

## Feasibility Study on Electrochemical Impedance Spectroscopy for Microbial Fuel Cells: Measurement Modes & Data Validation

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Electrochemical impedance spectroscopy (EIS) is in potential a powerful tool for the in depth analysis of microbial fuel cells (MFCs). To prevent the risk of drawing false conclusions from invalid EIS measurements we investigated the feasibility of this method on an MFC by checking: linearity, causality, stability and finiteness. EIS application under steady state conditions was partly feasible. For further application EIS on MFCs we recommend to: (1) use the constant anode or cathode potential measurement mode with a fast couple at the counter electrode; (2) record the polarization curve and measure at different amplitudes to check the linearity condition; (3) perform preliminary measurements to reveal measurement presets; (4) apply prolonged pretreatment to facilitate the stability criterion; (5) perform duplicate measurements to examine the stability; (6) use a broad frequency range to validate the finiteness criterion; (7) use a statistical based validation check based on the Kramers-Kronig transformation.

### 1. Introduction

The microbial fuel cell (MFC) converts bio-degradable material in wastewaters, organic residues, sediments and living plants directly into electricity (1,2,3,4). Like a chemical fuel cell, an MFC consists primarily of an anode and a cathode. At the anode, electrochemically active microorganisms act as biocatalyst: they oxidize organic matter and transfer the released electrons to the electrode. These electrons are released at a high energy level at the anode, which results in an electron flow from anode to the cathode. Here usually oxygen is reduced to water. The energy in the electrons can be used as electricity or used for bio-catalyzed hydrogen production (5).

To study the bio-electrochemical properties of MFCs, polarization curves, power curves, current interrupt, and cyclic voltammetry are used (3). By these methods, the internal resistance and the maximum possible power production can be estimated, and the way bacteria transfer their electrons can be investigated (3). The application of electrochemical impedance spectroscopy (EIS) on MFCs is not commonly done, although this tool was widely applied in chemical fuel cell, corrosion studies, and studies on membrane behavior of living cells (6,7,8). Impedance spectroscopy is based on the principle that a small alternating voltage or current is applied to the cell at different

frequencies, and the resulting alternating current or voltage is measured (8). The phase shift that exists between voltage and current provides then information about the resistances and other electrochemical properties of the interface under research. For chemical fuel cells, EIS is used to identify potential losses of the single fuel cell components like the anode, cathode, and electrolyte, with the purpose to identify the limiting processes which determine the performance of the fuel cell. EIS can be a useful method for MFC investigations because MFCs have essential similarities with chemical fuel cells. There are however also essential differences between microbial and chemical fuel cells. For MFCs, application of impedance spectroscopy is challenging because of the presence of a consortium of living microorganisms which can change in composition and behavior. Preliminary application of EIS on MFCs in our lab showed that measurements results could lead into chaotic, irreproducible results. This showed the necessity to validate EIS results on MFCs before analyses and interpretation of impedance data. Until now, in the most comprehensive study of EIS on MFCs (9,10,11), the impedance measurements were not validated and sometimes inexplicable results were obtained. When data validation is not done, there is a risk of drawing false conclusions. EIS results can be validated by checking them with four criteria which must be met for valid impedance results: linearity, causality, stability, and finiteness (12,13,14). This validation can be done by graphical and calculation methods, like the Kramers-Kronig transformation (12,13,14). In this study, we investigate the feasibility of EIS application on a MFC by using three different measurement modes (constant cell voltage, constant cell current, constant anode potential) with two different cathode reactions (oxygen and  $\text{Fe(CN)}_6^{3-}$  reduction) along with the data validation using the four criteria for valid impedance measurements.

## 2. Experimental approach

### 2.2. Microbial fuel cell design

The MFC consisted of two plexiglass plates with a flow channel, two flat electrodes, and two plexiglass support plates as used in (15). The two plates with a flow channel were separated by a cation exchange membrane (FKB, Fumatech, Braunschweig, Germany). The other side of the flow channel faced the electrode. Both anode and cathode were flat  $\text{Al}_2\text{O}_3$ -blasted graphite plates (Müller & Rössner GmbH & Co., Troisdorf, Germany), which also served as the current collector. The surface area of the flow channel, and thus the effective projected surface area of the electrodes, was  $22 \text{ cm}^2$ , and the volume of the flow channel was 33 mL.

### 2.2. Microorganisms and medium

The MFC was inoculated with the effluent of another MFC run on acetate (16). The anode conditions were directed into a steady-state (stable current production at the applied external resistance) by continuous feeding with potassium acetate (final concentration: 20 mM), phosphate buffer ( $\text{K}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$ ; final concentration: 20 mM; pH 7.0) and a nutrient solution adapted from reference (17). The hydraulic retention time of the anode compartment was 14 days. Both anode and cathode fluids were circulated continuously by peristaltic pumps.

### 2.3. Microbial fuel cell operation

The MFC was placed in controlled climate chamber at 30 °C. Both anode and cathode compartments contained a reference electrode (Ag/AgCl, 3M KCl, +205 mV vs NHE). Potentials of anode, cathode and reference electrodes were collected via a Fieldpoint AI-110 module on a PC during MFC operation with a resistor every minute, and during the impedance measurements every second.

The MFC was started with a resistor of 1000  $\Omega$  and a phosphate buffer (20mM) in the cathode. After a 32 days of operation and measurements, the catholyte was replaced for 16 days with a  $\text{Fe}(\text{CN})_6^{3-}$  solution (50 mM) in 20 mM buffer to be able to compare a slow cathode reaction (oxygen reduction at graphite) with a fast cathode reaction (ferricyanide reduction). Thereafter, the catholyte was replaced with phosphate buffer again.

### 2.4. Impedance measurements and validation

#### Equipment and connection modes

The used potentiostat was an IVIUMSTAT (IVIUM, Eindhoven, The Netherlands) Electrochemical Interface & Impedance Analyser which was controlled with a computer equipped with software (IviumSoft, IVUM, The Netherlands). Hereby we applied a small alternating voltage or current to the interface at different frequencies, and measurement of the alternating current or voltage (8). We tested 3 different connection modes for the impedance measurements on the MFC: (1) constant cell voltage, (2) constant cell current and (3) constant anode potential. For the constant cell voltage and constant cell current measurements, the anode was the working electrode and the cathode was the counter and reference electrode. For the constant anode potential measurements (3 electrode set-up), the anode was the working electrode and the cathode the counter electrode. The reference electrode placed in the anode compartment was then used as the reference electrode. We used these 3 different connection modes so we could examine their appropriateness for microbial fuel cells.

#### Conditions for impedance measurements

Before impedance measurements, a polarization (I-V) curve was made by chronoamperometric measurements with the potentiostat. The MFC was then controlled at different cell voltages for 10 minutes with a pre-treatment of 5 minutes at the first applied cell voltage. The anode potential was set so that the maximum value was 0 mV vs Ag/AgCl. This was done because preliminary measurements showed that long-term MFC operation at high anode potentials influenced the performance at the initial (lower) anode potential. During the impedance measurements the applied cell voltage and cell current were always between 0 and the maximum cell voltage or cell current achieved with the polarization curve. During impedance measurements with constant anode potential, this potential was always kept in the range of the anode potential during operation with an external resistor and 0 mV vs Ag/AgCl. So, instead of applying open cell voltages (He et al., 2006; He et al., 2007) we applied potentials and currents similar to MFC operation conditions. This way, data interpretations can be related to the conditions which were actually present during MFC operation.

Impedance measurements were done in the frequency range of 40 – 0.003 Hz. In this way, the measurement time was reduced to a practical period of approximately 20 minutes. Before the impedance measurements, a pretreatment of 5 minutes at the applied current or potential was used to start the measurement at stable conditions. In between the

repetitive measurements, the applied currents and potentials were kept constant at these same conditions. Amplitudes of 0.1 mA (constant cell current) or 10mV (constant cell voltage and constant anode potential) were used as a standard.

#### Impedance measurement validation

Impedance measurements were validated by checking them with the four criteria which must be met for valid impedance results (12,13). These criteria were: (1) Linearity, (2) Causality, (3) Stability, and (4) Finiteness.

At first, a system is linear if the response to a sum of individual inputs is equal to the sum of individual responses. In normal EIS practice, a small (1 to 10 mV) AC signal is applied to the interface under research. With such a small potential signal the system is most likely linear. The linearity was here checked by analyzing the linearity of the recorded polarization curve, which should show linear behavior around the applied potentials or currents. However, a linear polarization curve does not guarantee linear impedance, and therefore the here applied check is an indication rather than a sound check.

Secondly, causality means that the system must be entirely determined by the applied perturbation, so that the output depends only on the present and past input values. This was checked by observing if no perturbations were visible before and after the measurement.

Thirdly, a system is stable when it remains stable unless excited by an external source and should return to its original state after excitation. This was checked by duplicate or multiple recording of the impedance spectra, which should be identical. In case of graphical method used by us, we defined that impedance spectra were identical when the Nyquist plots tended to overlap and the trend of multiple recordings were similar.

Fourthly, in theory the system impedance must be finite valued over the entire frequency range from near 0 and near  $\infty$ . Hereby it must be noted that the actual frequency measurement range is firstly limited by measurement apparatus and secondly by the practical measurement time. The finiteness was checked by analyzing the real impedance value which must tend to a constant real value for the border frequencies.

### 3. Results and discussion

#### 3.1 Polarization curves & linearity check

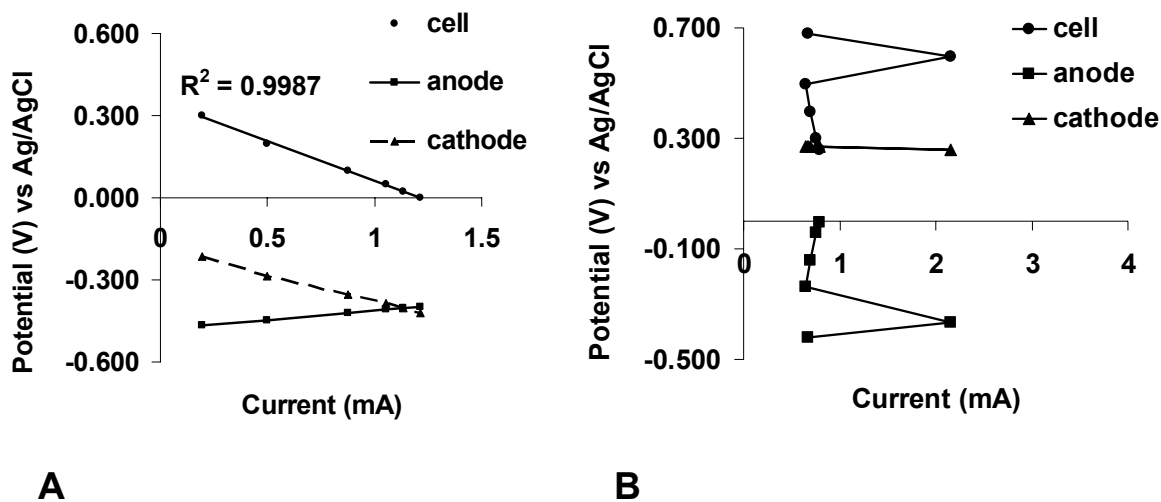


Figure 1. Polarization curve of MFC with oxygen reduction (1A) and  $\text{Fe}(\text{CN})_6^{3-}$  reduction (1B).

Figure 1A shows the polarization curve of the MFC with oxygen reduction before the EIS measurements. The cell voltage decreased linearly ( $R^2 = 0.9987$ ) with increasing current density, which indicates that the MFC shows linear behavior when poised at all cell voltages shown in the polarization curve. So, it was indicated that the MFC with oxygen reduction may meet the linearity criterion.

Figure 1B shows the polarization curve of the MFC with  $\text{Fe}(\text{CN})_6^{3-}$  reduction before the EIS measurements. The MFC might show linear behavior between a cell voltage of 0.7 V and 0.6 V, but too little data points were obtained to ensure this. So it cannot be verified with this polarization curve if the linearity criterion was met with  $\text{Fe}(\text{CN})_6^{3-}$  reduction.

Care should be taken with interpretation of the polarization curves. During the recording of the polarization curves, it may take several hours before the current becomes constant, depending on the poised potential and size of measurement steps.

A linear polarization curve does not guarantee linear impedance. Linearity is therefore also generally tested through the application of different amplitudes, which should result in the same impedance (13). An additional method to check the linearity is to visualize and verify the quality of the sinusoidal current or potential response of the system during EIS. Whenever irregular sinusoidal situations occur, the system may not meet the linearity criterion (13). Therefore we recommended to verify the quality of the sinusoidal response during EIS measurements and measure with different amplitudes as sound linearity checks.

## 3.2. Constant cell voltage, oxygen reduction

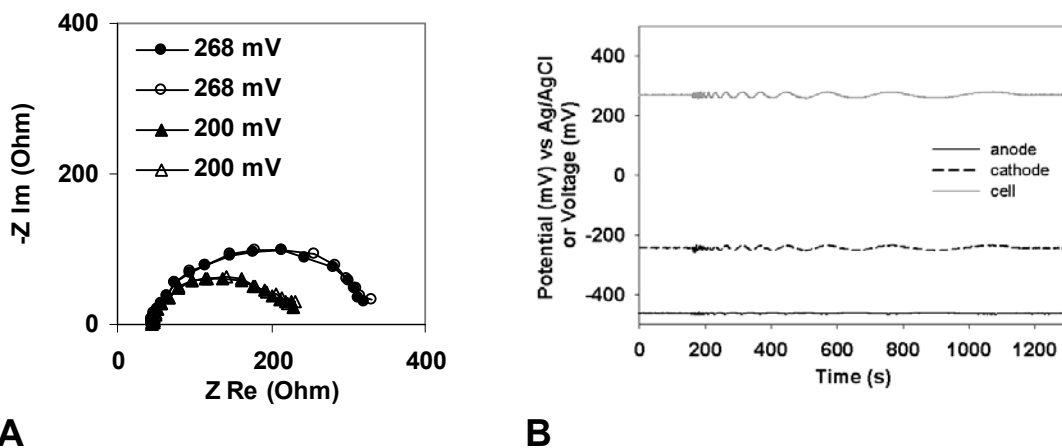


Figure 2. (A), Nyquist plots of EIS measurements at constant cell voltage and with oxygen reduction at the cathode (B). Cell (light grey), cathode (dark grey) and anode (black) potentials during the second EIS scan (C).

Figure 2A shows impedance measurements at a constant cell voltage and with oxygen reduction at the cathode. The Nyquist curves show good duplicates at the applied cell voltage similar to steady-state operation of the MFC with an external resistance (0.268 V) and also at a lower applied cell voltage (0.2 V). This shows that the stability criterion was met. Figure 2B shows the cell voltage, cathode potential, and anode potential recorded every second, before, during and after impedance measurements. From Figure 2B we can see that mainly the cathode potential was fluctuating, while the alternating voltage had only a small effect on the anode potential. The magnitude of the fluctuations is an indication of the reaction rate, and the most fluctuating (slowest reaction) will determine the impedance under constant cell voltage measurement. We therefore see that the oxygen reduction cathode prevails in the Nyquist plots.

### 3.3. Constant cell voltage, $\text{Fe}(\text{CN})_6^{3-}$ reduction

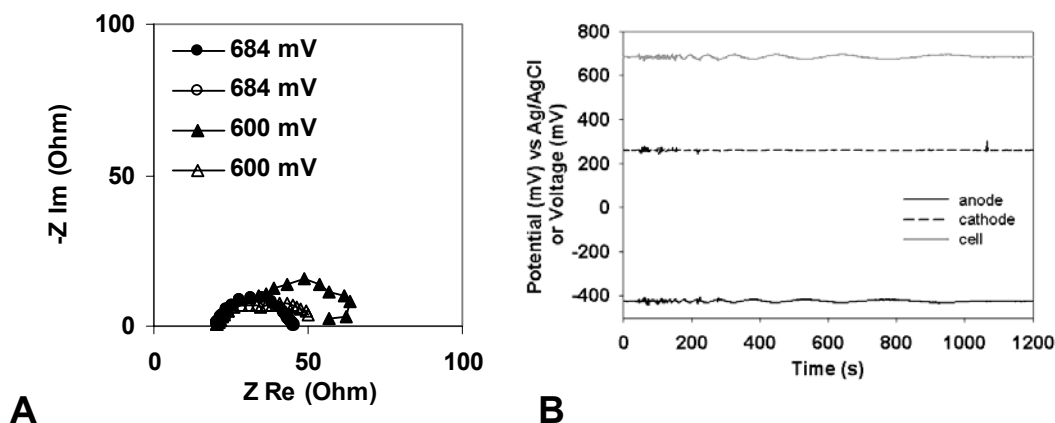


Figure 3. Polarization curve before EIS measurements (A), Nyquist plots of impedance measurements at constant cell voltage and with ferricyanide reduction at the cathode (B). Cell (light grey), cathode (dark grey) and anode (black) potentials during the second scan (C).

Figure 3A shows the results of the impedance measurements at a constant cell voltage and with  $\text{Fe}(\text{CN})_6^{3-}$  reduction at the cathode. The Nyquist curves at the cell voltage equal to the cell voltage during steady-state operation of the MFC showed reproducible results thus the stability criterion was met. Since also no perturbations before and after the measurement were seen and the border frequencies were finite, the causality and finiteness criterion were also met. At lower cell voltage (higher currents) than during operation with a resistor, the Nyquist curves did not show good duplicates, which means that no stable situation was reached during these impedance measurements. A longer pre-treatment period of e.g. several hours may be enough to stabilize the system. In Figure 3B we see that now the cathode potential shows almost no fluctuation, contrary to the situation with oxygen reduction at the cathode. This is a result of the fast reduction reaction of  $\text{Fe}(\text{CN})_6^{3-}$ . So, the Nyquist plots show the impedance of the anode.

## 3.4. Constant cell current, oxygen reduction

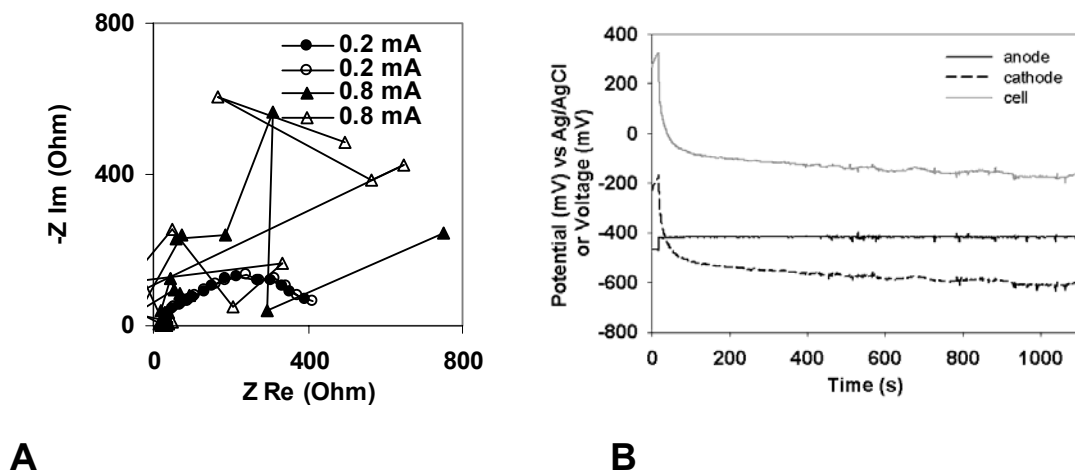


Figure 4. Nyquist plots of impedance measurements at constant cell current and with oxygen reduction at the cathode (A). Cell (light grey), cathode (dark grey) and anode (black) potentials during the third scan (B).

Figure 4A shows the results of the impedance measurements at a constant cell current and with oxygen reduction at the cathode. These measurements were performed right after the constant cell voltage with oxygen reduction measurements (Figure 2.1A). The Nyquist curves at the cell current equal to the cell current during steady state operation of the MFC showed reproducible results. In all measurements, the cathode fluctuated more than the anode, which implies that the oxygen reduction reaction was the most limiting and thus prevailed in the Nyquist plot. At higher currents, Nyquist curves did not show good duplicates. Especially at a current of 0.8 mA the Nyquist plots were chaotic. The measured negative  $\text{Re}(Z)$  are theoretically not possible, therefore we suppose that the negative  $\text{Re}(Z)$  values were due to instrumental artifacts of the presets of potentiostat in combination with the used MFC. The measurements coincided with a slow drop of the fluctuating cathode potential during the EIS measurement. Finally the cathode dropped below the anode potential during repetitive impedance measurements (Figure 4B). So, the cathode of the MFC was thus not stable at high currents. This effect was also sometimes observed during the measurements at a constant anode potential (data not shown). Care must be taken that the selected current should not be higher than the electron acceptor can accept at that moment. Otherwise energy will be supplied by the potentiostat, which makes these kinds of measurements meaningless. This problem can be solved by using a fast cathode reaction (e.g. reduction of  $\text{Fe}(\text{CN})_6^{3-}$ ).

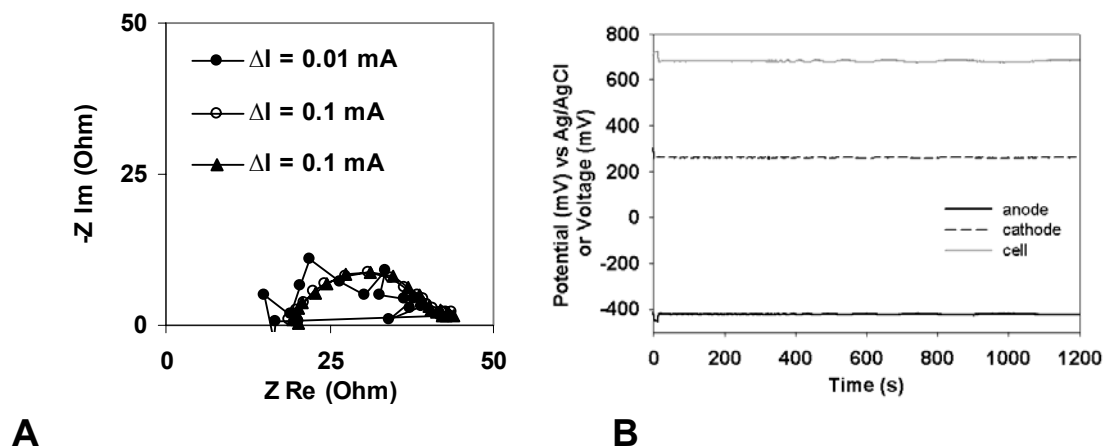
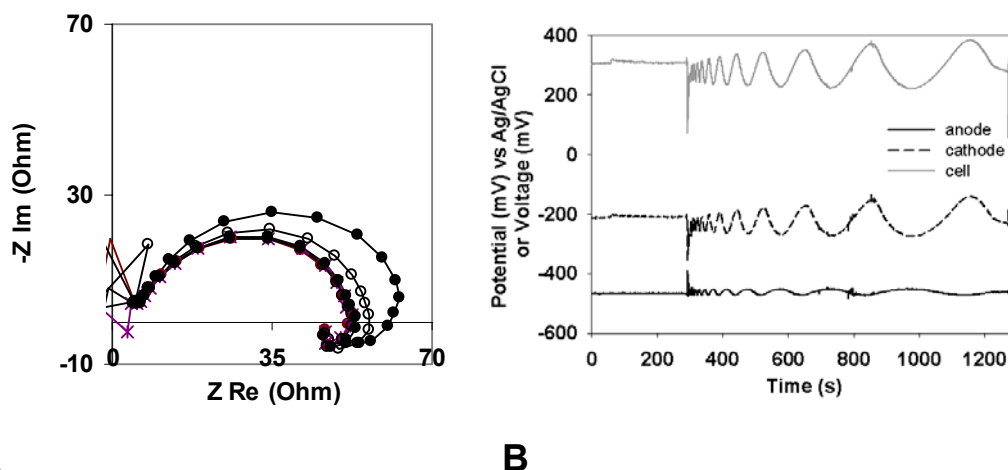
3.5. Constant cell current,  $\text{Fe}(\text{CN})_6^{3-}$  reduction

Figure 5. Nyquist plots of impedance measurements at constant cell current and with ferricyanide reduction at the cathode (A). Cell (light grey), cathode (dark grey) and anode (black) potentials during the third scan (B).

Figure 5A shows the results of the impedance measurements at a constant cell current and with  $\text{Fe}(\text{CN})_6^{3-}$  reduction at the cathode. These measurements were performed just after the constant cell voltage with ferricyanide reduction (Figure 3.2A). The first chosen amplitude of 0.01 mA did not result in smooth Nyquist curves (figure 5A, scan 1), because the amplitude was too small to produce detectable fluctuations in the potentials. Therefore a higher amplitude of 0.1 mA was chosen for the following measurements. Only Nyquist curves at the current equal to the current during steady state operation of the MFC with an external resistance showed reproducible results and thus met the stability criterion. No perturbations were seen before and after the measurement, so the causality criterion was met. The finiteness criterion was not clearly met since the border of the low frequency impedance was not finite. Figure 5B shows that the anode and cathode potentials had smaller fluctuations than during impedance measurements at constant cell voltage. The anode and cathode potential both fluctuated and therefore both electrode properties may be present in the Nyquist plots. This measurement mode was not suitable to distinguish between the anode and cathode impedance.

## 3.6. Constant anode potential, oxygen reduction



**A** **B**  
 Figure 6. Nyquist plots of impedance measurements at constant anode potential and with oxygen reduction at the cathode (A). Cell (light gray), cathode (dark grey) and anode (black) potentials during the second scan (B).

Figure 6A shows the results of six impedance measurements at a constant anode potential (-0.482 V vs Ag/AgCl) and with oxygen reduction at the cathode. These measurements were performed just after the constant cell potential with oxygen reduction measurements (Figure 3.1A) and thus the linearity criterion was met. The potentiostat seemed to have difficulties controlling the anode potential as illustrated in Figure 6B where some spikes in the anode potential can be seen. Furthermore we see that the recorded sinusoidals are not ideal which indicates non linear response (13). Since the first 3 Nyquist curves (Figure 6A) were not the same as the last 3 curves, the MFC was indeed not in a steady state during the first 3 measurements. The scattered initial points of the Nyquist plots were due to instrumental artifacts and should be removed before further data analysis. The instability was furthermore confirmed by the data of cathode potential and cell voltage which decreased approximately 10 mV during each of these measurements (Figure 6B) and remained stable during the last 3 recordings (data not shown). As a result of the connection mode, the anode potential was alternated during the impedance measurements, and as a consequence the cathode and cell potential were alternating too (Figure 6B). As the anode potential was controlled here, we see the characteristics of the anode in the Nyquist plots, even though the cathode potential was fluctuating more than the anode potential and does not measure the counter electrode (cathode).

### 3.7. Constant anode potential, $\text{Fe}(\text{CN})_6^{3-}$ reduction

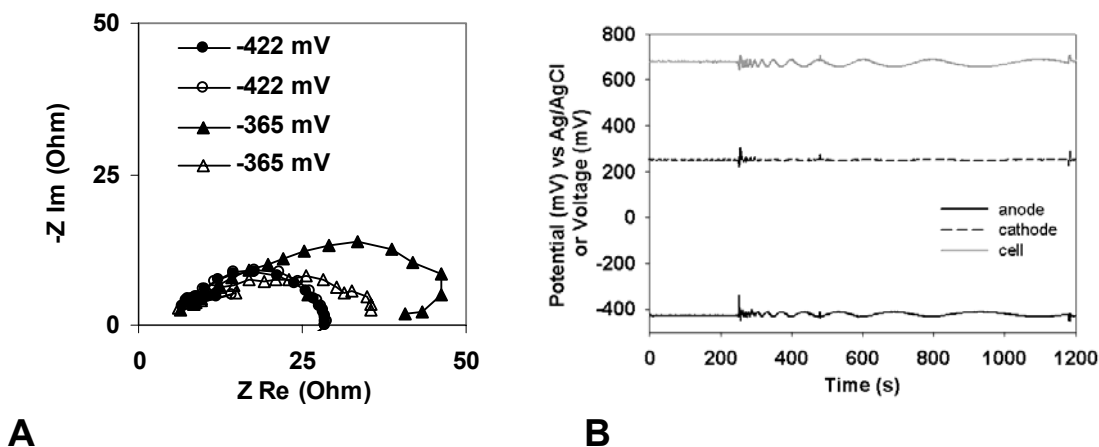


Figure 7. Nyquist plots of impedance measurements at constant anode potential and with ferricyanide reduction at the cathode (A). Cell (light grey), cathode (dark grey) and anode (black) potentials during the first scan (B).

Figure 7A shows results of the impedance measurements at a constant anode potential and with  $\text{Fe}(\text{CN})_6^{3-}$  reduction at the cathode. These measurements were performed just after the constant cell voltage with ferricyanide reduction (Figure 3.2A). The Nyquist curves present good duplicates at the anode potential set at the anode potential (-0.422 V vs Ag/AgCl) during steady-state operation of the MFC with an external resistance. At higher anode potentials, and thus higher currents, the Nyquist plots did not show good duplicates, which means that no stable situation was reached during impedance measurements. This was in line with the impedance measurements at constant cell voltage with  $\text{Fe}(\text{CN})_6^{3-}$  reduction at the cathode (Figure 2B) where an increased anode potential (increased current) also resulted in non-reproducible measurements. Comparison with Figure 5A shows that the resistances are in the same range ( $Z_{\text{Re}}$  between 28 and approx. 50  $\Omega$ ), which confirms that both figures show the anode behavior.

### 3.8 Summary of criteria for valid EIS results

For each criterion it was depicted in table 1 whether the criterion was met or not.

**Linearity:** As explained in paragraph 3.1, it was indicated that the MFC with oxygen reduction met the linearity criterion, however, as discussed, additional methods are needed to ensure linearity. With  $\text{Fe}(\text{CN})_6^{3-}$  reduction it could not be indicated whether the criterion was met.

**Causality.** In the figures 3B – 7B, no perturbations were seen in the signal before and after the measurement. Since no perturbations were seen, we can safely assume that the measured signal was caused only by the excitation of the potentiostat, and thus the causality criterion was met in all situation. Causality can also be measured using coherence which is the signal to noise ratio (Lasia, 1999).

**Stability.** When measuring at steady-state conditions, good duplicates were obtained. When measuring at higher potentials than at steady-state however, it was found that the

EIS results were not reproducible. Thus, the stability criterion was only met when measuring at steady-state conditions.

**Finiteness.** The finiteness criterion was in most cases met for the high frequency border value but not for the lower frequency border. The finiteness at the low frequency border is influenced by for example the capacitance of the system, the size of the electrode, and the presence of mass transfer limitations. So there are circumstances that the finiteness will not be reached (13, 14). Prolonged measurements at lower frequencies could show if the system's impedance is finite. However as mentioned before, it may be possible that the overall impedance is too large to be measured with the EIS equipment at the lowest possible frequency.

**TABLE 1.** Overview Measurement Modes & Data Validation

Measure ment mode	Cathode reduction reaction	Impedance determined by:	Linearity	Causality	Stability	Finiteness
Constant cell voltage	Oxygen	Cathode	N	Y	Y	N
	$\text{Fe(CN)}_6^{3-}$	Anode	N	Y	Only when steady-state for the bio- anode	Y
Constant cell current	Oxygen	Cathode	N	Y	Y	N
	$\text{Fe(CN)}_6^{3-}$	Anode	N	Y	Only when steady-state for the bio- anode	N
Constant anode potential	Oxygen	Anode	N	Y	Only when steady-state for the bio- anode	?
	$\text{Fe(CN)}_6^{3-}$	Anode	N	Y	Only when steady-state for the bio- anode	N

Y = yes; N = no

Even in the most comprehensive EIS measurements on MFC studies, it was not reported that data was checked on the four criteria which must be met for valid impedance results (9,10,11,18). Therefore, this study is the first work which validated EIS measurements on a MFC.

We applied a graphical methodology which was not completely statistically validated since deviations in perturbations, and Nyquist plots were not expressed in statistical information like standard deviations and root-means-square errors (13). In the future we can improve the graphical check to meet higher statistical quality. We recommend to use the Kramers-Kronig method (13). Nevertheless, the applied method here was appropriate for a fast validity check on EIS results. This is especially practical during the EIS measurements. In one survey one can graphically determine whether EIS data tend to valid or invalid EIS results. From this, measurement presets can be changed or one may choose to wait with further measurements until the system is stable. So, depending on the goal of the specific study one may combine a graphical check with a more thoroughly statistical secured check with e.g. the Kramers-Kronig transformations (12,13,14).

#### 4. Conclusions

EIS application on a MFC was partly feasible by using three different measurement modes: constant cell voltage, constant cell current and constant anode potential.

This because, non of all the EIS results showed clearly that all four criteria were met at the same time. However, looking to all measurements, all four criteria may be met by combining several measurements, which indicated that EIS application of MFCs can meet all four criteria. When measured at steady state operation conditions, the best EIS results were obtained. It was revealed that oxygen reduction cathodes may cause problems on the stability of EIS measurements. These problems were not observed with  $\text{Fe}(\text{CN})_6^{3-}$  reducing cathodes.

Since the MFC was generating a similar current before and after the EIS measurement, we saw that EIS is useful for further non-destructive MFC characterization. It was easily possible to generate invalid impedance data and therefore EIS data must always be validated before data interpretation.

By measurement of anode and cathode potentials, and cell voltage, during impedance measurements we were able to analyze which processes prevailed in the Nyquist plots.

Towards further EIS application on MFC we have recommendations.

We recommend always to check EIS results from measurements on a MFC on the four criteria which must be met for valid impedance results: linearity, causality, stability, and finiteness. Without data validation, there is a risk of drawing false conclusions from invalid data. Towards successful EIS application on MFCs we recommend:

1. to use the constant anode or cathode potential measurement mode with a fast couple at the counter electrode for studying a specific electrode.
2. to record the polarization curve to check the linearity condition. Hereby we recommended, additional linearity checks, by verification of the quality of the sinusoidal response during EIS measurements and measurement at different amplitudes to ensure that the impedance amplitude is independent of the input potential amplitude.

3. to perform preliminary measurements to reveal measurement presets.
4. to apply a prolonged pretreatment up to several hours at the specific potential or current to facilitate the stability prerequisite.
5. to perform duplicate measurements to show whether measurement data describe stable conditions or not.
6. to use a broad enough frequency range to validate the finiteness criterion.
7. to use a statistical based validation check based on the Kramers-Kronig transformation (13, 14).

By following this approach we expect that EIS in combination with the common electrochemical methods will be very useful for future analyses of the bioelectrochemical properties of MFCs.

#### Electrochemical impedance spectroscopy (EIS) is in potential a powerful tool for the in depth analysis of microbial fuels cells

Now we indicated that EIS application on MFC can be feasible, the next challenge is the interpretation of the results. Aimed qualitative MFC experiments with EIS measurements should be combined with modeling to extract information of the appearing phenomena. Simplified equivalent circuit models can be used to guide the development of meaningful mechanistic models based on physical grounds. Direct interpretation from hypothetical equivalent circuits as done by e.g. He (9) is thus a fallacious method if you really want to understand what is happening. Our future goal is to reveal the rate limiting processes and subsequently improve this process to increase the MFC power output.

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