Microbial fuel cell power overshoot studied with microfluidics:
from quantification to elimination

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Abstract

Power overshoot can hinder determination of maximum power densities in microbial fuel cells (MFCs). In this work, a microfluidic approach was used to study overshoot in an MFC containing a pure culture of electroactive biofilms (EAB) containing Geobacter sulfurreducens. After 1-month operation under constant flow of an ideal nutrient medium, the MFC health began to degrade, marked by voltage loss and the appearance of anomalies in the power density curves. One such anomaly was a chronic power overshoot, accompanying a loss of both measured power and current density on the high-current side of the power density curve. The degree of power overshoot was quantified while certain flow-based interventions were applied, notably the shear erosion of the EAB outer layer. Next, two approaches to acclimation were demonstrated to treat the remaining overshoot. The standard approach, which acclimates the MFC to high currents before a standard polarization test, eliminated the remaining overshoot and returned maximum power densities to initial levels, but maximum current density remained lower than the initial
level. A microfluidic-assisted “long-hold polarization test” enabled efficient in situ acclimation of each external resistor during the measurement. Despite the health-compromised MFC, this method provided long-term stability during the polarization test, resulting in power and current density measurements that exceeded those made on the healthy MFC using the standard polarization test. We conclude that slower electron transfer kinetics in unhealthy MFCs can provoke overshoot by prolonging the time to reach steady state during the polarization test, but a properly designed measurement overcomes this problem.

1. Introduction

Microbial fuel cells (MFCs) are a high-priority sustainable-energy technology due to their ability to deliver usable current through an external circuit while simultaneously biodegrading organic molecules in waste streams or natural environments.[1-3] For maximum power output, the external and internal resistances should be matched.[2] Alternatively, sacrificing power for operation at high current under a low external resistance could be a route to achieving high rates of substrate degradation. Regardless of the application, reliable assessment of performance during long-term operation is essential. To this end, a polarization test is usually a key measurement, providing polarization curves (voltage versus current density) and power density curves (power density versus current density).

Power overshoot is a common anomaly in MFCs that has been linked to measurements that are obtained from electroactive biofilms (EABs) that have not stabilized to new conditions during polarization tests.[4] This overshoot (referred to as “type D”) is characterized by tandem reductions in power and current when moving from low to high current densities in the power...
density curves and is usually, but not exclusively, observed on the high current density side after the maximum power peak.[5] The presence of overshoot often correlates with underestimation of power and current outputs at some positions on the power density curve. A different type of overshoot (referred to as “type M”) occurs when polarization tests are conducted too rapidly and results in artificially high power outputs.[6, 7] No matter the type, overshoot is understood to inhibit accurate evaluations of maximum power. Overshoot can be reversibly triggered in well-performing MFCs by exposure to low nutrient concentrations, low ionic strengths, weak buffers or low pH values,[7, 8] but in some underperforming MFCs, chronic overshoot can persist even during exposure to ideal nutrient medium conditions. Little is known about the origins of this behaviour, and authors often refer to such MFCs as “unhealthy”. The role of convection in overcoming diffusion limitations in nutrient delivery is usually not considered an important factor because chronic overshoot can persist under stirring.[8]

Other than some interesting systems-level control routines, the most generalized experimental strategy for dealing with overshoot is to acclimate the MFC to high current prior to a standard polarization test.[9] This approach (termed pre-acclimation) provides time for the EAB to adapt, but it is not clear by which mechanism(s) the measured performance is improved or why the effect is only temporary. In considering this question, a focus should be placed on the measurement methodology. Whether carried out by switching external resistors or linear sweep voltammetry, polarization tests are not always straightforward for MFCs because the time required to reach stable behaviour may be too long relative to the experiment, especially under the influence of certain EAB health states, which can result in some anomalies in power density curves.[10] Because the measurement process itself continuously perturbs the EAB away from
steady state, the result may be a slow approach to a long-term stability based on biological adaptation (gene transcription, growth, etc.). As such, measurements collected with standard polarization tests, which range from minutes to hours per external resistor, may not be representative of long-term operation. Thus we arrive at the problem of untangling the inherent performance of the MFC from the influence of the polarization measurement.

Deeper studies are required to address remaining fundamental questions about the factors contributing to power overshoot in MFCs and ensuring that the measurement does not influence the results. Of specific interest is the role of an improper measurement in producing overshooting power density curves and, relatedly, the connection among acclimation, time to reach stability, and the measured performance. In designing such studies, pure-culture G. sulfurreducens could be an ideal EAB because it can simplify interpretation (compared to mixed-species EABs) by eliminating fluctuations in interspecies populations[11] and by limiting electron transfer to direct mechanisms only.[12, 13] Precise control over operational conditions is also required to probe the overshoot effect and potentially cure it. Microfluidic microbial bioelectrochemical systems have been used to study the effect of flow rate, chemical composition of the nutrient solution, and other experimental conditions on measurable performance indicators, such as EAB electrical outputs power production, kinetic parameters, and EAB pH.[14-17] When applied to MFCs, certain efficiencies can be realized in a microfluidic format, such as reduced start-up time, ability to eliminate the need for ion exchange membranes, optimization of biofilm formation and improved outputs and assay studies via high parallelization.[18-22] The recent availability of microfluidic MFCs that are reliably operational over a timescale of months[23] provides the opportunity to finally study overshoot in mature MFCs vis-a-vis solution concentrations,
convection and other related hydrodynamic effects under highly controlled conditions. In addition to eliminating the concentration cycling that occurs in batch-fed MFCs, continuous-flow microfluidic electrochemical cells can allow accurate prediction and manipulation of concentration gradients,[24] thus presenting an opportunity to validate whether overshoot is indeed correlated to nutrient availability. A microfluidic approach can also be used to manipulate the EAB via controlled application of shear stresses over a large range of values.

In this work, a microfluidic MFC with a pure-culture *G. sulfurreducens* was observed while it transitioned from a healthy to an overshooting state. We conducted a quantitative analysis of the degree of overshoot following the application of different flow rates, including those high enough to cause shear erosion of the outer biofilm layers. EAB erosion cured some, but not all, of the anomalies in the power density curve. Pre-acclimation to low external resistances before standard polarization tests resulted in maximum power density measurements that matched those of the healthy MFC (before displaying overshoot) but the maximum current density was still reduced. Finally, a new microfluidic-assisted long-hold polarization test for *in situ* acclimation at all external resistances improved the measured maximum power and maximum current densities, even beyond those made on the healthy EAB using a standard polarization test measurement. This approach shows promise for accurate studies of the influence of the polarization test parameters on the measured results and points to the fundamental importance in making measurements on EABs that have attained long-term stability.

2. Materials and methods

2.1 Fluidic simulations and calculations
Calculations of shear stress were obtained from a closed-form calculation, assuming an empty channel:[25]

\[ \tau = \eta \left[ \frac{Q\lambda}{2AD_h} \right] \]  
(Eqn. 1)

where the viscosity of water is \( \eta = 8.90 \times 10^{-4} \) Pa s, \( Q \) is the volumetric flow rate (converted to units of \( m^3 \) s\(^{-1} \)), \( A \) is the cross-sectional area (\( m^2 \)), and \( D_h \) is the hydraulic diameter (\( m \)) of the channel, which is given as:

\[ D_h = \frac{4A}{P} \]  
(Eqn. 2)

where \( P \) is the cross-sectional perimeter length (\( m \)). From Eqn. 1, \( \lambda \) is a unitless shape factor, which has the following form for a rectangular duct with height (\( h \)) and width (\( w \)):[25]

\[ \lambda = \frac{24}{[(1-0.351h/w)(1+h/w)]^2} \]  
(Eqn. 3)

Details for computational simulations of the anolyte and catholyte co-flow properties and concentrations are presented in the Supplementary Materials.

2.2 Electrochemical measurements

Voltage measurements across external resistors (\( R_{\text{ext}} \)) were collected using a potentiostat (VersaSTAT 4, Princeton Applied Research, USA) while continuously flowing a side-by-side laminar co-flow anolyte and catholyte across each respective electrode. Standard polarization tests in this work monitored the electrical discharge versus time after cycling from OCV to \( R_{\text{ext}} \) (with \( R_{\text{ext}} \) ranging from 300 and 3.5 k\( \Omega \)) with a cycling period of approximately 1 to 2 hours. Switching between OCV and \( R_{\text{ext}} \) at this time scale strongly
limits any acclimation to the different applied $R_{\text{ext}}$ values during the polarization tests (i.e., avoiding in situ acclimation). Therefore, reaching stable voltages during standard polarization tests marks a short-term stability at each $R_{\text{ext}}$ value. To achieve a long-term stability at each $R_{\text{ext}}$, a so-called “long-hold” polarization test was conducted in which $R_{\text{ext}}$ was applied for up to 15 hours after switching from OCV. This allowed acclimation to occur at each position on the power density curve (i.e., achieving in situ acclimation).

Independent of the polarization test type used (standard or long-hold), we converted the voltage drop ($V$) across $R_{\text{ext}}$ to current $I$ (A) using Ohm’s law ($V=IR_{\text{ext}}$) and normalized by the solution-exposed anode area $A$ (30 mm$^2$) to obtain the corresponding current densities $\bar{I}$ (A m$^{-2}$) and power densities $\bar{P}$ (W m$^{-2}$):

$$\bar{I}=\frac{I}{A}=\frac{V}{A\cdot R_{\text{ext}}} \quad \text{(Eqn. 4)}$$

$$\bar{P}=\bar{I}\cdot V=\frac{P\cdot R_{\text{ext}}}{A} \quad \text{(Eqn. 5)}$$

The $V$ versus $\bar{I}$ curves (polarization curves) and $\bar{P}$ versus $\bar{I}$ curves (power density curves) were created using Eqns. 4 and 5, respectively.

The accepted definition of overshoot is a simultaneous reduction in both $\bar{P}$ and $\bar{I}$ when moving from low to high to low external resistance on a power density curve, i.e., when $\Delta \bar{P} < 0$ and $\Delta \bar{I} < 0$ occur between any consecutive data pairs in a power density curve. From Eqn. 5, it is sufficient to note that $\Delta \bar{I} < 0$ meets this definition because $\Delta \bar{P}$ is then necessarily negative as well. Therefore, the simplest way to quantify overshoot is to measure the absolute overshoot with the parameter $\Delta \bar{I}_{\text{OS}}$ (A), which is a positive value in the presence of overshoot:
\[ \Delta I_{OS} = I_{max} - I_f \]  
(Eqn. 6)

where \( I_{max} \) is the maximum current density measured during a polarization test, and \( I_f \) is the final current density obtained at the lowest applied external resistance. We also define a unitless measurement parameter called the overshoot factor \( \theta_{OS} \), which ranges from 0 (no overshoot) to 1 (complete loss of current):

\[ \theta_{OS} = \frac{\Delta I_{OS}}{I_{max}} = 1 - \frac{I_f}{I_{max}} \]  
(Eqn. 7)

We express the overshoot factor as a percentage \([\Theta_{OS} \%) = \theta_{OS} \times 100\%\]. A power density curve with no overshoot has \( I_f = I_{max} \) and both \( \Delta I_{OS} = 0 \) and \( \Theta_{OS} = 0 \).

2.3 Microfluidic MFC design and operation

Moulds used to create the microfluidic MFC were fabricated via photolithography using laminated photoresists and graphite electrodes (Isomolded Graphite Plate, GraphiteStore.com Inc., USA). The membraneless MFC device contains electrodes that were positioned flush with the top PDMS wall in the popular side-by-side arrangement (Supplementary Materials, Figure S1). Other details of the microfabrication and device design can be found in the Supplementary Materials (Section S1) and in the literature.[23]

The inoculum consisted of a subculture of \textit{G. sulfurreducens} (wild type, strain PCA, ATCC® 51573) in a nutrient medium with 40 mM sodium fumarate as a soluble electron acceptor. The nutrient medium consisted of a 10 mM NaC\(_2\)H\(_3\)O\(_2\) (sodium acetate) solution, a trace mineral supplement (10 mL/L) (ATCC® MD-TMS™), and a vitamin supplement (10 mL/L) (ATCC® MD-TMS™) in a sodium phosphate buffer (3.8 mM NaH\(_2\)PO\(_4\), 30 mM NaHCO\(_3\), 1.3 mM KCl, 28 mM NH\(_4\)Cl). No oxygen scavengers were used in this work. The MFC anode was inoculated with the
subculture by syringe pump (PhD 2000, Harvard Apparatus, MA) at a flow rate of $Q_a=0.5 \text{ mL h}^{-1}$ for two hours. To promote growth of the EAB after the inoculation, the same solution was used except that it was sterile and contained no sodium fumarate. A pH 7.0 sodium phosphate buffer containing 30 mM $K_3\text{Fe(CN)}_6$ (ferricyanide) was used as the catholyte solution to ensure that the cathode kinetics did not contribute to overshoot. At every stage, the catholyte formed a co-flow with the anolyte ($Q_c=Q_a=0.5 \text{ mL h}^{-1}$). The laboratory temperature was $23 \pm 0.5^\circ\text{C}$. Other details related to bacterial preparation are identical to those of previous studies.[23] Following the experiment, scanning electron microscopy (SEM) was applied on the EAB. The pumping system was used to deliver a fixant before sample extraction. See the Supplementary Materials for more details on sample preparation and SEM measurements.

3. Results

3.1 Experimental timeline (transition to compromised performance and hydrodynamic interventions)

We begin with a survey of the MFC voltage across the external resistor during the transition between the normal and health-compromised states, followed by the results of interventions that were made. Almost immediately after inoculation, the voltage began to increase exponentially due to growth of the EAB (Figure 1a). After 3 days, the voltage reached a constant level at 610 to 650 mV. At this time, the external resistor was reduced from $R_{\text{ext}}=50 \text{ k}\Omega$ to 30 k$\Omega$, and the MFC remained under flow conditions for 5 days while monitoring to ensure that a steady state was reached. Initially, the voltage was recorded every 4-8 days (Figure 1b), and the power density and polarization curves were acquired every week. The maximum power density
remained relatively constant at $\bar{P}_{\text{max}}=370 \text{ mW m}^{-2}$, and no overshoot was observed (Supplementary Materials, Section S2).

At 42 days, the voltage across the 30 kΩ external resistor had decreased and continued to drop, reaching 50% of its original value at $t=50$ days. Because the laminar flow in the microchannels promotes strong concentration gradients, we used simulations to investigate the possibility that the voltage loss occurred because some portions of the electrode experienced reduced activity due to local nutrient limitations (Supplementary Materials, Section S3). A simulation of the acetate concentration above the anode showed that acetate was the most depleted near the downstream portions of the anode due to continuous consumption during the liquid transit across the upstream portions. Nevertheless, the concentration never fell below 9 mM at any position on the anode. As this is well above the concentration threshold between first- and zero-order kinetics (3-5 mM),[26, 27] we conclude that access to substrate molecules was not a factor in the decreased cell voltage. A similar simulation showed that ferricyanide concentrations remained above 25.5 mM everywhere on the cathode, which is sufficient to avoid bottlenecks in reduction kinetics.[28] It is also doubtful that the observed voltage loss was due to EAB erosion because the shear stress was only (0.5 mPa) at the flow rates until this point, which is well below the values used in previous work.[23, 29] The microfluidic MFC was also designed to prevent $O_2$ contamination and ferricyanide crossover to the anolyte flow stream, but we do not rule out accidental contamination during syringe replacement or from slow $O_2$ leaks through faults in the device gas-protection layer, fluid connects or even through the porous graphite electrodes. Other possibilities include biological contamination and metabolically inactive outer layers, as recently reported. [30]
We exploited the ability of the microfluidic MFC to apply a large range of shear stresses to investigate whether the outer layers comprised of older cells were the cause of the poor MFC performance by testing this hypothesis. At $t=50$ days, we applied a shear stress of 7.5 Pa via three consecutive 3-second flow pulses ($Q_a=Q_c=250$ mL min$^{-1}$) to detach the EAB outer layers. Although no published data are available on the critical shear stress threshold for erosion of *G. sulfurreducens* biofilms, these applied flow rates correspond to shear stresses that were more than three times higher than the highest reported shear stress for EAB erosion in other microfluidic MFCs.[31] After the high-shear flow pulses, a continuous high flow of $Q_a=Q_c=8$ mL min$^{-1}$ (equivalent to 240 mPa) was applied for three minutes, and then the system was allowed to settle under a flow rate of $Q_a=Q_c=0.5$ mL h$^{-1}$ (0.5 mPa) for two days. Upon resumption of measurements across the 25 kΩ external resistor (reduced from 30 kΩ to encourage biofilm regrowth), a non-zero voltage (80 mV) was observed (*Figure 1c*). Based on a previous study, we believe that this signal was related to a remaining biofilm seed layer.[32] In that study, the same graphite electrode material was used for microfluidic electrochemical impedance and confirmed a quantity of residual *Pseudomonas* biofilms following shear erosion of the biofilm outer layers, possibly aided by the electrode surface roughness. The voltage initially recovered to 225 mV approximately one day after the measurements were restarted ($t=53$ days, marked as phase i in *Figure 1c*), followed by a slow recovery over the next 20 days (phase ii), and finally, a steady-state potential of 450 mV was attained (phase iii). It is likely that the initial current increase during phase i was due to the recovery of the biofilm metabolism after re-establishment of the initial co-flow conditions and washout of anolyte and catholyte solutions that may have crossed over to the opposite electrode compartment. The slower recovery in phase ii was likely due to biofilm
regrowth from the residual *G. sulfurreducens* seed layer. When the MFC reached steady state in phase iii (*t*=61 days), the voltage remained lower than the pre-overshoot voltages (at *t*=42 days).

Accounting for the reduced external resistance of *R*_{ext}=30 kΩ at *t*=42 and *R*_{ext}=25 kΩ at *t*=61 days, this voltage indicates that the MFC was still partially health compromised.

![Figure 1](image)

**Figure 1.** (a) Voltage growth profile across external resistor *R*_{ext}=50 kΩ starting immediately after the end of a 5-hour inoculation process; inset shows semi-log plot of (a) with a highlighted section from 2 to 40 hours (star) indicating the exponential growth period. (b) Voltage across *R*_{ext}=30 kΩ from intermittent voltage measurements between 5 and 50 days showing the onset of a reduction in voltage after approximately one month. Colour-coded arrows indicate the time at which the power density curves in Figure 2 were obtained. (c) Voltage recovery starting 2 days after application of high shear force under external resistor *R*_{ext}=25 kΩ showing three recovery phases i, ii, and iii; inset shows zoom of (c) on the first three hours, displaying recovery during phase i. Arrows in (c) mark three time points (*t*=61, 92 and 109 days) when power density and polarization curves were obtained. The time *t*=0 starts after inoculation. Total flow rate (Q_{T}=1 mL h^{-1}) was generated from Q_{a}=Q_{b}=0.5 mL h^{-1}.

### 3.2 Power density curves demonstrating overshoot

Next, we investigated the drop in MFC performance that occurred after one month by evaluating the power density curves (**Figure 2**). Standard polarization tests were conducted after the MFC potential began to drop (at 42 days), and the polarization and power density curves
were generated according to Eqns. 4 and 5. The cycling time between the OCV and different applied $R_{ext}$ values was 1 to 2 hours, which was sufficient for a healthy MFC of the same design to reach a stable value in the short-term.[23] Initially ($t=42$ days), the power density curves showed a typical monotonic approach to maximum power density ($P_{max}=360$ mW m$^{-2}$), and no overshoot was observed at high current densities, similar to measurements observed during the first month.

As the MFC performance became further degraded (after $t=45$ days), two different anomalies were observed in the power density curves. The first was a precipitous reduction in $P$ near the former apex of the power density curve (similar to type M overshoot), and the second was a classic current overshoot at low external loads, $R_{ext}<10$ kΩ (similar to type D overshoot). We consider these two phenomena separately and go beyond the usual definition of overshoot ($\Delta I<0$, $\Delta P<0$ in a power density curve) by quantifying its strength. At $t=45$ days, the measured absolute overshoot was $\Delta I_{OS}=54$ mA m$^{-2}$, which is equivalent to an overshoot factor $\Theta_{OS}=6\%$. At $t=48$ days, the absolute overshoot more than tripled to $\Delta I_{OS}=173$ mA m$^{-2}$, with a corresponding increase in the overshoot factor to $\Theta_{OS}=19\%$. This change corresponds to a more than tripled $\Delta I_{OS}$ and $\Theta_{OS}$ in three days, signaling a rapid decline in the health state of the MFC.

The second anomaly was the local drop in power density at the apex of the power density curve observed at $t=44$, 45 and 48 days. A similar anomaly has been linked to polarization tests that are conducted too quickly, producing unpredictable power density readings.[9] This effect is usually accompanied by a corresponding reduction in current density at the same position, thus meeting the definition of type M overshoot.[7] Changes in $P$ at 25 kΩ are shown in Figure 2b in a $\Delta P$ versus $I$ curve and can be compared to the power density curve in Figure 2a. Insofar as $\Delta P$ and
ΔĪ determine the presence of power overshoot, we plot ΔP versus ΔĪ (Supplementary Materials, Section S4). It is clear that while Ī continuously increases (i.e., ΔĪ > 0, always) even as ΔP becomes negative near the former power density apex (R_{ext}=25 kΩ), the values of Ī at 25 kΩ and ΔĪ between 40 and 25 kΩ are both progressively reduced. From these trends, it appears likely that further degradation of the MFC performance would have resulted in ΔĪ < 0 (classic type M overshoot) for the data pair marked 3 in Figure 2a, similar to the data pairs marked 1 and 2.

Figure 2. Results from MFC polarization test and overshoot analysis during the transition from a healthy state to an overshoot state. (a) Power density curves at t=42 days (black), t=45 days (red), and t=48 days (blue). Based on the previously estimated internal resistance of 25 kΩ, the external resistors used were R_{ext}=300, 150, 70, 40, 25, 15, 10, 8, and 3.5 kΩ. Power densities measured at two critical external resistances (25 and 10 kΩ) are highlighted (black arrows). Absolute overshoot (ΔĪ_{OS}) is exemplified at t=48 days with a horizontal arrow (red). (b) ΔP versus Ī for the same data in (a). Data in (a) and (b) share the same colour coding and x-axis. Regions 1, 2 and 3 indicate data pairs in all three power density plots in (a) that correspond to ΔP in (b).
3.3 The role of polarization test stability in measurements featuring power overshoot

To investigate the relationship between electron transfer and the anomalies observed in the power density curves in Figure 2, we inspected the raw discharge profiles generated during the standard switched resistor polarization tests. At t=42 days (before overshoot), a rapid exponential approach to stable values occurred during discharge from OCV across each of the connected external resistors. This same observation was true for all $R_{ext}$ values except for $R_{ext}=3.5\ k\Omega$, where the system was still discharging before the voltage was recorded (Figure 3a). In contrast, discharge was usually incomplete during the standard polarization tests acquired at t=45 and 48 days (during overshoot), regardless of the $R_{ext}$ (e.g., Figure 3b at t=48 days). In the latter case, except for the highest applied external resistors ($R_{ext}=300, 150,$ and $100\ k\Omega$), the voltage versus time discharge profiles were abnormal and had not stabilized before switching back to OCV in advance of the next applied value of $R_{ext}$. This information is shown in greater detail in the inset to Figures 3a and 3b for the discharge across 25 kΩ. Based on the voltage versus time discharge profiles, the local decrease in the power density curve at $R_{ext}=25\ k\Omega$ and the overshoot at $R_{ext}<10\ k\Omega$ were likely artifacts related to insufficient hold times during standard polarization tests due to worsening electron transfer kinetics at the bioanode. [4, 7, 33] This can be due to related changes in charge transfer components,[34] including changes to the number and types of cytochrome proteins and conductive pili that may be optimal for those conditions,[35] bioanode capacitance, [36] electrical resistance of the bulk biofilm, [37, 38] or charge transfer resistance from the EAB to the electrode.[26] Coincident with the proposed slower electron transfer kinetics at t=48 days was a reduction in OCV by approximately 50 mV compared to that at t=42 days (Figures 3a and 3b). Loss of OCV could be the result of a loss of
cytochrome c charge carriers or a reduced EAB metabolism, thus supporting the hypothesis of an unhealthy EAB slowing electron kinetics.

**Figure 3.** Voltage versus time discharge profiles from polarization tests at t=42 (before overshoot), 48 (during overshoot) and 61 days (three days following EAB erosion) are shown in (a), (b) and (c), respectively. Insets in each figure show a close-up view for a single transition from OCV to R_{ext}=25 kΩ in (a) and (b) and from OCV to R_{ext}=20 kΩ (c). Resistor values used in (a) and (b) were R_{ext}=300, 150, 70, 40, 25, 15, 10, 8, and 3.5 kΩ. Resistor values in (c) were R_{ext}=300, 150, 80, 50, 30, 20, 15, 8, and 3.5 kΩ. Cycle time was between 1 and 2 hours for each OCV/R_{ext} pair.

We also examined the polarization test (voltage versus time) discharge profiles after erosion of the outer EAB layers (t > 61 days). Data for t=61 days (Figure 3c) were representative of measurements at t=92 and 109 days (data not shown). The polarization curves did not show significant differences in slope in the ohmic region during phases ii or iii, indicating that the internal resistance was stable (Supplementary Materials, Section S5). The discharge curves returned to an exponential decay, resulting in a short-term stability being reached more often before switching back to OCV in the 1- to 2-hour cycle time under a standard polarization test. We conclude that removal of the outer EAB layers resulted in improved electron transfer kinetics. Likely related to this was the disappearance of the anomalous power loss at 25 kΩ in the
corresponding power density curve (Figure 4). However, overshoot was still observed at low external loads ($R_{\text{ext}} < 8 \, \text{k}\Omega$). The average absolute overshoot between $t=61$ and $t=109$ days was measured as $\Delta I_{\text{OS}} = 88.7 \pm 7.1 \, \text{mA} \, \text{m}^2$ (with the corresponding average overshoot factor of $\Theta_{\text{OS}} = 13 \pm 2\%$), which is a reduction from the overshoot at $t=48$ days ($\Delta I_{\text{OS}} = 173 \, \text{mA} \, \text{m}^2$, $\Theta_{\text{OS}} = 19\%$). Also, $I_{\text{max}}$ after shear erosion was lower than was observed while overshoot was present. This is likely related to the reduced biomass after erosion, but the internal resistance also appeared to be higher after erosion compared to before onset of overshoot (Supplementary Materials, Section S5). Taken together, shear erosion made some improvements in the MFC performance, but it is clear that the MFC was still in a health-compromised state.

We tested the hypothesis that moderate (sub-erosion) increases to flow and the related reductions in diffusion barriers could reduce or even eliminate $\Delta I_{\text{OS}}$ because overshoot is situated in the concentration-limited regime of the polarization curve. We limited the maximum flow rate to $Q_{\text{T}} = 10 \, \text{mL} \, \text{h}^{-1}$ because beyond this value, the returns on the MFC output are diminishing.[23] In addition to an expected increase in $P_{\text{max}}$ and $I_{\text{max}}$ (Figure 4), the increased flow rate significantly reduced the absolute overshoot to $\Delta I_{\text{OS}} = 40 \, \text{mA} \, \text{m}^{-2}$ (with a corresponding overshoot factor of $\Theta_{\text{OS}} = 5\%$). Inspection of the corresponding polarization curve indicated a small reduction (5 kΩ) in the internal resistance over the value obtained at $Q_{\text{T}} = 1 \, \text{mL} \, \text{h}^{-1}$ (36 kΩ) (Supplementary Materials, Section S5). This was corroborated by calculation of the internal resistance (not shown).[2] Previously, in a two-chamber *G. sulfurreducens* MFC with continuous anolyte replacement, higher internal resistances and lower power were measured at high flow rates.[39] This may reflect amplified stresses, e.g., those due to flow-enhanced transport of low
concentration $O_2$ in solution to the EAB, as other literature examples typically show an increase to power with flow.\cite{29,40}

### 3.4 Pre-acclimation to a low external resistance

In the final part of the study, we tested whether microfluidic MFCs could benefit from acclimation to high current conditions via a low $R_{\text{ext}}$ (3.5 kΩ) either before standard polarization tests (pre-acclimation) or during microfluidic-assisted long-hold polarization tests \textit{(in situ acclimation)}. In the first approach, the MFC was pre-acclimated for 4 days to one of three external resistors ($R_{\text{ext}}=150, 20, \text{and } 3.5 \text{ kΩ}$) followed by a standard polarization test. Each experimental condition was applied three times in random order to prevent bias in the results. Pre-acclimation at the lowest external resistor ($R_{\text{ext}}=3.5 \text{ kΩ}$) was most efficient in improving outputs. Specifically, $\overline{P}_{\text{max}}$ recovered to pre-overshoot levels ($\overline{P}_{\text{max}}=370 \text{ mW m}^{-2}$), and the overshoot was eliminated ($\overline{I}=\overline{I}_{\text{max}}$). However, the $\overline{I}_{\text{max}}$ value of 810 mA m$^{-2}$ after pre-acclimation was still 20% lower than that at $t=42$ days (before the onset of overshoot) and was nearly equivalent to $\overline{I}_{\text{max}}$ at $t=45$ and 48 days (during overshoot). We do not believe that this drop in current is the same as has been reported during MFC start-up \cite{41} because in this case the MFC was already mature. Rather, it is likely the result of the declining EAB health and loss of biomass after shear erosion. \cite{42} For comparison, we combine the pre-acclimated power density and polarization curves along with those data obtained at $t=42$ days in Figure 4. Pre-acclimation to higher resistances had the predictable effect of lowering $\overline{P}_{\text{max}}$ and $\overline{I}_{\text{max}}$.\cite{9} Commensurate increases to internal resistance were observed (Supplementary Materials Section S6).
Figure 4. (a) Power density curves (a) and polarization curves (b) obtained at different times and conditions. In both curves, the performance before onset of overshooting behaviour is reproduced from Figure 2a as a reference (t=42 days, black dotted curve). Average results after shear erosion at total flow rate $Q_T=1\text{ mL h}^{-1}$ are shown (t=61, 92, 109 days, green solid curve); results after increase of total flow rate to $Q_T=10\text{ mL h}^{-1}$ (t=110 d, green dashed curve); and average results after 4 days of pre-acclimation at $R_{ext}=3.5\text{ k} \Omega$ at flow rate $Q_T=1\text{ mL h}^{-1}$ are shown with error bars (t=115 to 120 days, orange). All error bars are the result of 3 separate measurements on different days.

3.5 In situ acclimation via microfluidic-assisted long-hold polarization tests

The beneficial effects of pre-acclimation to a single low external resistor before a standard polarization test might be partially reversed while the EAB readapts to higher loads during the measurement. As well, the 1- to 2-hour relatively rapid cycle time between OCV and $R_{ext}$ could achieve long-term stability related to bacterial adaption to different currents during the polarization test. Another approach is in situ acclimation in which the polarization at each $R_{ext}$ is held for a long duration so that every point in the power density curve is fully and individually acclimated, thus achieving long-term stability. The accurate and constant flow from the microfluidic MFC eliminates nutrient cycling and the related voltage cycling that occurs in batch MFCs. Thus, microfluidic MFCs can provide a unique perspective on acclimation dynamics. We
acquired the discharge curves during long-hold polarization tests with dwell times of up to 15 hours following the switch from OCV to selected \( R_{\text{ext}} \) values, and each measurement was repeated three times at two different flow rates (\( Q_T = 1 \) and 10 mL h\(^{-1}\)). In all cases, the voltage stabilized at a minimum value over a relatively short timescale (termed \( t'_s \)). For \( R_{\text{ext}} < 50 \) k\( \Omega \), the voltage eventually increased to a new (acclimated) voltage in a long time-scale stabilization (termed \( t''_s \)). This time to adjust to high current demand is probably due the same effect involved in the current recovery observed previously in the power density curves near the end of the experiment.\[39\] Both \( t'_s \) and \( t''_s \) increased with decreasing \( R_{\text{ext}} \), but interestingly, applying higher flow rates reduced these times. As an example, Figures 5a-c below show three discharge curves across external resistances of \( R_{\text{ext}} = 3.5, 30, \) and 300 k\( \Omega \). A plot of the measured \( t''_s \) as a function of \( R_{\text{ext}} \) under low- and high flow rates (Figure 5d) shows that the acclimation times are 6 to 11 hours at \( Q_T = 1 \) mL h\(^{-1}\) but only 4 to 7 hours at \( Q_T = 10 \) mL h\(^{-1}\). We estimate a 4.5- to 18-times reduction in experimental time using the flow-assisted long-hold approach compared to multi-cycle acclimation over three fill/deplete cycles per external resistor in a bulk MFC.\[10\] We believe that studies focused on the degree and timing of the voltage recovery can provide insights into the specific nature of the biological process(es) that occur in response to acclimation, e.g., biofilm growth, cytochrome or other protein expression, and shifts in metabolic states, among other factors.

We constructed power density and polarization curves (Figures 5e and 5f) from long-hold polarization tests based on the voltages obtained after “virtual hold times”, \( t'_s \) and \( t''_s \), each repeated for flow rates \( Q_T = 1 \) and 10 mL h\(^{-1}\). In all cases, \( P_{\text{max}} \) was obtained at \( R_{\text{ext}} = 20 \) k\( \Omega \). At \( Q_T = 1 \) mL h\(^{-1}\), the \( P_{\text{max}} \) value after hold time \( t'_s \) was 346 mW m\(^{-2}\) (slightly lower but within the standard
deviation of the average value (370 mW m$^{-2}$) obtained from a standard polarization test following constant acclimation to 3.5 kΩ). This value increased to 383 mW m$^{-2}$ under flow rate Q$_{t}$=10 mL h$^{-1}$. At hold times of t$_{s}$', absolute overshoot persisted, with Δī$_{OS}$=205 (and the corresponding overshoot factor of Θ$_{OS}$=24.5%) at Q$_{t}$=1 mL h$^{-1}$ and 74 mA m$^{-2}$ (Θ$_{OS}$=8.5%) at 10 mL h$^{-1}$. In contrast, the overshoot was fully eliminated for power density curves that were constructed using voltages at t=t$_{s}$ from the polarization test voltage versus time profiles. In addition, $\bar{P}_{\text{max}}$ and $\bar{I}_{\text{max}}$ were improved ($\bar{P}_{\text{max}}$=405 mW m$^{-2}$ and $\bar{I}_{\text{max}}$=1038 mA m$^{-2}$ at Q$_{t}$=1 mL h$^{-1}$), even compared to the pre-overshoot values obtained using the standard (non-acclimated) measurement protocol. Increasing the flow rate to Q$_{t}$=10 mL h$^{-1}$ had a beneficial effect on $\bar{P}_{\text{max}}$ and $\bar{I}_{\text{max}}$ ($\bar{P}_{\text{max}}$=436 mW m$^{-2}$ and $\bar{I}_{\text{max}}$=1215 mA m$^{-2}$). Made possible by the microfluidic-assisted long-hold approach, in situ acclimation especially favours higher measurements of $\bar{I}$ at low R$_{\text{ext}}$ (including high values of $\bar{I}_{\text{max}}$), leading to an unusual shape of the curves in Figure 5. We do not believe that this shape is an artifact of the technique, but rather, it is the true form of a properly acclimated MFC. This view is supported by the similar $\bar{I}$ values obtained using the 4-day pre-acclimation approach at R$_{\text{ext}}$=3.5 kΩ (before the subsequent standard polarization tests) and from similar power density curve shapes in multi-cycle acclimation conducted in bulk MFCs.[10]
Figure 5. MFC voltage after switching from OCV to external resistance of (a) 3.5 kΩ, (b) 30 kΩ, and (c) 300 kΩ. Each figure includes results under total flow rates of $Q_T=1 \text{ mL h}^{-1}$ (solid) and 10 mL h$^{-1}$ (dashed). Arrows in (a), (b) and (c) show the time after reaching the lowest voltage ($t_s'$, red), and (a) and (b) indicate only the acclimation time sufficient to observe full voltage recovery ($t_{s''}$, blue). Vertical black scale bars indicate the vertical range corresponding to the marked voltage. (d) Time required to reach the short- and long-term voltage stability ($t_{s'}$, $t_{s''}$) versus $R_{\text{ext}}$ from 50 kΩ to 3.5 kΩ for total flow rates of $Q_T=1 \text{ mL h}^{-1}$ (solid) and 10 mL h$^{-1}$ (hollow). For eye guidance, solid and dashed lines indicate exponential fits for $Q_T=1 \text{ mL h}^{-1}$ and 10 mL h$^{-1}$, respectively. Reconstructed power density (e) and polarization curves (f) for total flow rates $Q_T=1 \text{ mL h}^{-1}$ (Δ) and 10 mL h$^{-1}$ (●). Reconstructions were created based on polarization test voltages obtained at time $t=t_{s'}$ (red curve) and $t=t_{s''}$ (blue curves). External resistors used were 300, 150, 80, 50, 30, 20, 15, 8, and 3.5 kΩ. Data were obtained between $t=150$ and 170 days. Each data point in (e) and (f) is the average of three points, but error bars (standard deviation in average) are shown only for (e).

We extended the advantage of the long-hold polarization test to construct and investigate power density curves at arbitrary hold times of $t < t_s'$ (i.e., to investigate the effect of polarization tests that were too fast). Power density curves from discharge curves obtained under $Q_T=1 \text{ mL h}^{-1}$ using virtual hold times of 2 hours or less were chosen to mimic standard polarization test conditions used in this work (Supplementary Materials, Section S7). The longest hold time was 2
hours \((t\approx t_s')\) and resulted in a power density curve that was nearly identical to the constructed power density curve in Figure 5e for \(t=t_s'\) \((Q_T=1 \text{ mL h}^{-1})\). This was expected because 2 hours was sufficient to obtain short-term stability. This “stable overshoot” does not represent a measurement error because stability was achieved, but it does represent an error in experimental design because it did not account for the voltage recovery after long-term stability was reached. A 90-minute hold time resulted in a worse overshoot (type D), and a further reduction in the hold time to 60 minutes caused a drop in power density near the apex of the power density curve, similar to that (type M-like overshoot) observed in Figure 2a after \(t=45\) days. Further reductions to virtual hold time resulted in power densities that were unrealistically high. Therefore, we confirm that conducting measurements before stability is reached in a standard polarization test can produce a range of power density curve irregularities. Use of a constant-flow MFC allows for real-time monitoring the voltage discharge during the polarization test to ensure proper long-term stability is achieved at each external resistor.

4. Discussion

To summarize the effects on the health-compromised MFC outputs after the applied interventions, we refer to Figure 6. After shear erosion of the EAB outer layers followed by a restabilization period of 10 days, \(P_{\text{max}}\) and \(I_{\text{max}}\) were much diminished compared to their pre-overshoot values. The power density curve anomaly at 25 kΩ was eliminated after removal of the outer biofilm layer due to a decrease in the settling time during the polarization test discharge profile. Therefore, we presume that the outer EAB layers formed an electrical and/or nutrient transport barrier. SEM imaging shows two strata (Supplementary Materials Section S8). The
lower stratum, which contained residual EAB following shear erosion, showed more extracellular polymeric substance, indicating stresses during growth, likely related to higher electron transfer resistance after transition to an unhealthy state. The upper stratum contained bacterial cells only (no extracellular polymeric substance), indicating that after shear erosion, the EAB regrew without such stresses. Except for some debris, SEM showed that the biofilm was uniform in height and displayed no cracks or crevices, indicating that the shear erosion was experienced evenly everywhere and not through any preferential flow paths. Although it is not shown in Figure 6, the overshoot persisted after shear erosion but with a reduced $\Delta I_{\text{OS}}$. Increasing the flow rate to $Q_T=10 \text{ mL h}^{-1}$ resulted in improvements to all figures of merit (including a further reduced $\Delta I_{\text{OS}}$). Next, we applied a 4-day pre-acclimation to 3.5 kΩ followed by a standard polarization test. This eliminated overshoot ($\Delta I_{\text{OS}}=0$) and returned $P_{\text{max}}$ to pre-overshoot levels, though $I_{\text{max}}$ still remained lower than the initial (pre-overshoot) levels. Pre-acclimation also produced better performance compared to waiting for short-term stability (at $t=t'_s$) for the same flow rates. Finally, a long-hold polarization test enabled in situ acclimation for each $R_{\text{ext}}$ after long-term stability was reached. This method maintained $\Delta I_{\text{OS}}=0$ and improved both $P_{\text{max}}$ and $I_{\text{max}}$ beyond those values obtained using the standard polarization test before the MFC overshoot began. By combining the in situ acclimation during a long-hold polarization test with an increased flow rate, $P_{\text{max}}$ and $I_{\text{max}}$ were further improved. We note that in the absence of any acclimation, the performance returned to the previously measured non-acclimated post-erosion levels.

Looking towards the future, when the integration of reference electrodes into microfluidic MFCs is more straightforward, we anticipate new studies that include EIS and voltammetry to more deeply investigate overshoot. Based on our observations, we believe that it is not the MFC
health state that causes the power overshoot but rather the related slower electron transfer kinetics that result in the polarization tests being conducted before EAB was adequately stable. This is proven by the ability to eliminate overshoot and even improve on MFC outputs over the initial (healthy) measurements using a long-hold technique on a health-compromised MFC. The reader is directed to the Supplementary Materials (Section S9) where we have summarized the terminology, conclusions and guidelines on proper conduction of polarization tests.

Figure 6. A summary of the effect of the acclimation methods on (a) maximum power density ($P_{\text{max}}$) and (b) maximum current density ($I_{\text{max}}$) following shear erosion at $Q_T=1 \text{ mL h}^{-1}$ (solid bars) and $Q_T=10 \text{ mL h}^{-1}$ (cross-hatched bars). The respective pre-overshoot values obtained at $t=42$ days are shown as horizontal dashed lines.

5. Conclusions

A pure-culture *Geobacter sulfurreducens* microfluidic MFC with a membraneless configuration was used to study overshoot behaviour under controlled flow rates. Power overshoot was observed in the power density curves from a standard polarization test when applying different external resistors (from high to low) at cycle times of 1 to 2 hours. This drop is most likely related to a slow response from the microbes during adjustment to the new
resistance. Eroding the outer EAB layers under strong shear forces improved the power density curve shape, but the overshoot at low $R_{\text{ext}}$ persisted. Moderate increases to the flow rate (below erosion levels) reduced overshoot, but its elimination could only be achieved via the \textit{in situ} acclimation approach. No intervention made could cure the MFC from its unhealthy state (based on persistence of overshoot in the absence of acclimation, reduced cell voltage and lower current compared to the mature healthy state), but long-hold polarization tests produced results that were better than the results obtained using the standard polarization test applied to the healthy state. We anticipate that \textit{in situ} acclimation could be applied to healthy MFCs for measurement results that are representative of long-term MFC operation. We conclude that the role of health state can cause challenges in properly conducting polarization test and that long-hold measurement are the best way to avoid artifacts such as overshoot in power density curves.

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\textbf{Declaration of Competing Interest}

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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