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Innovative Statistical Interpretation of *Shewanella oneidensis* Microbial Fuel Cells Data

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Sofia Babanova[a], Orianna Bretschger[b], Jared Roy[c], Andrea Cheung[d] , Kateryna Artyushkova[a] and Plamen Atanassov[a]

The last decade of research has made significant strides toward Microbial Fuel Cells (MFCs) practical applications; however, design improvements and operational optimization cannot be realized without equally considering engineering designs and biological interfacial reactions. In this study, the main factors contributing to MFC`s overall performance and their influence on MFCs reproducibility are discussed. Two statistical approaches were used to create a map of MFCs components and their expanded uncertainties, *Principal Component Analysis* (PCA) and *Uncertainty of Measurement Results* (UMR). PCA was used to identify the major factors influencing MFCs and to determine their ascendency over MFC operational characteristics statistically. UMR was applied to evaluate the factors' uncertainties and estimate their level of contribution to the final irreproducibility. In order to simplify the presentation and concentrate on the MFC components, only results from *Shewanella spp*. were included; however, a similar analysis could be applied for any DMRB or microbial community. The performed PCA/UMR analyses suggest that better reproducibility of MFC performance can be achieved through improved design parameters. This approach is exactly opposite to the MFC optimization and scale up approach, which should start with improving the bacteria-electrode interactions and applying these findings to well-designed systems.

1. Introduction

1.1. Microbial fuel cells

With the decreasing water supply and the increasing population growth, the need for alternative water sources and water recovery is becoming one of the major problems facing humanity. Microbial Fuel Cells (MFCs) are a technology that can address these issues through providing self-powered, sustainable "bioreactors" for wastewater purification. MFCs are attracting worldwide attention, driven by the idea that they can produce renewable energy from various wastes and wastewaters. However the reported energy densities associated with MFCs are very low relative to chemical fuel cells and MFCs have not yet been proven as practical devices for electricity production. This is due to the fact that the scaling up process of these devices is not trivial. For example, what are thought as "acceptable losses" at the lab scale become parasitic when scaled up to practical applications. Three major steps must be undertaken in order to implement MFCs as a viable technology for energy recovery during wastewater treatment: i) identify and isolate the factors having the biggest impact on MFC performance; ii) scale up the technology with an emphasis on material design and interfacial biological reactions; iii) understand the factors behind the variability of MFC power generation.

1.1.1 Identify and isolate the factors having the biggest impact on MFC performance

Many research efforts have been dedicated to the development and improvement of biofuel cells, and specifically the fundamental understanding of extracellular electron transfer processes, materials selection, and design optimization. Based on the gained knowledge through the years, the generated power and current densities from laboratory MFCs have increased significantly (from 0.05 mA/m^2 to 1000 mA/m^2). However, after 100 years of research in this field, scientists and engineers have not yet realized dramatic improvements in energy densities and/or huge achievements as practical devices. One of the primary reasons why this has not yet occurred is that most researchers have applied a "one by one" approach: understanding and optimization of one parameter at a time. Here, we propose a multi-parameter approach, based on *principal component analysis* (PCA), that provides information on how MFCs can be optimized based on understanding how multiple parameters amplify each other and improve the performance.

The parameters determining MFC performance are represented in Figure 1. They can be divided into three major groups: the

electrode compartments, the fuel cell design and the electrochemical methods used to characterize MFCs. Each of these three groups contains a set of parameters, some of them have been studied in detail (type of microorganisms [1-3], extracellular electron transfer (EET) mechanisms [4-10], biofilm formation [5, 11, 12], anode materials [13, 14], etc.), others have only been described (volume of the compartments [15-17], area of the electrodes [18] cross-section, cathode material [19, 20], etc.) and some have rarely been considered (influence of the electrochemical methods).

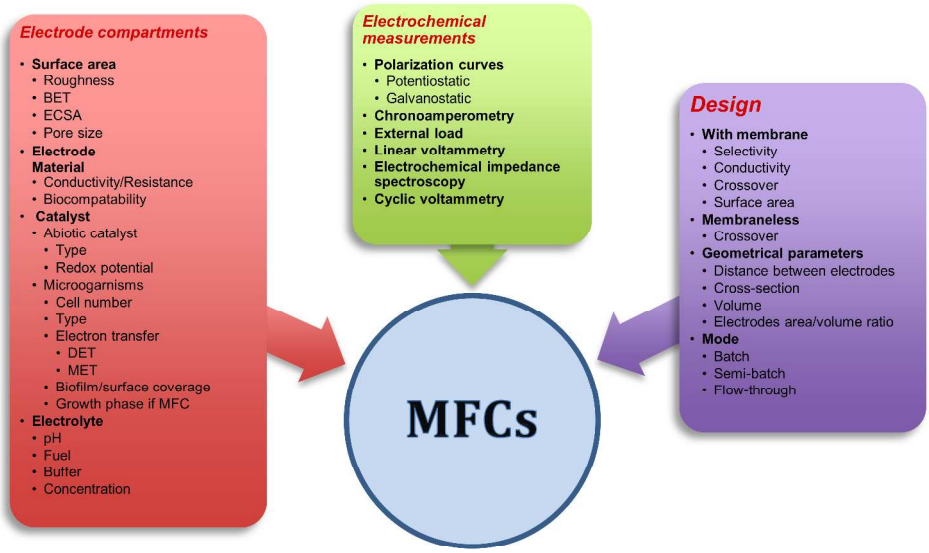

Figure 1: Factors determining MFC`s performance

The importance of the type of the microorganisms used in MFCs has been considered to be the major factor for MFC operation [2, 21-23]. Dissimilatory metal reducing bacteria (DMRB) have proven to be the most appropriate microorganisms for MFC applications due to their ability to reduce insoluble terminal electron acceptors [2, 22, 24]. *Geobacter* and *Shewanella* species are two DMRB that are widely studied in MFCs [4, 9, 12, 24-29]. Although they may have different electron transfer mechanisms, they have one major feature, which makes them similar - the presence of outer-surface *c*-type cytochromes that can transfer electrons directly to a solid electron acceptor (e.g. iron and manganese oxides, and MFC electrodes) [9, 24]. After this discovery was reported, many researchers conducted detailed studies of these phenomenon [4, 5, 30-32].

The direct electron transfer (DET) mechanism appeared as the second most important parameter to MFC performance, which strongly depends and is determined by the type of the microorganism and the environmental conditions [10, 22, 33]. In order to increase the EET rate, the electrode-bacteria interactions gained more attention and subsequently different electrode materials and electrode surface modifications were explored [13, 34-36]. As a result, it was observed that bacteria could colonize and develop biofilms preferably on some electrode surfaces and less on others [34, 37].

Biofilm formation appears as the third main factor in MFC performance. It is regulated by the bacteria type, environmental conditions and, as we just mentioned, by the anode material, etc. [11, 37-39]. It was established that during MFC operation, biofilm development is strongly influenced by the potential of the anode electrode [39, 40].

The type of microorganisms, their mechanisms for EET, and biofilm formation are three parameters that are clearly correlated and, therefore, the study of each of them separately does not provide significant meaning. This holds true especially when other parameters also play a role in determining MFC performance.

In most studies, one parameter has been varied and the rest are kept constant. This is a good approach only if the unvaried factors are independent from the factor varied. However, this is not usually the case. For example, varying the type of the microorganism influences the EET ability and mechanism, biofilm development, substrate used, system stability, operating conditions, etc. If a claim is made that changing the anode material is changing the bacteria-electrode interactions, one must also consider that the real surface area, material hydrophilicity, conductivity, porosity, etc. are also altered. As a consequence the observed effect, for example, an increase in the recorded current, can be due to several different contributing factors such as increased real surface area of the electrode, or increased electrode conductance, or increased porosity at meso/macro-scale, etc. Therefore, a new analysis approach must be developed and applied to MFCs in order to take into account the intra-factor connections and separate individual factor influence from the overall combinatorial impact. Such an approach is *Principal component analysis* (PCA).

Principal component analysis provides the ability to identify the major factors influencing the MFC`s output and statistically determine their ascendency over the operational characteristics. PCA is a multivariate statistical tool, commonly used to reduce the dimensionality of large data sets and extrapolate patterns out of them [41, 42]. This method expresses the data in a way to highlight their similarities and differences [41-43] and visualize the factors (variables), called loadings in PCA, responsible for correlations and anticorrelations among samples [44]. PCA transforms a number of correlated or possibly correlated variables into a number of uncorrelated ones, called *principal components* [45]. The first principal component has the largest possible variance and the second, orthogonal to the first, has the largest possible inertia [43].

PCA in combination with Design of experiments (DOE) was successfully applied for the analysis and improvement of an enzymatic gas-diffusional cathode [46]. This study showed that each of the used statistical methods, DOE and PCA, could provide valuable information regarding the different factors and their associated contribution and correlation, and finally predict “an optimal” system

configuration based on those factors that contribute most significantly and how factors combine to affect performance.

1.1.2. Scale up the technology with an emphasis on material design and interfacial biological reactions

All optimization procedures explored in lab-scale MFCs are considered successful when an increase of the system performance is observed relative to a control sample [13, 18, 47, 48]. This approach does not take into account losses due to poor design, nor does it consider the nature of the interfacial biological reactions. Therefore, the scaling up process of these devices becomes problematic since the unaccounted losses at lab scale become tremendous issues when scaled up to commercial products.

Very often the observation made using lab prototypes are not applicable for practical commercial installations. This can be due to differences in biofilm microenvironment when a jump from small MFCs, or half-cell measurements, to large-scale systems is carried out. Alternatively, scale-up issues can be due to differences in the design, parameter ratios or physical environment that can influence the character of the interfacial biological reactions. For example, half-cell experiments do not take into account the influence of the cathode, the crossover, the MFC design, the presence of a membrane, etc. It is not surprising that when the studied electrodes are transferred into MFCs, totally different phenomena are observed and dramatically change the system characteristics and behavior.

1.1.3. Understanding the factors behind the variability of MFC power generation

One of the main requirements that a practical device should satisfy is reproducibility. Reproducible MFCs results so far are a rare phenomenon mostly due to the complexity of these systems and the lack of knowledge of what exactly is causing this irreproducibility. Although we all try to reproduce operational conditions identically, the materials, the loadings, the design, etc., the uncertainty of the gained results is still not acceptable. In recent years, a step ahead was taken when most of the researchers in this field started to represent the results as an average from at least three replicates along with their standard deviation [4, 25, 40, 49, 50]. The *mean* value as well as the standard deviation are not the most appropriate estimates when MFCs are involved, especially at the level of small data sets (< 20) usually available from MFCs studies [51]. These statistical parameters are representative only when the data possess a normal/Gaussian distribution. Based on only three results the distribution cannot be determined, outliers cannot be recognized and, as a result, a value deviating dramatically from the true value cannot be estimated as representative for the system. A for more appropriate approach is the utilization of Robust statistics. Robust statistics is used to process small data sets or data that are unevenly distributed [51]. The data estimators in this case are the median and normalized median of absolute deviation (*MADN*).

The Uncertainty of Measurement Results (UMR) is a well-known statistical tool in analytical chemistry [52]. The uncertainty of a result is a parameter that combines random and systematic errors and provides a realistic range of values within which the true value of a measured quantity lies. The term uncertainty is used to characterize the inaccuracy of a measurement result, whereas the term error is used to characterize the components of the uncertainty [53]. The representation of the operational characteristics with their expanded uncertainty is in agreement with the chemical metrology requirements and allows direct comparison of the obtained data with the results obtained by other experimenters.

Recently the uncertainty was introduced in the area of MFCs by Babanova et al. [54]. In their study, the expanded uncertainty of the main operational characteristics (OCV, maximum current and power) of yeast-based MFCs were evaluated. Using the bottom up approach by creating an uncertainty budget, the uncertainty of the different MFC parameters was estimated and showed that the main factor contributing to higher irreproducibility of results was the differences in electrode resistances. Preliminary selection of electrodes with resistances in the interval of 6-7 Ω led to notable decreases in the uncertainty of operational characteristics. For example, the uncertainty decreased from 19 to 13 % for OCP, from 42 to 14 % for maximum power and from 46 to 13 % for maximum current. This approach was also used by Roy et. al. for the characterization of *Shewanella MR-1* anodes [40]. Roy and Babanova observed that the expanded uncertainty of the measured current was extremely high, especially, at more positive potentials where a higher current is produced. After the current was normalized to the electrochemical accessible surface area (ECSA) the uncertainty of the current density was significantly decreased. This was even more applicable when a biofilm was formed, and the ECSA became the limiting factor. These two studies show that the material aspect of the MFC is the most significant factor causing irreproducibility of results. Due to the complex structure and inhomogeneity of the electrode materials, the differences in their real surface area as well as their resistances play a major role for MFC performance reproducibility.

In this study, the main factors contributing to MFCs overall performance and their influence on the reproducibility of results are discussed. Two statistical approaches, PCA and UMR, were used to create a map of MFC components and their expanded uncertainties [54]. PCA was used to identify the major factors influencing MFC performance and statistically determine their ascendency over MFC operational characteristics. UMR was applied to evaluate the factors' uncertainties and estimate their level of contribution to the final irreproducibility. The study relied on diverse data sets collected at collaborating institutions and reported in the literature. In order to simplify the presentation and concentrate on the MFC components, only results from *Shewanella spp*. were included [8, 14, 26, 29, 40, 55, 56]; however, a similar analysis could be applied for any DMRB or microbial community.

2. Statistical analysis

2.1. Principal component analysis

Methods for analyzing data sets for the main sources of correlation and variation can be of critical importance in structure-to-property studies. PCA is one of the simplest multivariate statistical analysis methods, allowing for the identification of similarities and differences between samples, resulting in the classification of samples into groups. PCA, as applied to chemical problems, can be used to reduce the number of variables and to assist with correlation, pattern recognition and prediction. At its most fundamental level, PCA visualizes the difference between samples (captured in scores) and "explains" why the samples are different (captured in loadings). PCA transforms original variables into new uncorrelated variables, called principal components using Singular Value Decomposition. Each principal component is a linear combination of the original variables (operational characteristics and electrode parameters). The first principal component (PC1) contains the maximum variance while the second principal component (PC2) has the second most variance, and, importantly, is uncorrelated with the PC1 and so on. The first output from PCA is loadings, which are the coefficients of the linear combinations of the original variables that generate the principal components. The second output, called scores, contains the coordinates of the original data in the new coordinate space. Biplots displaying both the loadings for each variable and the scores for each sample in a single plot for the PC1 and PC2 are used to visualize the

clustering of samples with respect to different characteristics that were the most or least significant for separating samples. Correlated variables and samples are located in the same quadrant on a biplot.
For PCA, parameters associated with the construction and operation of MFCs were combined into the two-dimensional matrix $D_{m,n}$, where m samples are arranged row-wise and n variables (parameters) column-wise:

$$\begin{pmatrix} a_{1,1} & a_{1,2} \dots & a_{1,m} \\ \vdots & \vdots & \vdots \\ a_{n,1} & a_{n,2} \dots & a_{n,m} \end{pmatrix}$$

The data matrix was autoscaled to have a mean of 0 and standard deviation of 1 to ensure equal weights (significance) of all parameters into the model. Singular Value Decomposition (SVD) is used to decompose the original data matrix $D_{m,n}$ into a set of loadings, V, which are a linear combination of the original measurable variables (Eq. (1)).

$$D_{m,n} = U_{m,n} * S_{n,n} * V_{n,n}^{T} \quad \text{Eq. (1)}$$

U is the score, which represents coordinates of the data matrix D in a new coordination system. S is a diagonal matrix, which contains information related to the criteria for component significance. The diagonal elements are the square root of the eigenvalues λ of the correlation matrix $Z=D^{T}D$. The primary principal components are those corresponding to the largest r eigenvalues and represent the set of r mathematical components that are required to reproduce the original data matrix D within experimental error E. The remaining principal components, each describing a low variance, represent the noise in the data set. 2-component model was created for all datasets.

For parameters, which cannot be ascribed a particular quantitative value (growth phase, EET, operation mode, electrochemical method, and membrane type) a binary code was used. For example, the parameter "*growth phase*" was split according to the different types of growth phases to "lag phase", "exponential phase" and "stationary phase", and these were used as separate variables in the PCA matrix (See Table 1-3, Supplementary Information). Therefore, when bacteria are in lag phase the variable "lag phase" is ascribed value of "1", the rest of the growth phase variables ("exponential phase" and "stationary phase") were ascribed "0". Samples with bacteria in exponential growth phase had "0" for lag phase, "1" for exponential phase and "0" for stationary phase. The same approach was used for the operation mode, which was divided into three separate variables: "batch mode", "semi-batch mode" and "flow mode"; for the electron transfer mechanism, divided into "MET", "mixed EET" and "DET", etc.

Principal component regression (PCR) was also used to predict the final response of a system using different values of parameters that are shown as being significant for the best MFC performance. PCR is a regression analysis technique that is based on PCA. It considers regressing the response or, the dependent variable on a set of independent variables (parameters) based on a standard linear regression model, but uses PCA for estimating the unknown regression coefficients in the model. In PCR, the principal components that are created by PCA are used as regressors. We have used 2 principal components in building a PCR model.

2.2. Uncertainty

According to International Vocabulary of Basic and General Terms in Metrology (VIM 3.9, taken from GUM) uncertainty of measurement is: "a parameter associated with the results of measurements, characterizing the dispersion of the values, which could be reasonably attributed to the measurand, based on the information used"[57], where measurand is a quantity, subject of a given measurement. The uncertainty of measurement is also called measurement uncertainty (u). This uncertainty characterizes the dispersion of quantity values for the measurand (x), obtained by experimentally derived information (Type A) or based on experience or other information (Type B). Usually the measurement uncertainty is expressed as a standard deviation (*SD*) in Gaussian statistics and as normalized median of absolute deviation (*MADN*) when Robust statistics is used[51, 53]. Which of the two mentioned statistics will be used depends on the form of the distribution function of the observations.

For a normal distribution of the observations Gaussian statistics is used and the arithmetic mean (usually abbreviated to the *mean*) of the observations is taken as an estimate of the true value of the measured quantity[53]. It should be noted that in Gaussian statistics the *mean*, *median* and *average* overlap and are representative for the set of data analyzed[52].

In case of a small number of replicates, there is no reason to assume a normal distribution of the measured values[51]. This especially holds true when the object of investigation is a biological system. In such case, the mean value and standard deviation are not good estimators of the quantity values for the measurand and corresponding measurement uncertainty. For observations following a different pattern or no pattern at all, data should be processed by mean of Robust or nonparametric statistics[51, 58]. According to this approach the median of all replicates ($\tilde{x}$) is an estimator of the measurand value (Eq. (2)). Uncertainty is related to the median of absolute deviations (MAD) estimated using Eq. (3)

$$\tilde{x} = median(x_i) \quad \text{Eq. (2)}$$

$$MAD = median\,(|x_i - \tilde{x}|) \quad \text{Eq. (3)}$$

When this value is divided by a factor of 0.6745 (Eq. (4)) it becomes compatible with SD.

$$MADN = \frac{MAD}{0.6745} \quad \text{Eq. (4)}$$

The value 0.6745 corresponds to the *MAD* of a random variable with standard normal distribution (N (0,1)). Hence for a random variable with distribution N (μ,s) is fulfilled: *MADN* = *SD*. *MADN* is a very useful robust estimation of *SD*, applicable in cases when the compliance of the experimental data with the normal distribution is doubtful as it is in most cases for data associated with MFCs or other bio-related systems.

Usually the analytical procedures are associated with the implementation of several steps and different parameters. Therefore, the final result implies the application of the one or more mathematical equations forming a measurement model. In such analytical procedures, combined uncertainty (u_c) is used to evaluate their uncertainties.

Two approaches are used for combined uncertainty evaluation, "bottom-up" and "top-down" approach[52]. In the bottom up approach combined uncertainty is obtained using the individual measurement uncertainty associated with the input quantities in the measurement model using the uncertainty propagation law (Eq. (5)).

$$u_c^2(y) = \sum_{i=1}^{n} \left(\left(\frac{\partial y}{\partial x_i} \right) u^2(x_i) \right) \quad \text{Eq. (5)}$$

Where: y is the measurand, $u_c^2(y)$ is the squared combined uncertainty of y and $u^2(x_i)$ is the squared measurement uncertainty of the input quantity x_i.

A completely different approach is the top-down method, which seeks to use the results of several tests, preferably from different laboratories, to give estimates of the overall uncertainty of the analytical procedure without necessarily trying to identify every

individual source of error[52]. For the evaluation of the combined uncertainties of the open circuit voltage (OCV), maximum power (P_{max}) and maximum current (I_{max}) of fuel cells, for example, the latter system outputs (OCV, P_{max} and I_{max}) can be obtained through proper electrochemical methods of at least three parallel measurements. Then the *SD* or the *MADN* can be calculated as representative for the measurement uncertainty, and they will be equal to the combined uncertainty due to the fact that the values of OCV, P_{max} and I_{max} are results of the overall system performance. In other words, the *SD* or *MADN* of as least three replicates of identical MFCs operational characteristics are comparable to their combined uncertainties.

The top-down approach is applicable in areas where data from properly run proficiency schemes are available, or when the bottom down approach cannot be used due to the lack of mathematical equations forming a measurement model. Therefore in the area of MFCs since there is no mathematical equation combining the factors affecting the system performance, the top-down approach is the only applicable method for uncertainty evaluation.

Finally, the expanded uncertainty (U) is used as an estimation of the half-width of the confidence interval of the final result[52]. It is a product of the combined uncertainty and so called "coverage factor" (real number larger than 1) Eq. (6). For clarity, the estimators of the measurand, and the corresponding uncertainties according to the two types of statistics based on the top-down approach are represented in Table 1.

$$U = k * u_c \qquad \text{Eq. (6)}$$

Typical value for the coverage factor is: $k=2$ for confidential level of 95%.

The final measurand value is then represented with its *mean* ± U, when Gaussian statistics is used, or with its *median* ± U in Robust statistics.

Table 1: Measurand (x) and the corresponding uncertainties according to Gaussian and Robust statistics based on the top-down approach

Estimator	Gaussian statistics	Robust statistics
x	*mean*	*median*
u (factor)	*SD*	*MADN*
u_c (output)	*SD*	*MADN*
U	*k*SD*	*k*MADN*

3. Results and Discussion

3.1. Principal component analysis

3.1.1. Different *Shewanella* strains

A set of results from two identical MFC systems, differing only in the analyte content (buffer type and lactate concentration) or *Shewanella* strain [26] were processed. Both of the MFCs were two chamber MFC systems with graphite felt anodes and oxygen reducing platinized graphite felt cathodes (geometrical surface area of 79 cm^2) with a Nafion membrane as a separator. Sodium phosphate buffer (100 mM, pH 7.0) with 20 mM lactate was used as the anolyte and catholyte in MFCs 1. The second set of MFCs (MFCs 2) was filled with a 50 mM piperazine-N,N'-bis(2-ethanesulfonic acid), known as PIPES buffer (pH 7.0) as the anolyte and catholyte, and 5 mM lactate as the electron donor at the anode. Both MFC 1 and MFC 2 were inoculated with the same *Shewanella spp*. cultivated for 48 hours. Several variables associated with the construction and operation of these MFCs, such as formed biofilm, bacterial growth phase, number of cells, concentration of the fuel source, buffer type, internal resistance of the MFCs, operational mode, electrochemical method used for MFCs characterization as well as the systems final response were taken into account and processed through PCA (Fig. 2). The response of the systems was evaluated by two electrochemical methods, polarization and discharge measurements. The MFCs open circuit voltage (OCV), maximum current (I_{max}) and maximum power (P_{max}) were the electrochemical parameters used for data comparison. In the represented PCA one of the key parameters, the biofilm coverage, was determined quantitatively based on the SEM images of the anodes taken after the electrochemical measurements [26]. The SEM images were processed using Image J. The images were segmented and transformed into binary images. The lines were filtered and the uneven background was corrected. The covered area by bacteria was calculated in percent (Table 2). The same approach was applied for biofilm evaluation in the subsequent data sets and PCA analysis. The uncertainty of this procedure was estimated to be 5.3%.

Table 2: Biofilm coverage (%) calculated on the base of SEM images

	MFC 1	*MFC 2*
ANA 3	33.56	21.35
CN 32	7.57	27.18
MR 1	52.47	54.22
MR 4	34.59	7.83
MR 7	8.38	39.12
PV-4	2.11	16.04
SB2B	1.63	39.66
W3-18-1	8.18	36.52

According to the PCA, *Principal Component* 1 (PC 1) separated the MFC samples and responses into two major groups depending on the buffer type, which indicated that this was the main parameter determining the MFCs performance (Fig. 2). The influence of the buffer type was indirect. The PIPES buffer promoted biofilm formation in a batch mode, preferably for strains in a lag phase. The pronounced positive effect of the PIPES buffer on the biofilm coverage suggested that *Shewanella* biofilm structure and development strongly depended on the analyte content, despite the identical carbon source. As a result of the simultaneous influence of these factors, an increased electrochemical performance was observed.

Obviously, the amount of the biomass attached to the anode surface was not the major factor determining the electrochemical output of those systems since *Shewanella* MR 1 developed more dispersed biofilm, having higher coverage than the rest of the *Shewanella spp*. and at the same time, the MFCs inoculated with MR 1 strain were not the best performing systems in terms of power and current generation. The influence of several factors had to be considered. Such factors included the cathode OCP. Surprisingly, the cathode`s OCP was slightly higher in PIPES buffer than in phosphate, which also contributed to the higher operational characteristics of MFCs 2. As it was expected the MFCs having higher internal resistance ($R_{int.}$) showed decreased values for the maximum current and power outputs. The position of the lactate as a variable on the PCA biplot confirmed the observations made by other researchers that the lower carbon source concentration promotes the development of more dense and robust biofilms [8, 11]. Roy et al. showed that *Shewanella oneidensis MR-1* secretes riboflavin in carbon-limited conditions most likely as a stress response [8]; therefore, the increased electrochemical activity with limited carbon source may have been a result of riboflavin production.

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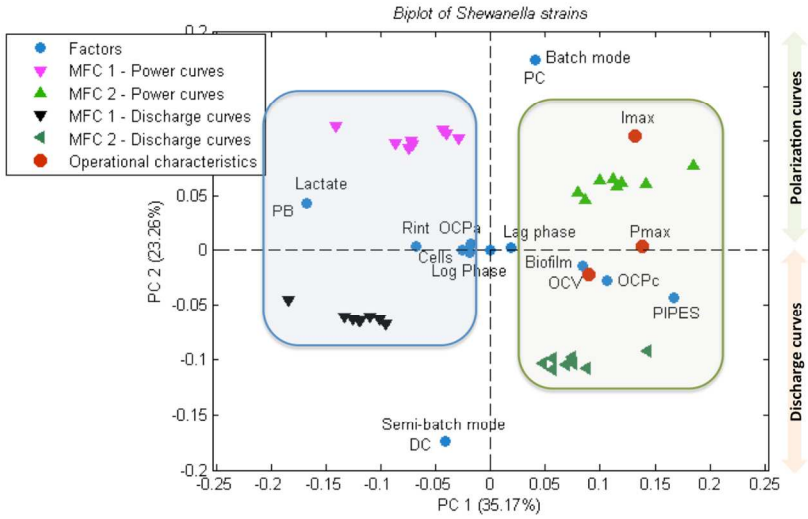

Figure 2: PCA biplot where scores (samples) and loadings (the variables) for MFCs with different *Shewanella* species are plotted on the first two components, where PC is power curves, DC – discharge curves, PB – phosphate buffer, R_{int} is the MFCs internal resistance, OCP_a is the open circuit potential of the anode and OCP_c is the OCP of the cathode.

Principal Component 2 (PC 2) separated MFCs according to the electrochemical method used to determine the MFC operational characteristics, with the polarization curves shown to have a positive effect. In general, the generated maximum power and current observed during the polarization experiments were higher than the currents recorded via discharge measurements due to the shortness of the experiments. The electrochemical methods used have a significant effect on the behavior of the system [59]. When a constant potential is applied (chronoamperometry measurement) the colonization rate, and growth yield of bacteria can change as a function of the applied potential. In the case of chronopotentiometry, a fixed current is applied to, or demanded from the system, which can stimulate or diminish a metabolic process [59]. Therefore, the bacterial metabolism and activity can be forced to provide the required current. When a fixed external resistance is applied, the evolution of the electrode's potential as a function of bacterial kinetics can be monitored. Bacterial communities adapt quickly to large fluctuations in the potential of the electron acceptor [59].

To avoid the influence of the electrochemical method used, the data were separated in two groups and processed separately. Group 1 contained only results from polarization curves (Fig. 3A) and Group 2, only from discharge measurements (Fig. 3B).

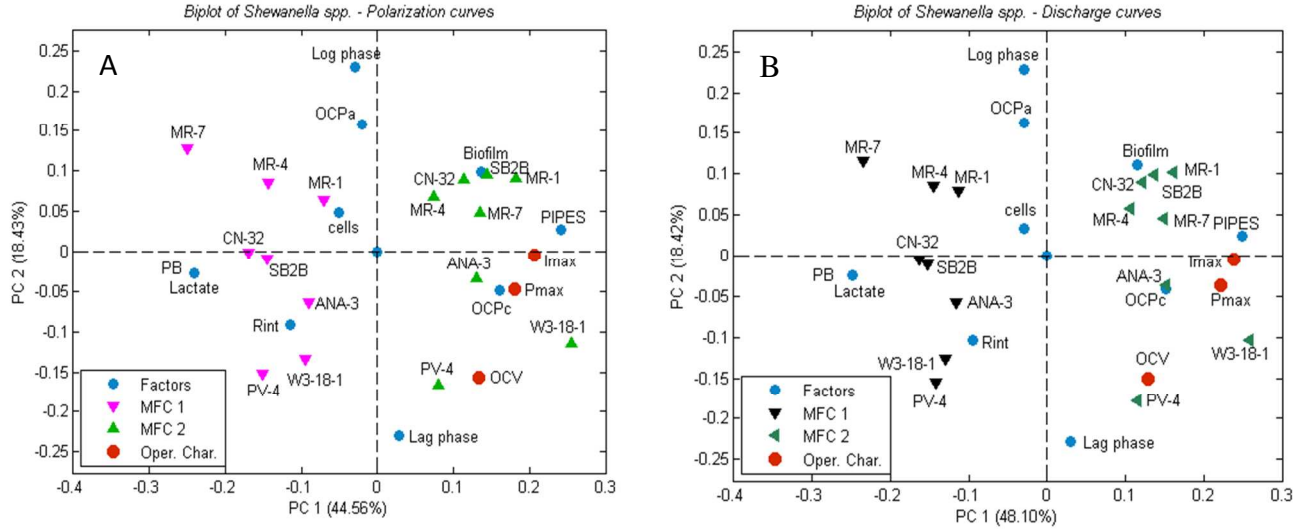

Figure 3: PCA biplot where scores (samples) and loadings (the variables) for MFCs with different *Shewanella* species are plotted on the first two components. A) Results from polarization curves and B) Results from discharge curves. PB means phosphate buffer, R_{int} is the MFCs internal resistance, OCP_a is the open circuit potential of the anode and OCP_c is the OCP of the cathode.

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After the influence of the electrochemical methods was eliminated, the position of the samples and the factors on the two biplots were notably identical. This indicated that the electrochemical behavior of the *Shewanella spp*. in the two MFC`s systems was not random. It was a result of the specific features of the species. All conclusions made based on Figure 2 (PCA analysis of different buffers) were clearly confirmed by Figure 3 and they hold true for both MFCs` systems, regardless of the electrochemical method used. Despite this observed consistency for the analyzed data set, it is important for researchers to bear in mind that directly comparing results obtained from different measurement procedures should be avoided.

3.1.2. Different anode designs with *Shewanella oneidensis MR-1*

In order to eliminate the influence of the specific bacterial features, data from MFCs with various designs but the same bacterial composition (*Shewanella oneidensis MR-1*) were collected from the literature [8, 14, 29, 40, 55, 56] and analyzed through PCA (Fig. 4). Only data relative to the anode factors and the anode electrochemical responses were taken into account (Table 2 Supplementary Information). Those factors were divided into six groups according to their main area of influence. The design parameter group included factors such as the anode compartment volume, anode geometrical surface area, anode resistance, electrode material and surface area/volume ratio. A second group was defined based on the mechanism of electron transfer. Several assumptions were made for this grouping including the conductivity of pili, the presence of riboflavin indicating MET, and electrochemical activity of biofilms. Determination of the electron transfer mechanism was done via empirical observation, such as the riboflavin concentration evaluated by spectrometry or cyclic voltammetry, the presence of pili, the stage of the developed biofilm.

The cultivation time, growth phase, biofilm coverage and the number of cells inoculated in the MFCs were combined as one group, named cultivation parameters. Another group was defined as the analyte set of parameters and included the buffer type and the carbon source. In all experiments, the analyte pH was 7.0, therefore, this factor was not included in the PCA. The current response from the MFCs anode compartments was collected by two main electrochemical methods, polarization [29, 40, 55] and discharge measurements [8, 40, 56], grouped as electrochemical methods, in three operational modes – batch [8, 14, 55, 56], semi-batch [40] and flow [29] mode – operational mode.

Based on the biplot (Fig. 4) a separation of the samples in three major groups based on the EET mechanism was observed. Group I has DET, group II mixed and group III mediated EET. PCA showed that the direct electron transfer is the preferential type of EET for the generation of higher current, followed by MET and mixed EET. In general, DET provides the lowest extracellular potential losses and, therefore, ensures high electron transfer efficiency [10]. Although DET is preferential, it cannot be observed without the existence of a biofilm. The same holds true for the mixed EET. Therefore, biofilm development is a necessary requirement for DET and mixed EET mechanisms, rather than MET, to occur. It was expected that the anodes using MET and DET simultaneously, referred here as mixed EET, would demonstrate higher electron transfer rates than *Shewanella* MET anodes and, as a result, increased current response would be recorded. The reason why this was not observed was the "artificial" nature of the *Shewanella* biofilms. Most of the anodes with mixed EET included in this study were created artificially by encapsulation of the bacterial culture in a silica-gel matrix [50, 56]. Thus, the amount of bacterial cells placed in direct contact with the electrode surface was fixed due to their entrapment in the silica matrix and at the same time, due to the presence of riboflavin in the introduced bacterial suspension. For these reasons, we assume mixed EET mechanism. This immobilization procedure provided the development of robust "artificial" biofilms but at the same time introduced diffusional limitations and decreased bacterial metabolic rates, which led to a decrease in the electrochemical anode performance in terms of generated current.

In addition to the EET mechanism, other important parameters showing a correlation to improved current response were: high geometrical surface area of the anodes, carbon felt as preferred electrode material, presence of fumarate in the anolyte, lactate as a carbon source, increased number of cells in exponential growth phase for MFCs inoculation and semi-batch mode of operation. The positive effect of the enhanced electrode surface area is a well-known and consistent observation [16, 60]. The anodes included in this study were based on carbon materials with different geometrical and real surface area, porosity, roughness, resistance, etc. Most likely the higher current densities observed when carbon felt anodes were used was due to the lowest material resistance (3.28 Ω) in comparison with the other anodes (10 – 45 Ω).

An interesting observation was that the presence of fumarate, which can be reduced by *Shewanella oneidensis*, had a positive effect on MFC current response. This most likely was a result of the enhanced biofilm formation when *Shewanella*`s had a soluble terminal electron acceptor to improve growth rates in the system. A similar conclusion was made by Rosenbaum et al. and separately by Biffinger et al [49, 61]. They observed that due to the higher levels of active biomass under aerobic conditions, an enhanced electrochemical performance of the system was recorded. Wu et al. showed that the presence of ferric citrate decreased the start up period and increased the current of a single-chamber MFC inoculated with *Shewanella oneidensis*. The researchers hypothesized that the higher biomass content in presence of Fe(III) indicated that Fe(III) plays a role in the biofilm formation most likely due to the ability of iron cations to regulate the biofilm development by iron acquisition-signaling genes [48]. Theoretically, the presence of multiple terminal electron acceptors, preferable for bacteria, would introduce a competition between the electrode and the introduced acceptor for the electrons released during the oxidation processes. It is possible that these observations are not contrary to the theory, and that the efficiency of the electron transfer to the electrode surface from each single bacteria was decreased, but as a result of the increased number of bacteria in direct contact with the electrode surface an increase in the overall electrochemical characteristics of those MFCs was observed.

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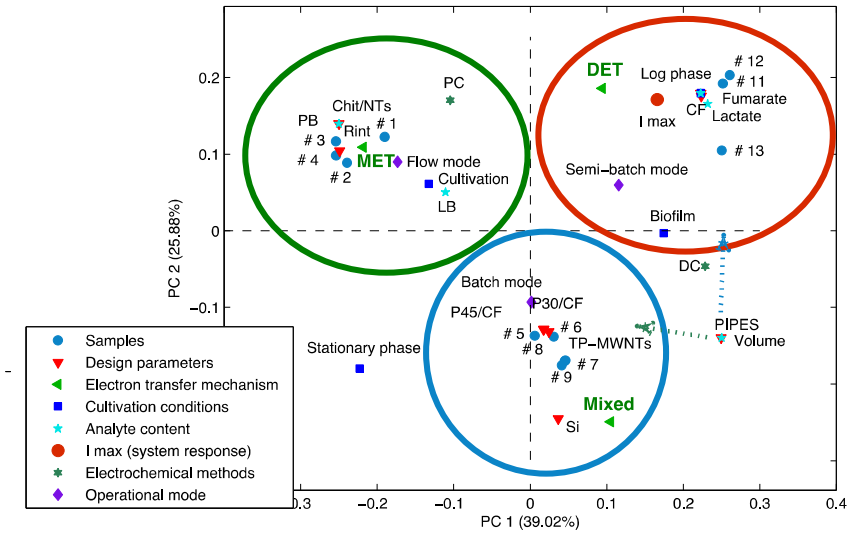

Figure 4: PCA biplot where scores (samples) and loadings (the variables) for different MFC`s anodes with *Shewanella oneidensis* MR-1 are plotted on the first two components. Acronyms: PB is for phosphate buffer, PC is polarization curve, DC – discharge curve, LB is Luria-Bertani medium, ChitNTs is chitosan-CNTs anode material, TP-MWNTs is multi-wall nanotubes grown on Toray paper, P45CF is modified graphite felt. The arrows show the contribution of the variables on the groups response.

An inoculation culture with a higher number of cells in exponential phase was found to be a key component for fast biofilm development. The growth phase at which the bacterial cells are harvested is an important parameter. If bacterial cells are in lag phase, they have not yet reached exponential growth and are still in adaptation and transition stage slowing down the process of their proliferation, development and biofilm formation. On the opposite end, if bacterial cells are in stationary or late stationary phase, the bacteria are decreasing in cell functions or are not as metabolically active. Based on this knowledge and the position of this parameter on the biplot (Fig. 4) it can be concluded that for *Shewanella MR-1* exponential growth phase is the most suitable for MFCs inoculation.

The last parameter that had to be evaluated was the mode of operation. According to the PCA analysis, both batch and semi-batch mode were appropriate for the development of robust and thick biofilms. The structure of the biofilm was clearly influenced by the physical growth environment and more precisely, the rate and direction of the flow [62]. At high flow rates and unidirectional flows, the influence of the increased sheared forces starts to play a significant role in the biofilm structure. The developed biofilms consist of elongated in the downstream direction cell clusters, different than the normal mushroom-shaped colonies. The stability of this biofilm is questionable, and a significant part of the upper layers cells are constantly detached from the biofilm. Therefore although the flow mode provides a constant supply of fuel and eliminates form the approximate environment the final product of metabolic processes, it is not the optimal mode of operation when MFCs are involved.

3.1.3. Different MFCs designs with *Shewanella oneidensis MR-1*

Often researchers may use a three-electrode half-cell setup for electrochemical characterization of a given electrode. As a result, important information regarding the impact of design parameters is missing. This is one of the reasons why observations made in such systems are not applicable to interpreting results from different cell designs and sizes. Very often the cathode of MFCs is used as a counter electrode without paying attention to its importance relative to the overall MFC operation. It should be noted that the electrochemical response of MFCs, as with all types of fuel cells, depends on, and is determined from, the properties of both the anode and the cathode. Despite the fact that the cathode is often not the biological part of the system, it is still an important component that should not be underestimated.

The data from *Shewanella oneidensis MR-1* MFCs with different designs and types of electrodes and electrode materials were collected and processed through a PCA analysis (Table 3 Supplementary Information and Fig. 5) [14, 15, 26, 29, 48, 49, 55, 61, 63-66]. According to PCA, the design parameters having the highest influence over the operational characteristics were: the surface area of the electrodes and the membrane, the volume of the compartments, carbon paper and carbon cloth as electrode materials and AMI or Nafion 117 membrane. Regarding the anodic solution and conditions, PIPES was again the preferred buffer with lactate or even glucose as carbon sources in aerobic conditions, or in the presence of ferric citrate after a longer adaptation period. The electrode areas and the compartment volumes had the highest impact on the maximum power, and the analyte content and anode material mostly impacted the maximum current.

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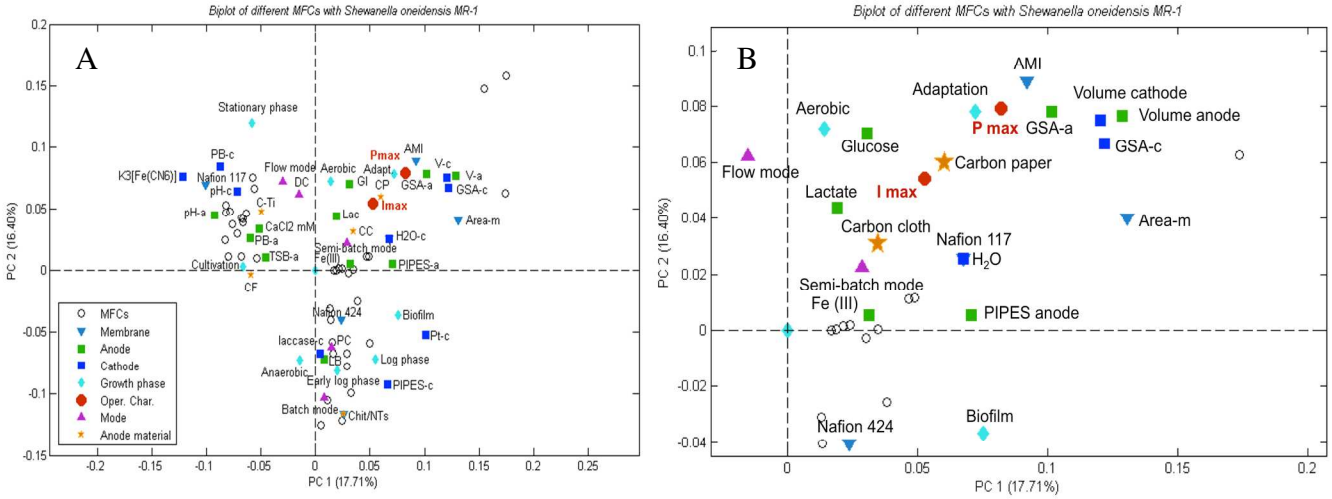

Figure 5: A) PCA biplot plotting loadings of the variables for different in construction *Shewanella MR-1* MFCs (scores) for the first two components; B) Zoom in upper right quadrant of the biplot.

Principal component 1 separates the variables into parameters having positive vs. negative impact on the MFCs current and power. The factors with positive impact when *Shewanella* MFCs were analyzed were: the electrodes and membrane surface area, volume of the compartments, developed biofilm, aerobic conditions, PIPES at the anode and the cathode, Pt at the cathode, adaptation period (longer), batch and semi-batch mode of operation, polarization curves as the electrochemical method, and inoculum source harvested at exponential growth phase. Additionally, the increase of the values of parameters with a negative effect, such as phosphate buffer at the anode and the cathode, flow mode of operation, stationary growth phase of the inoculated bacteria, higher pH in both of the compartments, anaerobic conditions, longer cultivation time, etc., led to a decrease of the generated from MFC current and power. For the parameters that have an expression of present/absent, such as which growth phase, what electrochemical method, EET mechanism, the position around PC1 determines if their presence is favorable/unfavorable for the system.

Given set of MFC results included in this analysis, those systems having large electrode and membrane areas, as well as larger compartment volumes, had the highest operational characteristics relative to the rest of parameters, which could have positive or negative effects according to PCA. This clearly showed that these three design parameters were the dominating factors when the necessity bacterial biofilm was developed.

In combination with PCA, the data was used to create a linear regression model via Principal Component Regression (PCR) analysis and predict the final response of a system having the same parameters but different values (Table 3). The values of the parameters were chosen based on the conclusions made by PCA of published results, such as large electrode and membrane surface area, large volumes of the two compartments, Pt at the cathode, exponential growth phase for the inoculated *Shewanella* culture, aerobic conditions in the presence of 10 mM ferric citrate, AMI membrane, operating in a semi-batch mode, when the maximum current and power were recorded by taking polarization curves.

Table 3: Components of optimized MFC and their values

	Lactate, mM	*Volume$_a$, cm^3*	*Volume$_c$, cm^3*	*Area$_{mem}$, cm^2*	*AMI-7001*	*GSA$_a$, cm^2*	*GSA$_c$, cm^2*	*Pt cathode*	*PIPES$_c$, mM*
Opt.	20	220	220	225	+	225	225	+	50
	Fe (III) mM	Carbon felt	Biofilm	Rint	pH$_a$	PIPES$_a$, mM	Cultivation, h	Early log phase	Semi-batch mode
Opt.	10	+	+	3.41	7.0	50	18	+	+
	PC	pH$_c$	Adaptation, h	Aerobic					
Opt.	+	7.0	24	+					

a – anode, c – cathode, h – hours, + utilization of the given factor

The components of the optimized MFC and their values were processed through PCR and Sample/Scores plots were created showing the predicted values of the generated maximum current and power (Fig. 6).

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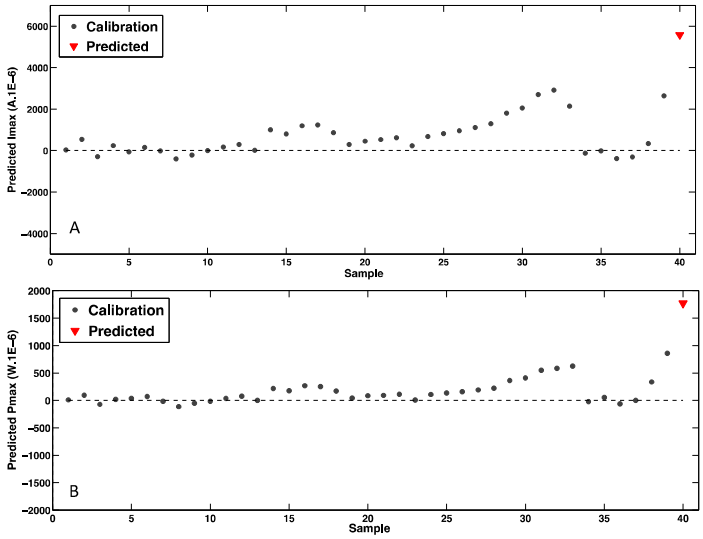

Figure 6: Sample/Scores plots for A) Imax and B) Pmax of the optimized MFC, determined by PCR.

The predicted magnitudes of the optimized MFC`s operational characteristics were notably higher than the scores data used for building the model, which supports the conclusions made by PCA. An experimental confirmation of the build model is required and will be performed as a part of future work.

3. 2. Uncertainty of Measurement Results

The expanded uncertainties of *Shewanella spp*. MFCs operational characteristics, internal resistance, optical density and number of cells inoculated represented in section 2.1.1. [26], were evaluated using results from at least three identical MFCs processed with Robust statistics. Only results from polarization measurements of the two systems were used for the uncertainty evaluation with the expanded uncertainties of the operational characteristics (*U (OCP)*, *U (Imax)* and *U (Pmax)*) as the main task of the uncertainty estimation. The uncertainty of the MFCs internal resistance *U (Rint)* and amount of inoculated cells *U (# cells)* were also calculated since these parameters were considered important for MFCs (Fig. 7).

A trend between uncertainties for MFCs` internal resistances, maximum generated current and power was observed. It was observed that the uncertainties for MFCs with high *U (Rint)* were also characterized with high *U (Imax)* and *U (Pmax)*. This was indicative for the significant role of the deviation in the MFCs internal resistance on the reproducibility of the MFCs` final output. No correlation was observed between the uncertainty of the number of cells used to inoculate the MFCs and the expanded uncertainties of the main operational characteristics, indicating that this parameter does not significantly contribute to irreproducibility of MFC performance results.

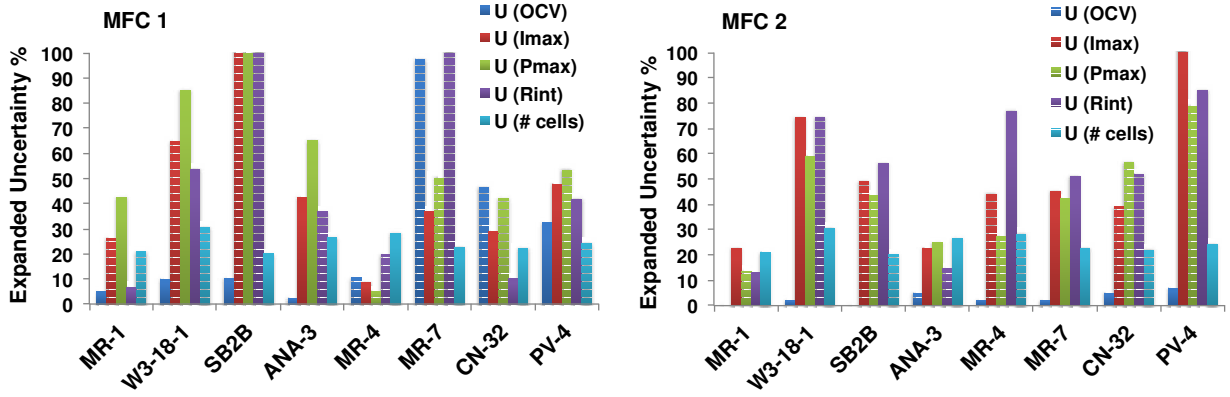

Figure 7: Expanded Uncertainties (%) of MFCs 1 and 2 operational characteristics (OCV, maximum current, maximum power) as well as the uncertainties of the systems internal resistances and amount of bacterial cells introduced in the systems [26]. The results for the expanded uncertainty estimation of P_{max} and I_{max} were taken from the performed polarization curves [26].

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In most of the studies included in these analyses, the optical density (OD) at 600 nm was used as a measure of cell density and corresponded to the amount of bacteria introduced in the MFC [1, 25, 26, 40, 48, 67]. One of the disadvantages of this approach is the heterogeneous nature of bacterial suspensions, which may lead to uneven bacterial loading [54]. This parameter is also used to trace out bacterial development and determine their growth stages. The PCA results suggested that the bacterial growth stage of the MFC inoculum is an important parameter for MFC performance. Therefore, we estimated the OD uncertainties (*U(OD)*) during *Shewanella spp*. development (Table 4 Supplementary Information and Fig. 8). Obviously *U (OD)* varies significantly with higher values at the beginning of the time dependent measurement. The uncertainties were in the range of 0.2 – 42.2 % with highest median value of 16.4 %.

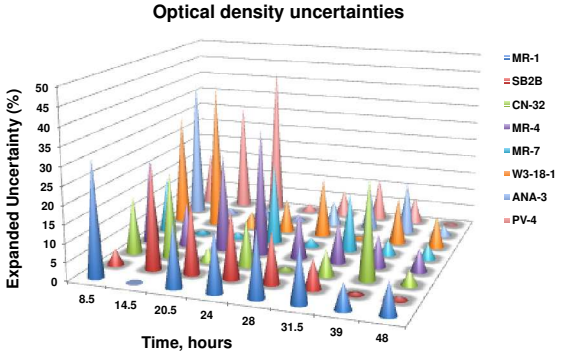

Figure 8: Variation of expanded uncertainties of $OD_{600\ nm}$ of *Shewanella spp*. during their cultivation and growth curves determination.

The median expanded uncertainties of the parameters shown in Figures 7 and 8 were calculated separately for each *Shewanella* strain, and the higher value was used as representative for the measurement procedure and the MFC design test (Table 4). The higher value was chosen in order not to underestimate the uncertainty.

Table 4: Expanded Uncertainties (%) of MFCs 1 and 2 operational characteristics (OCV, maximum current, maximum power) as well as the uncertainties of the systems internal resistances and amount of bacterial cells introduced in the systems [26]

sample/ U	*OCV*	*Imax*	*Pmax*	*Rint*	*# cells*	*OD*
MFC 1	10.81	40.32	52.55	39.80	23.86	16.40
MFC 2	2.60	45.20	43.27	54.44	23.86	16.40
Overall	10.81	45.20	52.55	54.44	23.86	16.40

In general the uncertainty of the open circuit voltage was 4-5 times lower in comparison with the uncertainties of the maximum current and power. The same observation was reported previously and can be explained by the lower number of factors influencing the system performance at open circuit when no current is flowing and the electrodes are in equilibrium [54]. The uncertainty of the *Imax* contains the *U (OCV)* and *U (Rint)* along with some other parameters. The *U (Pmax)* includes in addition both, the *U (OCV)* and *U (Imax)*. Therefore, the expanded uncertainties of the MFCs operational characteristics can be arranged in the following descending order: *U (Pmax) > U (Imax) > U (OCV)*.

The results forming these uncertainty evaluations were expanded to include previous uncertainty calculations of the ECSA and electrode resistance ($R_{el.}$) of carbon felt materials [40, 54] (Fig. 9).

The calculated expanded uncertainties for the MFCs` operational characteristics and some important parameters suggest two basic conclusions: i) the maximum current and power values had significant uncertainties; and ii) these uncertainties were due mostly to differences in the electrodes real surface area followed by the differences in the MFCs internal resistance (Fig. 9). These conclusions are not surprising since these parameters were determined by PCA to have a significant impact on MFC performance. Therefore, these analyses suggest that the problem of high uncertainty in MFC data must be addressed using a bottom-down approach to lower the uncertainty associated with deviations of the main design parameters (e.g. real surface area, electrode resistance, total internal resistance, etc.), and then focus on the biological component of the system.

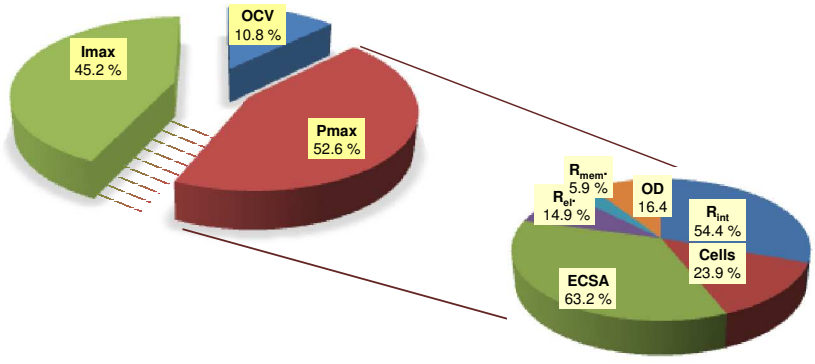

Figure 9: Uncertainties of the MFCs operational characteristics and several MFCs parameters. The uncertainty values in the pies are not normalized to 100%.

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4. Conclusions

The last decade of research has made significant strides toward practical applications; however, design improvements and operational optimization cannot be realized without equally considering engineering designs and biological interfacial reactions. This PCA/UMR approach enables a predictive capability to optimize biology and engineering simultaneously. Based on the performed statistical analysis the following conclusions were withdrawn:

i) The buffer type is a main parameter determining the MFCs performance;

ii) Lower carbon source concentration has a positive impact on MFCs performance, most likely due to promote the development of more dense and robust biofilms;

iii) The electrochemical methods used have a significant effect on the system behavior. Therefore, direct comparison of results obtained from different measurement procedures should be avoided;

iv) A separation of the samples in three major groups based on the EET mechanism was observed showing that the direct electron transfer is the preferential type of EET for the generation of higher current, followed by MET and mixed EET.

v) Biofilm development is a necessary requirement for DET and mixed EET mechanisms, rather than MET, to occur;

vi) Other important parameters showing a correlation to improved current response are: high geometrical surface area of the electrodes and membrane, high compartment volume, presence of soluble terminal electron acceptor in the anolyte (ferric citrate, fumarate, oxygen), increased number of cells in exponential growth phase for MFCs inoculation, longer adaptation period for bacteria and semi-batch mode of operation;

vii). The electrode areas and the compartment volumes had the highest impact on the maximum power, and the analyte content and anode material mostly impacted the maximum current;

viii) The uncertainty of the open circuit voltage of *Shewanella* MFCs was 4-5 times lower in comparison with the uncertainties of the maximum current and maximum power. The expanded uncertainties of the MFCs operational characteristics can be arranged in the following descending order: *U (Pmax) > U (Imax) > U (OCV)*.

ix) The uncertainties of the MFCs operational characteristics are due mostly to differences in the electrodes real surface area followed by the differences in the MFCs internal resistance.

As a summary, the PCA/UMR analysis of published MFC results suggest that better reproducibility of MFC performance can be achieved through improved design parameters. This approach is exactly opposite to the MFC optimization and scale up approach, which should start with improving the bacteria-electrode interactions and applying these findings to well-designed systems.

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Notes

[a] Chemical and Nuclear Engineering Department, Center for Emerging Energy Technologies, University of New Mexico, Albuquerque, NM 87131

[b] Microbial and Environmental Genomics, J. Craig Venter Institute 4120 Torrey Pines Road, San Diego, CA 92037

[c] Center for Bio/Molecular Sci. & Engr., Naval Research Laboratory, Washington, DC 20375

[d] Environmental Engineering and Science, Stanford University, Stanford, CA 94305

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