could be limited by the amount of EAB, because the reactions occurring at the anodes were just related to redox proteins.

To determine the midpoint potential in voltammograms of the biofilms, the first derivative of each voltammogram was plotted as a function of the potential. Examination of the first derivative of the CV for biofilms enriched with and without azide revealed one predominant redox couple centered at -0.37 ± 0.01 V *vs.* Ag/AgCl (Fig. 2B), which was close to midpoint potentials reported for periplasmic cytochrome c (PpcA, -0.37 V *vs.* Ag/AgCl) and OmcB (-0.39 V *vs.* Ag/AgCl) purified from *G. sulfurreducens*.[19,28] Thus, the similarity in midpoint potential between anode biofilms enriched with and without azide suggests that the presence of azide did not alter the extracellular electron transport pathways compared to those of the controls. Moreover, the bacteria dominant in the biofilms may contain genes encoding PpcA or OmcB, which function as electron transfer carriers by direct contact with the anode. CV scans of the spent medium, as well as of the fresh medium without acetate, showed similar voltammograms (data not shown) without any apparent peaks, indicating that no self-produced mediators appeared in these reactors. This further demonstrated that current generation was dependent on membrane-bound proteins in the biofilms.

3.3 Microbial diversity estimation and community distribution

The two samples yielded 14 819 (0 mM azide) and 18 327 (1.5 mM azide) qualified sequencing reads with an average length of 507 ± 3 bp. These reads were clustered into 5882 and 3972 operational taxonomic units (OTUs) with a distance limit of 0.03. To evaluate community diversity, rarefaction curves were generated based on the pyrosequencing data set. In addition, estimators, such as the Shannon index and Chao 1, as well as Good's coverage, were calculated for each sample (Table 1).

The shape of the rarefaction curves shows that new phylotypes would continue to emerge even after 15 000 reads, as neither of the curves tends to reach a plateau (Fig. 3). However, the coverage value for both samples was more than 73%, which indicated that an evaluation based on pyrosequencing could reflect the community compositions.[29] In the control, the Shannon index was 8.0, which was higher than that (7.0) of the azide-acclimated MFCs (Table 1), indicating higher microbial diversity in the control reactor. Although rarefaction curve analysis for the biofilms showed that the community of the control was more diverse than that of the azide-acclimated MFCs, only a few genera of bacteria dominated the anode biofilms enriched with and without azide.

To classify the bacterial community developed on the anodes, qualified reads were further assigned to known phyla, families and genera. Three dominant phyla (*Proteobacteria*, *Chlorobi* and *Bacteroidetes*) were identified on the anodes of the control and azide-acclimated reactors. *Proteobacteria* accounted for 85.2% of the population in the control (Fig. 4A), and 75.3% in the azide-acclimated MFC (Fig. 4B). The other two phyla, *Chlorobi* and *Bacteroidetes*, present in the azide-acclimated reactors accounted for 9.1% and 8.0%, which contrasted with the figures (5.7% *Chlorobi*; 3.6% *Bacteroidetes*) for the control reactors. Furthermore, a certain number of sequences were assigned to unclassified phyla. The relative abundances of each family on the anode were analyzed and are compared in Fig. 4C. The results show that *Geobacteraceae* was abundantly present in the controls (60.9%) and azide-acclimated reactors (69.8%). However, other major bacterial families, such as *Ignavibacteriaceae* and *Comamonadaceae*, present in both control and azide-acclimated reactors showed significant differences in relative abundance. *Comamonadaceae* made up 13.8% of the community in the control, much higher than the amount (0.2%) in the azide-acclimated reactor, while the amount of the family *Ignavibacteriaceae* present in the former was visibly lower than that in the latter (Fig. 4C). *Comamonadaceae* is a family of the *Betaproteobacteria*, which have been confirmed to be capable of reducing oxygen in wastewater.[30] Thus it seems that adding azide to an acetate medium might facilitate enrichment in *Ignavibacteriaceae*.

Classification of sequences at genus level could, from the perspective of bacterial function, give a description of biofilm communities.[31] It is clear that the anodes with and without azide were mainly colonized by *Geobacter*, with a relative abundance of 69.3% for the azide-acclimated reactor, and 60.0% for the control (Fig. 4D). *Geobacter* species are typically

Table 1 Various estimators for evaluation of community diversity and richness

Sample	Reads	OTU	Shannon	ACE	Chao 1	Coverage
Azide-enriched	18 327	3972	7.048188	16 324.13	9892.806	0.865226
Azide-free	14 819	5882	7.985024	27 594.53	16 324.43	0.730414

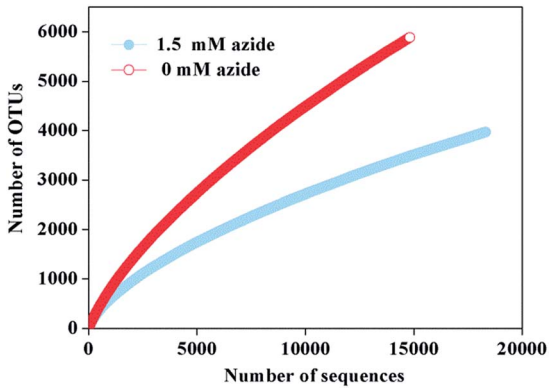

Fig. 3 Rarefaction analysis of sequences for the anode samples with and without azide. OTUs = operational taxonomic units, defined by clustering sequences with a 3% pairwise distance threshold.

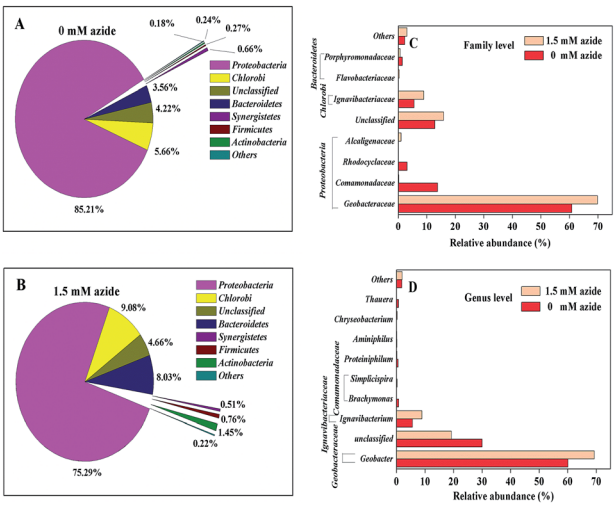

Fig. 4 Distributions of bacterial populations enriched on the anodes at phylum level (A and B); family level (C); and genus level (D).

found in acetate-fed MFCs, which are used to enrich EAB with electrodes as electron acceptors.[32] Apart from those sequences identified as unclassified (30.0%, 0 mM; 19.2%, 1.5 mM), a certain number of sequences were assigned to *Ignavibacterium* (5.5%, 0 mM; 8.9%, 1.5 mM). As noted above, the microbial community in all samples mainly consisted of *Geobacter* and *Ignavibacterium*, as well as unclassified genera, with an average percentage of 64.7 ± 6.6% for *Geobacter* and 7.2 ± 2.4% for *Ignavibacterium*. The similarity in microbial community structure between anode biofilms enriched with and without azide showed excellent agreement with that for the waveforms produced in the CV test.

4 Discussion

As noted above, rarefaction curve analysis, as well as the diversity index, showed that the community of azide-enriched biofilms was much less diverse than that of the control. *Comamonadaceae*, for example, were detected on the control, making up 13.8% of the population. Being aerobic, they were hardly detected on the azide-enriched anode. Obviously, the presence of azide restricted the diversity by effectively eliminating aerobes. Even so, the community on both anodes was mainly dominated by microbes very similar to *Geobacter*. In both cases, the distributions of microbes at the genus level were very similar, with *Geobacter* (60.0%, 0 mM; 69.3%, 1.5 mM) and *Ignavibacterium* (5.5%, 0 mM; 8.9%, 1.5 mM) (Fig. 4D). The results conclusively demonstrate that the presence of azide in air-cathode MFCs did not significantly alter the structure of the microbial community as compared to controls. This is different from communities in two-chamber MFCs continuously fed with and without azide, as the bacterial diversity and composition on an azide-acclimated anode in two-chamber MFCs were much lower than in controls.[12,13] The factors that affect the microbial community in the single- and two-chamber reactors are more complicated, because the reactor configuration and operating mode (single-chamber in fed-batch mode and two-chamber in continuous-flow mode), as well as the external resistance (1000 Ω for the single-chamber and 10 Ω for the two-chamber reactor), make it difficult to know which factors actually determine the microbial community. The difference in the composition of two-chamber MFCs with and without azide was possibly due to the inoculum used during the start-up period, because they were inoculated and operated for different times.

Previous studies have suggested that members of *Geobacteraceae* are typically found in acetate-fed MFCs, with most sequences related to *Geobacter*. As two representative members of *Geobacteraceae*, *Geobacter sulfurreducens* and *Geobacter metallireducens* were initially known to be capable of reducing Fe(III) oxide before being confirmed to be capable of directly transferring electrons to an anode.[33] Furthermore, alternative electron acceptors, such as fumarate and azide, are used for anaerobic respiration by *G. sulfurreducens*.[13,16] In the case of *Geobacter* species, the available evidence indicates that direct electron transfer to the anode is mainly related to various outer-membrane c-type cytochromes (*e.g.*, OmcZ), whereas it is still unknown whether these cytochromes can react with soluble electron acceptors, such as azide. In fact, an individual bacterium can use multiple electron transport chains, often

simultaneously, because electrons can enter the chain at three levels (a dehydrogenase, the quinine pool and a mobile cytochrome carrier), which correspond to successively smaller Gibbs free energy changes for the overall redox reaction between the electron donor and acceptor. Therefore, there exists the possibility that azide reduction can be achieved *via* other chains instead of *via* c-type cytochromes. Together with the similar waveforms and peak currents of CVs (Fig. 2A), this assumption may account for the relatively uniform distribution of microbes whether enriched with azide or not.

Even so, the influence of azide on current generation was evident during long-term operation, since current generation from the azide-acclimated reactors was found to gradually increase (Fig. 1). In MFCs, current generation depends on the response of EAB enriched on the anodes. In the case of anodes highly enriched with *Geobacter* species, the increasingly developed layers of cells do not cause a significant limitation in cell–cell transfer of electrons to the anode.[27] Several reports have shown that current generation by *Geobacter* species is positively correlated with biomass. Therefore, the gradual increase in current generation in azide-acclimated MFCs is thought to be due to an increase in biomass. Because the current generation from the azide-acclimated reactors was still lower than from the controls, even at the end of the experiment, the biomass on the azide-acclimated reactors should be less than that on the controls. This assumption was demonstrated in two-chamber MFCs, where a similar trend in current generation was also observed, with 0.487 μg DNA per g electrode extracted from the control and 0.3856 μg DNA per g electrode from the azide-acclimated reactor at the end of the experiment (data not published). Theoretically, the energy required for microbial growth depends on the electron donor and acceptor available in a biochemical system. According to the assumption that azide can enter the electron transport chain and accept electrons at a low level (except for the NADH dehydrogenase and the outer-surface cytochromes of the terminal complex), corresponding to low redox potentials, there will be less energy captured for bacterial growth in the presence of azide. This assumption was in agreement with the lower current generation and peak current of CVs compared to those of the controls. In addition, the enrichment process was slower in the presence of azide, also probably due to molecular oxygen diffusing through the MEA, which might be removed by aerobic respiration in the control MFCs.

5 Conclusions

This study mainly investigated the effects of azide on current generation and the microbial community in air-cathode MFCs. The results show that the enrichment process was much slower in the presence of azide when compared to the control. Correspondingly, the maximum current density gradually increased in the presence of azide, but was still below that of the control. Biofilms acclimated with and without azide were found to produce similar voltammograms, but the difference lay in the current intensity of the predominant peaks. Pyrosequencing indicated that biofilm communities enriched with and without azide were almost similar and dominated by bacteria very similar to *G. sulfurreducens*, although the community of the azide-enriched biofilm showed less diversity. The microbial community structure was not significantly altered in the presence of azide compared to that of the controls. The differences in current generation and peak current of CVs were thought to be due to the biomass yields developed on the anodes.

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