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High Biofilm Conductivity Maintained Despite Anode Potential Changes in a *Geobacter*-Enriched Biofilm

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Abstract

This study systematically assessed intracellular electron transfer (IET) and extracellular electron transfer (EET) kinetics with respect to anode potential (E_{anode}) in a mixed-culture biofilm anode enriched with *Geobacter* spp. High biofilm conductivity (0.96–1.24 mS cm⁻¹) was maintained during E_{anode} changes from -0.2 to +0.2 V versus the standard hydrogen electrode (SHE), although the steady-state current density significantly decreased from 2.05 to 0.35 A m⁻² in a microbial electrochemical cell. Substantial increase of the *Treponema* population was observed in the biofilm anode at $E_{\text{anode}}=+0.2$ V, which reduced intracellular electron-transfer kinetics associated with the maximum specific substrate-utilization rate by a factor of ten. This result suggests that fast EET kinetics can be maintained under dynamic E_{anode} conditions in a highly conductive biofilm anode as a result of shift of main EET players in the biofilm anode, although E_{anode} changes can influence IET kinetics.

Introduction

Extracellular electron transfer (EET) is a unique electron-transfer step for anode-respiring bacteria (ARB) to complete anaerobic respiration using conductive solids.^{1–3} This specific electron transfer is important for bioremediation of hazardous contaminants in subsurface environments,^{4, 5} resource recovery wastewater treatment technologies,^{6, 7} and bioelectronics.^{8, 9} Among them, resource recovery wastewater treatment using microbial electrochemical cells (MxCs) has gained significant attention in environmental engineering as energy and water issues become significant in recent society, moving toward sustainable wastewater treatment.

High current density is essential for improving benefits of MxC-utilized wastewater treatment, but identification of a rate-limiting step is still unclear owing to complex electron-

transfer processes in biofilm anodes. Electron transfer from a soluble electron donor to solid conductors can be divided into intracellular electron transfer (IET) and EET. The IET kinetics are commonly characterized with the Monod equation.^{10–14} Several studies reported IET kinetic parameters with apparent half-saturation substrate concentration (K_s^{app}), apparent maximum specific substrate utilization rate (q_{max}^{app}), or q_{max}^{app} multiplied with active biofilm density (X_f) for biofilm anodes.^{11–17} To obtain high current density from MxCs, biofilm anodes should be enriched with ARB having small K_s^{app} and high $q_{max}^{app} X_f$. For instance, K_s^{app} and $q_{max}^{app} X_f$ values range from 119–176 gCOD m³ (COD: chemical oxygen demand) and 8×10^5 – 1×10^6 gCOD m³ d^{−1}, respectively, in *Geobacter*-enriched biofilm anodes generating high current density (ca. 10 A m^{−2}).^{11, 13}

Many parameters can influence the IET kinetics, including pH, substrate type, substrate concentration, anode potential (E_{anode}), anode materials and configurations, and the presence of soluble electron acceptors (e.g., sulfate, oxygen, and nitrate).^{11, 13–15, 18–26} pH mainly affects ARB activity.^{25, 26} In comparison, most of other factors directly or indirectly influence microbial community structures on biofilm anodes, changing the IET kinetics.^{18, 21, 27} Among them, E_{anode} is a known important parameter for selection of kinetically efficient ARB, such as *Geobacter* genus, from mixed culture.^{18–21} Literature showed that negative E_{anode} [−0.05 to −0.15 V; all E_{anode} values are reported versus the standard hydrogen electrode (SHE)] enriched *Geobacter* spp. from mixed culture.^{18, 19} More diverse microbial communities in biofilm anodes were established at positive E_{anode} (+0.02 to +0.37 V) where IET kinetics becomes sluggish.^{18, 19} For instance, Hamelers et al. reported an increase of K_s^{app} from 0.37–2.2 mm acetate in a mixed culture biofilm anode for E_{anode} increase from −0.2 to −0.1 V.¹⁷ However, there are limited studies quantitatively evaluating E_{anode} effects on IET kinetics simultaneously with biofilm community analysis, especially for mixed-culture biofilm anodes that will be mainly applied for field applications, despite of significant E_{anode} impacts on the both parameters. Interestingly, literature suggested that a more diverse ARB community built at positive E_{anode} would diversify EET pathways, along with decrease of current density.^{18, 28–32} This finding implies that E_{anode} can also affect EET kinetics, but no literature has quantitatively investigated this important aspect in mixed-culture biofilm anodes.

Several EET mechanisms (i.e., direct contact, mediator, electron hopping, Ohmic conduction, or combined routes) have been proposed,^{1–3, 31} but most of the recent studies have commonly indicated the importance of electronically conductive EET in biofilm anodes producing high current density over several A m^{−2}.^{1, 8, 33–37} For instance, biofilm conductivity (K_{bio}) for pure culture of *Geobacter sulfurreducens* or *Geobacter*-enriched cultures ranged from 0.25 to 5 mS cm^{−1}, along with high current density (0.9–10 A m^{−2}).^{8, 35, 38} This result indicates that Ohmic conduction plays a vital role of EET in the biofilm anodes. Modeling works also supports the importance of Ohmic conduction for EET in the biofilm anodes,^{10, 39} such as the Nernst–Monod equation [Eq. 1]:

$$j = 0.14(1 - f_s^0)q_{max}^{app}X_fL_f\frac{S}{K_s^{app} + S}\left\{\frac{1}{1 + \exp\left[-\frac{F}{RT}(E_{anode} - E_{KA})\right]}\right\} \quad (1)$$

where j is the current density ($A\ m^{-2}$), 0.14 is the conversion factor for converting substrate flux to the current density ($0.14\ A=1\ g_{COD}\ d^{-1}$), f_s^0 is the fraction of electron used for cell synthesis ($f_s^0=0.1$),^{10, 11, 13} q_{max}^{app} is the maximum specific substrate utilization rate ($g_{COD}\ g^{-1}_{VSS}\ d^{-1}$), X_f is the concentration of active ARB in the anode biofilms ($g_{VSS}\ m^{-3}$), L_f is the biofilm thickness (m), K_s^{app} is the apparent half-saturation concentration of acetate ($mg_{COD}\ L^{-1}$), S is the acetate concentration ($mg_{COD}\ L^{-1}$), E_{anode} is the anode potential (V), E_{KA} is the halfsaturation potential at $j=0.5\ j_{max}$ (V), R is the ideal gas constant ($8.3145\ J\ mol^{-1}\ K^{-1}$), F is the Faraday's constant ($96\ 485\ C\ mol^{-1}\ e^{-1}$), and T is temperature (298.15 K).

The Nernst–Monod equation, which assumes Ohm's law governs EET,¹⁰ characterizes EET kinetics with E_{KA} . This equation well matches sigmoidal patterns of current density to E_{anode} change under substrate non-limiting conditions.^{10, 11, 13} Literature reports that E_{KA} ranges from -0.12 to $-0.24\ V$ (vs. SHE) for biofilm anodes, producing high current density.^{11–13, 18, 40} The model approaches using the Nernst–Monod equation suggested that K_{bio} would be higher than $0.5\ mS\ cm^{-1}$ for conductive biofilm anodes generating approximately $10\ A\ m^{-2}$, but there are no experimental evidences directly to support this estimation.¹³

Although conductive EET is significant for the biofilm anodes generating high current density, the information on E_{anode} effects exclusively on EET kinetics is very limited. Torres et al. assessed E_{KA} and cyclic voltammograms (CVs) for biofilm anodes grown at different E_{anode} conditions, based on the Nernst–Monod equation, and reported better EET kinetics for the biofilm anode grown at $E_{anode}=-0.15\ V$ than that at $E_{anode}=+0.37\ V$.¹⁸ However, E_{KA} data would be insufficient to support conductive EET without experimentally quantified biofilm conductivity. Previous works based on the Nernst–Monod equation have not quantitatively assessed biofilm conductivity and EET kinetics in biofilm anodes at different E_{anode} .^{10–14} To better understand kinetic dynamics in biofilm anodes caused by E_{anode} , we need detailed, quantitative data on both IET and EET kinetic parameters involved in current generation for MxCs.

Herein, we report IET (K_s^{app} and $q_{max}^{app} X_f$) and EET (K_{bio} and E_{KA}) kinetic parameters, along with microbial community structures, at steady-state current density in an MxC run with two different E_{anode} conditions. We then discuss the implications of the IET and EET kinetic parameters, along with biofilm community, to identify kinetic bottlenecks responsible for the decline of current density at positive E_{anode} .

Results

Current density, biofilm thickness, and biofilm conductivity

The steady-state current density was $2.05\pm0.05\ A\ m^{-2}$ at $E_{anode}=-0.2\ V$ (Figure 1). The current density significantly decreased by $0.35\pm0.004\ A\ m^{-2}$ at $E_{anode}=+0.2\ V$, although

equivalent operating conditions were kept, except for E_{anode} . The average biofilm thickness (L_f) was $34 \pm 5 \mu\text{m}$ at $E_{\text{anode}} = -0.2 \text{ V}$, which was comparable to an average of $L_f = 39 \pm 9 \mu\text{m}$ at $E_{\text{anode}} = +0.2 \text{ V}$ (Table 1). K_{bio} was high at $1.24 \pm 0.24 \text{ mS cm}^{-1}$ at $E_{\text{anode}} = -0.2 \text{ V}$, which slightly reduced to $K_{\text{bio}} = 0.96 \pm 0.21 \text{ mS cm}^{-1}$ at $E_{\text{anode}} = +0.2 \text{ V}$. High K_{bio} was maintained despite E_{anode} changes, although the steady-state current density decreased substantially at $E_{\text{anode}} = +0.2 \text{ V}$. Observed biofilm conductance ($G_{\text{bio}}^{\text{obs}}$) ranged from $G_{\text{bio}}^{\text{obs}} = 0.69 \pm 0.14$ to $0.79 \pm 0.15 \text{ mS}$ in two gold anodes bridged by biofilms (see Figure S1), while abiotic gold anodes showed trivial conductance of $G_{\text{control}} = 0.06 \pm 0.008$ to $0.06 \pm 0.002 \text{ mS}$ (see Table S2). This high $G_{\text{bio}}^{\text{obs}}$ clearly proved that the two gold anodes were connected by biofilms (see Experimental Section and Supporting Information for further details).

Microbial community analysis

Biofilm community structures were identified with a total of 158 565 rRNA sequences. Detailed information on the bacterial community analysis was provided in the Supporting Information (See Table S1). *Geobacter* genus, one of the most kinetically efficient ARBs, was predominant (96 %) for the biofilm anode at $E_{\text{anode}} = -0.2 \text{ V}$. *Geobacter*'s population substantially decreased to 44 % in the biofilm at $E_{\text{anode}} = +0.2 \text{ V}$ in which the population of *Treponema* spp. became significant (40 %) in the biofilm, along with small numbers of the *Pseudomonadaceae* family (5.3 %) and *Aminiphilus* genus (3.3 %; see Figure 2).

Estimation of the half-saturation potential

Figure 3 shows sigmoidal shapes of CVs for two E_{anode} conditions. E_{KA} was determined at $-0.230 \pm 0.003 \text{ V}$ at $E_{\text{anode}} = -0.2 \text{ V}$ and slightly increased by $-0.197 \pm 0.008 \text{ V}$ at $E_{\text{anode}} = +0.2 \text{ V}$ (see Table 1). This small change of E_{KA} is consistent with the K_{bio} trend, implying that EET would not limit the electron-transfer rate from a soluble electron donor (acetate) to the anode in the highly conductive biofilm anode, despite of substantial decline in current density.

Monod kinetic parameters

We estimated the best-fit values for Monod kinetic parameters of K_s^{app} and $q_{\text{max}}^{\text{app}} X_f$, using Equation (1) and measured parameters (effluent acetate concentration, biofilm thickness, halfsaturation potential, and current density). At $E_{\text{anode}} = -0.2 \text{ V}$, the best-fit K_s^{app} was 156 gCOD m^{-3} , which was 67 gCOD m^{-3} at $E_{\text{anode}} = +0.2 \text{ V}$. The best-fit $q_{\text{max}}^{\text{app}} X_f$ was $6.4 \times 10^5 \text{ gCOD m}^{-3} \text{ d}^{-1}$ at $E_{\text{anode}} = -0.2 \text{ V}$, but it was very small at $6.9 \times 10^4 \text{ gCOD m}^{-3} \text{ d}^{-1}$ at $E_{\text{anode}} = +0.2 \text{ V}$.

Discussion

Shifts of bacterial community in the biofilm anode

The bacterial community in the biofilm anode was shifted from *Geobacter*-dominant (96 %) to a relatively diverse structure (*Geobacter* 44 %, *Treponema* 40 %, *Aminiphilus* 3.3 %, and *Pseudomonadaceae* 5.3 %) after E_{anode} was changed from -0.2 to $+0.2 \text{ V}$. This substantial change of biofilm community occurred only after 2 weeks. Abundance of *Geobacter* spp. at

$E_{\text{anode}} = -0.2$ V is consistent to the literature reporting enrichment of *Geobacter* spp. from the mixed culture at negative E_{anode} (-0.05 to -0.2 V).^{18, 19, 21} There are no studies clearly proving EET for *Treponema* spp., one of the dominant bacteria at $E_{\text{anode}} = +0.2$ V, but they have been frequently identified in biofilm anodes.^{41–46} Owing to limited understanding of *Treponema*'s EET, previous works interpreted the presence of *Treponema* genus in biofilm anodes as non-EET function, such as homoacetogenesis.^{42, 44–46} We operated the dual-chamber MxC with acetate medium, so hydrogen₂ would be unavailable for *Treponema* genus in the biofilm. Hydrogen might be diffused from the cathode to the anode. However, this small amount of hydrogen diffused to the anode might have commonly occurred for the two E_{anode} conditions, which cannot account for the substantial increase of *Treponema*'s population only for $E_{\text{anode}} = +0.2$ V. Hence, our result implies that *Treponema* spp. would be one of the main EET players at positive E_{anode} , although more study is essential to prove this hypothesis.

Intracellular electron-transfer kinetics

The estimated K_s^{app} (156 gCOD m^{-3}) for *Geobacter*-enriched biofilm at $E_{\text{anode}} = -0.2$ V falls within K_s values from 119 to 176 gCOD m^{-3} for biofilm anodes dominated with *Geobacter* genus.^{11, 13, 47} The estimated $q_{max}^{app} X_f$ of $6.4 \times 10^5 \text{ gCOD m}^{-3} \text{ d}^{-1}$ was close to $q_{max}^{app} X_f$ values from 8×10^5 to $1 \times 10^6 \text{ gCOD m}^{-3} \text{ d}^{-1}$ in *Geobacter*-enriched biofilm anodes.^{11, 13} X_f is computed at $29460 \text{ gVSS m}^{-3}$ (VSS: volatile suspended solids) for the biofilm anode, given that q_{max}^{app} is $22 \text{ gCOD g}^{-1} \text{ VSS d}^{-1}$.¹¹ This relatively low X_f , which is approximately 60 % of $X_f = 50000 \text{ gVSS m}^{-3}$ in the literature,¹¹ would partially account for the maximum current density of $2.05 \pm 0.05 \text{ A m}^{-2}$ lower than approximately 10 A m^{-2} in the literature.^{11, 13} In addition, this small X_f of *Geobacter* spp. estimated for the biofilm anode suggests less biocompatibility of gold electrodes for formation of *Geobacter* biofilm.^{48, 49} Assuming q_{max}^{app} of $22 \text{ gCOD g}^{-1} \text{ VSS d}^{-1}$ ^[11, 15] and 50 % of active biomass cell (i.e., VSS) is proteins,^{50, 51} the electrontransfer rate was estimated at $2.6 \times 10^{16} \text{ electrons mg}^{-1} \text{ proteins s}^{-1}$, which is comparable to literature values ranging from 1×10^{16} to $5 \times 10^{16} \text{ electrons mg}^{-1} \text{ proteins s}^{-1}$ for pure-culture *G. sulfurreducens* biofilms.³⁵ This calculation supports high metabolic activity of *Geobacter* genus in the biofilm anode at $E_{\text{anode}} = -0.2$ V.

At $E_{\text{anode}} = +0.2$ V, the best-fit K_s^{app} and $q_{max}^{app} X_f$ were small at 67 gCOD m^{-3} and $6.9 \times 10^4 \text{ gCOD m}^{-3} \text{ d}^{-1}$, respectively. The K_s^{app} term controls how early current density is saturated to substrate concentration. In comparison, the $q_{max}^{app} X_f$ term regulates the maximum current density. Hence, these small K_s^{app} and $q_{max}^{app} X_f$ terms at $E_{\text{anode}} = +0.2$ V indicate kinetic advantages of ARB only at current density smaller than the saturated current density of 0.35 A m^{-2} . Owing to diverse bacterial community in the biofilm anode at $E_{\text{anode}} = +0.2$ V, we could not conclude which parameter (q_{max}^{app} or X_f) primarily limits current density. However, our result clearly indicates that $q_{max}^{app} X_f$ is a main rate-limiting factor for current density at $E_{\text{anode}} = +0.2$ V. Substantial increase of *Treponema*'s population (40 %) in the biofilm anode at $E_{\text{anode}} = +0.2$ V suggests that *Treponema* spp. would be related to low K_s^{app} and $q_{max}^{app} X_f$ for the biofilm anode at this positive E_{anode} .

Extracellular electron-transfer kinetics

High K_{bio} of 0.96–1.24 mS cm⁻¹ was measured for the biofilm anode to E_{anode} change; the K_{bio} was constantly high at 0.96 mS cm⁻¹, along with negative E_{KA} (−0.197 V) at $E_{\text{anode}} = +0.2$ V. The K_{bio} is higher than 0.25 mS cm⁻¹ in a mixed-culture biofilm anode enriched with *Geobacteraceae* family.³⁸ This high K_{bio} supports Ohmic conduction for EET in the biofilm anode.^{8, 10, 35} For pure culture of *Geobacter sulfurreducens*, K_{bio} showed a sigmoidal pattern to E_{anode} variation (−0.6 to 0.4 V),^{8, 35} suggesting Ohmic conduction for EET to E_{anode} dynamics in the pure-culture biofilm anodes. Our study first proved that high K_{bio} is conserved to E_{anode} change in the mixed-culture biofilm anode under steady state, although biofilm community structure and IET kinetic parameters were significantly changed at $E_{\text{anode}} = +0.2$ V (see Table 1). Other literature reported that E_{anode} changes affected biofilm conductivity for *Geobacter sulfurreducens* pure culture in non-ohmic manner, suggesting redox conduction (electron hopping or tunneling).^{62, 63} Although our study likely contradicts to redox conduction, the highly conserved K_{bio} during E_{anode} changes (from −0.2 to +0.2 V) cannot completely rule out EET through redox conduction; redox conduction EET follows the Ohm's law when potential gradient throughout biofilm anodes is small, like our study (<0.5 V).⁶³ Biofilm resistance, reciprocal of its conductance, measured with two-probe method includes contact resistance, which suggests that an intrinsic K_{bio} for the biofilm anode might be higher than 0.96–1.24 mS cm⁻¹. For instance, Malvankar et al.⁸ reported that K_{bio} quantified with four-probe method was higher than that with two-probe one. No studies, however, have quantified contact resistance for biofilm anodes, probably owing to complex biofilm-electrode interface. Instead, literature has used charge-transfer resistance for the biofilm-electrode interface.^{38, 64} ARB having higher K_{bio} consistently showed lower charge-transfer resistance.^{38, 64} Comparing two-probe with four-probe measurements would be important for quantification of contact resistance, understanding of anode respiration, and improvement of K_{bio} accuracy, which is our on-going research. It is speculated that there might be a threshold population of main EET players (*Geobacter* spp. in this study) in mixed culture biofilms to keep high K_{bio} . We observed relatively constant K_{bio} with respect to E_{anode} increases when the population of *Geobacter* spp. was over 44 % in the mixed-culture biofilm anode. More studies are essential to understand the threshold ARB population in mixed-culture biofilm anodes.

The experimental CVs at the two E_{anode} conditions matched CVs simulated with the Nernst–Monod equation [Eq. (1)], as shown in Figure 3. The Nernst–Monod equation assumes Ohm's law for EET.¹⁰ Hence, the small deviations between experimental and simulated CVs support Ohmic conduction for EET in the biofilm, which is consistent with the high K_{bio} that was maintained at the two different E_{anode} values. EET conduction mechanisms are still on debate between metallic-like and redox conduction for the *Geobacter sulfurreducens* biofilm anode.^{8, 9, 33–37, 60–63} Malvankar et al.⁸ proposed the metallic-like conduction of a *Geobacter sulfurreducens* biofilm anode by showing the conductivity increase to temperature decrease. This metal-like conduction attributed from π – π stacking of aromatic amino acids in *Geobacter*'s pili or nanowire.^{8, 65} In contrast, recent publications reported that the conductivity of *Geobacter sulfurreducens* biofilms increased with increasing temperature and water content,^{60, 61} supporting redox conduction among extracellular cofactors (e.g., cytochromes) for EET. It seems that *Geobacter sulfurreducens* biofilms would have multiple

conduction mechanisms for EET to electrodes, which implies that EET conduction mechanisms would be more complicated in *Geobacter*-enriched mixed culture biofilms. The two conduction models have reported K_{bio} increase with increasing anode potential, but in different manners.^{8, 61} K_{bio} increased to the maximum at E_{anode} equal to the formal potential of an EET cofactor for redox conduction.⁶¹ For metallic-like conduction, K_{bio} follows a sigmoidal pattern, such as turnover CVs (no substrate-limiting conditions).⁸ However, the two conduction models can commonly support “Ohmic conduction for EET” in biofilm anodes having small potential gradient. For our study, the potential gradient throughout the biofilm anode was relatively narrow at 0.1–0.5 V (acetate potential $\approx$ -0.3 V). For this reason, we cannot conclude which conduction mechanism primarily accounts for EET in the biofilm. However, our study proves that mixed-culture biofilm anodes can maintain Ohmic nature of EET despite E_{anode} changes for efficient anode respiration.

Conclusions

An increase in the anode potential (E_{anode}) significantly reduced the steady-state current density from 2.05 to 0.35 A m⁻² in the microbial electrochemical cell (MxC), along with substantial changes of the bacterial community in the biofilm anode. At $E_{\text{anode}} = +0.2$ V, the intracellular electron transfer (IET) kinetic parameter of apparent maximum specific substrate utilization rate ($q_{\text{max}}^{\text{app}}$) multiplied with the active biofilm density (X_f) for biofilm anodes decreased to approximately ten-fold less than at $E_{\text{anode}} = -0.2$ V. In comparison, extracellular electron transfer (EET) kinetics was relatively conserved in the biofilm anode, showing small change of half-saturation potential (E_{KA}) and biofilm conductivity (K_{bio}) values for E_{anode} increase. Our study suggests that fast EET kinetics would be well conserved in a highly conductive biofilm anode under E_{anode} dynamic conditions, although E_{anode} increases can slow down IET kinetics, mainly owing to changes of the main EET players in biofilm anodes.

Experimental Section

Microbial electrochemical cell configuration and operation

Two identical dual-chamber MxCs were constructed with plexiglass (Figure 4 a). Two gold anode electrodes (9.5 mm×15 mm×10 μ m) on a glass base with a non-conductive gap of 50 μ m was designed and fabricated specifically to measure the K_{bio} (Figure 4 b and 4 c); the total geometric surface area of the two anodes was 2.85 cm². A porous graphite plate (Isomolded Graphite Plate 203101, Fuel Cell Earth, USA) was used as the cathode and anion exchange membrane (AMI-7001, Membranes International Inc., USA) was placed between the anode and the cathode chambers as separator. The working volumes of both chambers were 15 mL. A reference electrode (Ag/AgCl reference electrode, MF-2052, Bioanalytical System Inc., USA) was placed within less than 1 cm from the anodes to control E_{anode} during experiments. We reported all anode potentials versus SHE.

The MxC was inoculated with 10 mL of anolyte from a mother MxC that had been operated for over 1.5 year using acetate medium,⁵² and was fed with the acetate medium (2628 $\pm$ 76 mg_{COD} L⁻¹) supplemented with 100 mm phosphate buffer (pH 7.25–7.4). The cathode

chamber was filled with tap water, producing hydrogen gas. After inoculation, the anode chamber was sparged with ultra-pure nitrogen (99.999 %) for 5 min, and then the E_{anode} was set at -0.2 V using a potentiostat (BioLogic, VSP, Gamble Technologies, Canada). Current was recorded at every 120 s using EC-Lab for windows v10.32 software on a personal computer connected to the potentiostat (BioLogic, VSP, Gamble Technologies, Canada). After operation in batch mode for 12 d, the acetate medium was continuously fed to the MxC at a flow rate of 5.8 mL h^{-1} using a cartridge-type peristaltic pump (Master Flex® L/S digital drive, Model 7523–80, Cole-Parmer, Canada) to maintain hydraulic residence time (HRT) of 2.6 h in the anode chamber (substrate non-limiting conditions). This short HRT can effectively wash out soluble mediators produced by ARB,^{13, 31} which can rule out or minimize the contribution of the mediators to current generation. After steady-state current density, we changed E_{anode} from -0.2 to $+0.2$ V under equivalent operating conditions. The MxC was operated in a temperature-controlled room at 26 ± 2 °C.

Biofilm conductivity measurement

We quantified biofilm conductivity for each E_{anode} , according to Equation 2 and measured biofilm conductance using the two-probe measurement method.⁸ For measurements of biofilm conductance, the anodes and the cathode were disconnected temporarily (open circuit mode). Then, a linear sweeping voltage of 0–50 mV in a step of 25 mV was applied across two gold electrodes using a source meter (Keithley 2400, Keithley Instruments, Inc., USA), and current for each voltage was recorded. The voltage ramp was applied at least in 4–6 cycles until a steady-state current–voltage (I – V) response was obtained. The duration between two cycles was approximately 1 min (Figure S2). Observed biofilm conductance ($G_{\text{bio}}^{\text{obs}}$) was calculated from steady-state I – V characteristics. Estimated $G_{\text{bio}}^{\text{obs}}$ values were comparable for every cycle, and we did not observe any transient high conductance for early cycles (Figure S2). We measured ionic conductance (G_{control}) with an identical, abiotic MxC (control) to account for ionic current across the non-conductive gap using the acetate medium. Intrinsic biofilm conductance ($G_{\text{bio}} = G_{\text{bio}}^{\text{obs}} - G_{\text{control}}$) was quantified to report K_{bio} using conformal mapping [Schwarz–Christoffel transformation; Eq. (2)]:⁵³

$$K_{\text{bio}} = \frac{G_{\text{bio}} \frac{\pi}{L}}{\ln\left(\frac{8L_f}{\pi a}\right)} \quad (2)$$

where L is the length of the electrodes (1.5 cm) and a is half of the non-conductive gap between two electrodes (50 μm).

Biofilm thickness measurement

L_f was measured using the method proposed by Bonanni et al. using the resistance change between the surface of biofilms and electrodes.⁵⁴ We used a stainless steel needle with a tip diameter of 100 μm as the microelectrode for biofilm thickness measurement. The microelectrode connected with a motorized micromanipulator (MM33, Unisense A/S, Denmark) was positioned close to the surface of the biofilm anode and then we stepwise moved the microelectrode toward gold anodes (see Figure 5). We moved the microelectrode

at a step size of 5 μm using Unisense SensorTrace Suite software (Unisense A/S, Denmark) on a personal computer connected with the motorized micromanipulator. For measuring L_f , we disclosed the anode chamber and decanted anolyte with a syringe, which means that the biofilm anodes were open to the air for the access of the microelectrode. When the microelectrode was open to air away from the biofilms, resistance was close to infinite value (open circuit). High resistance in the range of $\text{M}\Omega$ was observed when the microelectrode touched the outmost layer of the biofilms, mainly owing to the water content in the biofilms. Resistance substantially decreased to $<1\ \Omega$ when the microelectrode touched the anode surface. We quantified L_f by monitoring the change of resistances in the biofilms using a multimeter (Fluke 179/TPAK, Fluke Electronics Canada LP, Mississauga, ON, Canada) connected to the microelectrode, as showed in Figure 5. During measurements, we added a few drops of the acetate medium on the surface of the biofilm to avoid dehydration. We completed the measurements in 20 min to mitigate adverse effects of oxygen on ARB. After quantifying L_f , we quickly assembled the MxC, filled anolyte, and operated the MxC under potentiostat mode. Current density was immediately recovered to the steady state. We measured biofilm thickness in quadruplicate for the steady-state current density for two E_{anode} conditions.

Estimation of the kinetic parameters

Under substrate non-limiting conditions ($2628 \pm 76\ \text{mg}_{\text{COD}}\ \text{L}^{-1}$ and $\text{HRT}=1.3\ \text{h}$), we estimated E_{KA} of the biofilm anodes at $E_{\text{anode}}=-0.2$ and $+0.2\ \text{V}$ with low-scanning CV at a scan rate of $1\ \text{mV}\ \text{s}^{-1}$.^{11, 13} E_{anode} was ramped between -0.4 and $+0.4\ \text{V}$ at a scan rate of $1\ \text{mV}\ \text{s}^{-1}$ using the potentiostat. The current and anode potential were recorded every 5 s using EC-Lab for windows v10.32 software on a personal computer connected to the potentiostat. To ensure the reproducibility of the CV data, each test was conducted in triplicate, and the average data is reported.

We estimated K_s^{app} and $q_{\text{max}}^{\text{app}} X_f$ for the biofilm anodes as the acetate concentration was changed for the MxC ($11\text{--}2750\ \text{mg}_{\text{COD}}\ \text{L}^{-1}$). According to the Nernst–Monod equation [Eq. (1)],^{10–13} we estimated the best-fit K_s^{app} ($\text{g}_{\text{COD}}\ \text{m}^{-3}$) and $q_{\text{max}}^{\text{app}} X_f$ ($\text{g}_{\text{COD}}\ \text{m}^{-3}\ \text{d}^{-1}$) with the relative least squares method^{11, 12} using MS 2016 excel solver.

Microbial community analysis

Microbial community structures were analyzed by targeting 16S rRNA. We collected biofilm samples with a sterilized spatula when the current density reached steady state for each E_{anode} . At $E_{\text{anode}}=-0.2\ \text{V}$, we collected samples from three different zones of the biofilm anode and the total RNA was extracted from the samples to better represent community structure. After conducting all experiments at $E_{\text{anode}}=+0.2\ \text{V}$, we collected the whole biofilms for RNA extraction. The color of biofilm was pink for both E_{anode} conditions, and we did not observe any significant difference in color. Total RNA were extracted from anode biofilm samples as previously described with some minor modifications.⁵⁵ Briefly, the AllPrep DNA/RNA Mini Kit (Qiagen GmbH, Hilden, Germany) was used to extract total nucleic acid. RNA was further purified using Ambion TURBO DNA-free DNase kit (Life Technologies, Grand Island, NY). The concentration and

purity of RNA were determined using Qubit RNA assay kits and the Qubit 2.0 Fluorometer (Life Technologies). We used barcoded 16S rRNA gene targeting primers (i.e., 515F and 806R)⁵⁶ and sequenced the targeted product (i.e., 291 bp) in both directions using an Illumina MiSeq PE250 approach. Sequence reads (16S rRNA-based) were processed and analyzed using Mothur software.⁵⁷ Sequence reads that did not fit the following criteria were discarded from further analyses: did not form contigs, deviated considerably from the expected PCR size product, identified as chimeras, had ambiguous bases, and had homopolymers greater than 7 bases long. Sequence reads were grouped at a 97 % similarity and the consensus sequences were then identified using Mothur and the Silva⁵⁸ database as a reference. Excel was used to determine the overall relative abundance of representative sequences at different taxonomic levels (e.g., class, order, family, genus). Rare members (less than 10 sequences) were excluded for the calculation of the relative abundance. Sequences were analyzed using Blast (<http://www.ncbi.nlm.nih.gov/BLAST>) and RDP classifier⁵⁹ further confirms their phylogenetic affiliation and to classify sequences at a low taxonomic level (genus and species) whenever possible.

Acetate and pH measurements

Acetate concentration was analyzed using a gas chromatogram (Model: Hewlett Packard HP 5890 Series II) equipped with flame ionization detector.⁵² All samples were analyzed in triplicate. The pH values were measured with a pH benchtop meter (PHB-600R, OMEGA, Canada) connected with a microprobe pH electrode (RK-55500-40, Accumet[®] MicroProbe[™] combination electrode, Cole-Parmer, Canada).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Current density over time during operation of the MxC at two different E_{anode} .

Figure 2.
Microbial community structures at different E_{anode} (vs. SHE).

Figure 3. Experimental (blue solid line; 1 mV s^{-1}) and simulated CV data (red dotted line). The simulated data was estimated using the Nernst–Monod equation.

Figure 4. Representative images of (a) a dual chamber MxS, (b) two gold anodes on a glass base with a non-conductive gap of 50 μm, and (c) a microscopic image of a non-conductive gap between two gold electrodes.

Figure 5.
Measurement setup for determining L_f .

Table 1.

Biological (IET) and electrochemical (EET) kinetic parameters.

E_{anode}	L_f	K_s^{app}	$q_{max}^{app} X_f$	K_{bio}	E_{KA}
[V]	[μm]	[$\text{g}_{\text{COD}} \text{m}^{-3}$]	[$\text{g}_{\text{COD}} \text{m}^{-3} \text{d}^{-1}$]	[mS cm^{-1}]	[V]
-0.2	34 $\pm$ 5	156	648 152	1.24 $\pm$ 0.24	-0.230 $\pm$ 0.003
+0.2	39 $\pm$ 9	67	69 591	0.96 $\pm$ 0.21	-0.197 $\pm$ 0.008