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Stability and reliability of anodic biofilms under different feedstock conditions: Towards microbial fuel cell sensors

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ABSTRACT

Stability and reliability of microbial fuel cell anodic biofilms, consisting of mixed cultures, were investigated in a continuously fed system. Two groups of anodic biofilm matured with different substrates, acetate and casein for 20–25 days, reached steady states and produced 80–87 μW and 20–29 μW consistently for 3 weeks, respectively. When the substrates were swapped, the casein-enriched group showed faster response to acetate and higher power output, compared to the acetate-enriched group. Also when the substrates were switched back to their original groups, the power output of both groups returned to the previous levels more quickly than when the substrates were swapped the first time. During the substrate change, both MFC groups showed stable power output once they reached their steady states and the output of each group with different substrates was reproducible within the same group. Community level physiological profiling also revealed the possibility of manipulating anodic biofilm metabolisms through exposure to different feedstock conditions.

1. Introduction

Microbial fuel cells (MFCs) are electrochemical devices that generate electricity via the metabolic activity of microorganisms, when breaking down a wide range of organic matter, including waste and wastewater. With the depleting fossil fuels and necessity of seeking alternative and sustainable technologies, the MFC technology has justifiably received increased attention from the scientific community. MFC applications primarily include electricity generation, wastewater treatment, hydrogen production and bio-sensing [13,19,23,24,29]. Further developments may include pollution treatment, resource recovery and powering of other remote equipment such as portable IT systems, environmental monitoring tools and medical support apparatus [15,17,18,25,31,42].

Amongst these applications, MFCs have gained attention as a new technique for microbial biosensors, which are analytical devices that combine biological recognition components with physicochemical signal transducers to convert the response to the analyte into a measurable signal [5,37,41]. Unlike optical transducers, producing optical properties such as adsorption, fluorescence, luminescence or refractive index, the transductive element of MFC biosensors converts biological responses to electrical signals [6]. MFC biosensors have competitive advantages including physical robustness, low cost, fast response, easy handling and portability [28,35]. So far, potential applications of MFC biosensors have focused mostly on environmental monitoring, which include BOD (biochemical oxygen demand) and VFA (volatile fatty acid) measurements, dissolved oxygen monitoring, and toxicity detection [1,4,7,21,39,47].

In an MFC, mono- or mixed species of microorganisms are attached to an anode in the form of biofilm (transducer) and the performance of an MFC biosensor greatly depends on the physiological state of the anodic biofilm, which is a dynamic system that changes its status upon the given conditions. Although many of the studies on MFC biosensors have employed single species due to their consistent response, using pure cultures is limited by the narrow range of utilisable substrates, which results in a rather narrow spectrum of substrate detection capacity in comparison with mixed cultures; this is in addition to the propensity of the mono-culture to get contaminated. It was reported that MFC biosensor produced lower maximum current (peak height) when real wastewater was tested compared to pure substrates such as acetate [9]. In this respect, using mixed cultures for MFC biosensors was suggested for the purpose of environmental monitoring [16].

Meanwhile, it is well understood that different substrates potentially have an impact on the structure and composition of the microbial community, which subsequently influences the MFC performance [14,20,22,30]. Furthermore and in addition to performance in terms of power generation and coulombic efficiency, the integral composition of the bacterial community enriched under a specific feedstock condition has a potential to acclimate to other substrates depending on the initial substrate type [3,48]. Therefore these various responses caused by different initial feedstock conditions could influence the selectivity, sensitivity

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and response time of an MFC biosensor. Further to these findings, it was successfully shown that a change of feedstock, after the initial maturing period with a starting feedstock, altered the MFC anode microbial composition [48]. However this particular study reported slow responses due to the relatively small anode electrode size (42 cm^2) compared to the working volume of the anode chamber (250 mL) and batch feeding operation (10–17 days for each feeding cycle). Continuous feeding operation could be a clearer and faster way to see the progress of the MFC anodic biofilm change. Continuous flow is also able to facilitate non-cumulative steady states as previously shown [27]. Bacterial cell metabolic adaptation would happen after repeated changes with exposure to a new substrate for prolonged periods, which could diminish the effect of initial carbon sources. A question still remains about how this transition would proceed in terms of biofilm metabolic activity and its power generating performance. Nevertheless, retaining a stable and reproducible response to analytes from anodic biofilms, is one of the key requirements when implementing the MFC technology to bio-sensing. The objective of this study was therefore to (1) understand the change of biofilm metabolic activity and power generating performance under different feedstock conditions, and (2) investigate the feasibility of obtaining stability and reliability of MFC anodic biofilms during the changes. For the current study, MFCs were fed continuously with two different substrates, acetate (carboxylic acid, monomer, non-fermentable) and casein (protein, polymer, hydrolysed into monomers). These two substrates were selected as exemplars of two completely different chemical compounds, in terms of their molecular structure, with one representing an accessible short-chain-sugar-based carbohydrate (acetate) and the other representing a more complex long-chain-sugar-based protein (casein).

2. Materials and methods

2.1. MFC design and operation

A total of 16 single-chamber MFCs with an anodic chamber volume of 6.25 mL as previously described [43], were used in this study. Plain carbon fibre veil electrodes (carbon loading: 30 g/m^2, PRF Composite Materials, UK) with 10 layers of 1 cm^2 (width: 1 cm, length: 1 cm) were used as anodes. A cation exchange membrane (CMI-7000, Membrane International, USA), 25 mm diameter, was placed between the anode and cathode frames. The cathode window was sealed with a punched circular Perspex sheet with 25 mm diameter in order to prevent the cathode from drying but still enabling oxygen contact from air. A hot-pressed activated carbon cathode electrode (with the same carbon fibre veil base as the anode) with a total macro surface area of 4.9 cm^2 was pressed onto the membrane. Stainless steel mesh was attached to the cathode to enhance current collection.

The MFCs were inoculated with activated sewage sludge supplied from the Wessex Water Scientific Laboratory (Saltford, UK). Prepared media with different sole carbon sources (acetate, casein, glutamine or glucose) were provided to the MFCs continuously at a flow rate of 1.89 mL/h using a 16-channel peristaltic pump (205 U, Watson Marlow, Falmouth, UK). The media contained the following ingredients: 3 mM PIPES buffer, 7.5 mM NaOH, 28 mM NH_4Cl, 1.34 mM KCl, 4.35 mM $NaH_2PO_4 \cdot H_2O$, 30 mM NaCl, 3 mM $MgCl_2 \cdot 6H_2O$, 6.8 μM CaCl2, Wolfe's vitamin solution, SL-10 trace element solution and COD value of 1150 ppm equivalent sole carbon source (1.48 g/L sodium acetate, 0.83 g/L casein, 1.17 g/L glutamine, 1.08 g/L glucose). Once the MFCs started producing >20 μW under an external load of 3 kΩ, the external load was changed to 1 kΩ and stayed the same throughout the study.

Occasional cleaning of the anode chambers was required when blockage in the anodic chamber inlets or outlets occurred due to bacterial overgrowth. Blockage was removed manually, after opening the MFC chambers, or was washed off by switching the media flow to a high rate (580 mL/h) for 2–3 min.

The experiment mainly consisted of three steps; (1) two groups of anodic biofilm from the same inoculum but matured with different substrates (acetate for group A and casein for group B) (2) once MFCs reached steady states in terms of power output and bacterial cell population (monitored in the perfusate), the two substrates were swapped (3) once new steady states were reached, the MFCs were switched back again to their original substrates, to monitor the microbial community response. Additionally a 3rd and 4th substrate, glutamin and glucose, were provided to both groups for 3 days each at the end of the study.

All chemical, biological and electrical measurements were conducted at least in triplicate and all experiments were carried out in a temperature controlled environment, at 22 ± 2 °C.

2.2. Biolog sample preparation and analysis

For studying biofilm metabolic pathway activity change, community level physiological profiling (CLPP) using Biolog AN plates (Biolog, Hayward, CA, USA) was employed. At the end of each stage, two MFCs from both groups were opened and one layer of the anodes was removed aseptically for the Biolog analysis. The anode sample was transferred into 40 mL of sterile phosphate buffered saline (PBS, Sigma-Aldrich, Dorset, UK) solution and re-suspended by rigorous vortex mixing for 3 min. The 96 wells of a Biolog AN plate were inoculated with 150 μL of each sample per well. Then the microplates were incubated anaerobically (10% CO_2 in oxygen free N_2) in a portable container at 30 °C to allow utilisation reactions to proceed along with tetrazolium colour changes, and the changes in colour intensity were measured at 590 nm every 24 h up to 120 h, using a Biolog Microstation in accordance with the Biolog operating protocol.

Average well colour development (AWCD) was calculated according to Garland and Mills [12], i.e., $AWCD = \sum(C - R) / n$ where C is the optical density of each well measured at 590 nm, R is the absorbance value of the control well (A1), and n is the number of substrates ($n = 95$). In order to compare a specific carbon source (acetate in this case) utilisation of the two groups at different stages, the raw difference data of the well containing acetic acid was divided by the AWCD of the plate, i.e. (C − R) / AWCD. As a measure of the degree of substrate utilisation (substrate richness) and diversity of extent of particular substrates utilisation (substrate evenness), the Shannon–Wiener index (SI) was used: $H = -\sum_{i=1}^{n} p_i(\ln p_i)$ where p_i is the proportion of a microbial activity on a particular substrate (OD_i) to the total microbial activity ($\sum OD_i$) and N is the number of substrates on a plate [10,45]. Plate readings at 24 h of inoculation were used to calculate AWCD and SI.

2.3. Indirect measurement of bacterial population

Viable counts were performed on non-selective nutrient agar (Oxoid, Basingstoke, UK) and the number of colony forming units per sample (cfu/mL for effluent and cfu/mm^2 for anodic biofilm) was calculated. A 1 mL volume of each sample was serially diluted to 10^{-6} and 100 μL from sample dilution 10^{-4}, 10^{-5} and 10^{-6} spread onto the non-selective recovery medium. All plates were incubated in an anaerobic cabinet (MK3 anaerobic workstation, Don Whitley, Shipley, UK) at 37 °C for 5 days.

The optical density at 600 nm wavelength of each undiluted 1 mL sample was measured using a spectrophotometer (model name: 6700, Jenway, Staffordshire, UK).

2.4. Polarisation measurement and power output calculations

Power output of the MFCs was monitored in real time in volts (V) against time using an ADC-24 Channel Data Logger (Pico Technology Ltd., Cambridgeshire, UK). Polarisation experiments were performed weekly by connecting a decade variable resistor box (Centrad Boite A Decades De Resistances DR07, ELC, France) between the anode and cathode

electrodes and changing the external resistance from 30 kΩ to 10 Ω in 5-minute intervals, after the MFCs had established a steady-state open circuit voltage at the start of the experiment. Maximum power output (P_{MAX}) and internal resistance (R_{INT}) were calculated from the power curves. The current (I) in amperes (A) was determined using Ohm's law.

2.5. Statistical analysis

In order to evaluate the influence of two MFC groups and substrates on the variation of power output, an ordinary two-way ANOVA analysis and an unpaired t-test were performed using GraphPad Prism 6 (GraphPad Software, California, USA). Power output data from 6 MFCs, each group during the last five days of stage 2 and 3, which were assumed as steady states, were averaged for every recording point (5-minute intervals) and superimposed. This resulted in 1440 data points each for the 4 different conditions (two groups and two substrates: group A-casein, group B-acetate, group A-acetate and group B-casein). Due to the large size of data set, a smaller data set ($n = 240$) was built by averaging 6 data points into 1 data point for each condition, which were sufficiently large to run a parametric test on these non-Gaussian data [32]. The unpaired t-test with Welch's correction was performed separately on group A in stage 2 and 3 (group A-casein and group A-acetate) pair and on group B in stage 2 and 3 (group B-acetate and group B-casein) pair, to measure any effect of the initial feedstock condition on the MFC response (power output) to the same analytes (substrates).

3. Results and discussion

3.1. System maintenance

Whilst running the 16 MFCs for over 3 months, catholyte accumulation occurred. Catholyte formation on the surface of the cathode electrode was first observed as droplets on the electrode and membrane surfaces, and then catholyte accumulated in the cathodic part to the extent that power output started to decrease. It is widely known that water is synthesised by the electrochemical reaction and electro-osmotic drag [33]. Although water is essential for the MFC operation, since it enables an electrolyte bridge between anolyte, membrane and the cathode, excessive water that is not appropriately removed is known to cause cathode flooding, which hinders the transfer of oxygen to the reactive site of the cathode [44]. Similar with other studies reporting catholyte formation in MFC cathodes, cathode flooding had a negative effect on power production [40,46]. However it did not seem to significantly affect power generation until a certain point, as illustrated in Fig 1. Water in the cathode formed gradually, but a significant voltage reduction was observed only when the water level had reached the very top of the cathodic part. The sudden voltage drop was therefore thought to be due to limited air exchange through the air holes, thus hindering the oxygen supply. When water was removed, power output instantly returned to its previous level. Alteration of the cathode design, such as with the addition of a drain channel for removing the accumulated water could resolve this.

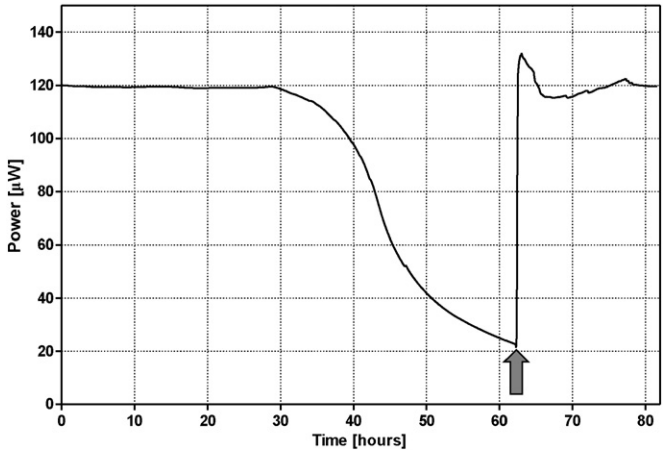

Fig. 1. Power output change during cathode flooding. The grey arrow indicates when water was removed from the cathodic part.

Once the MFCs had matured and showed stable performance under the given feedstock, blockage in the anodic chamber inlets or outlets was observed occasionally mainly due to the overgrowth of bacteria attached inside the anode chamber wall, suspended in the anolyte and the consequent increase of extracellular polymeric substance (EPS), which required periodic cleaning of the anodic chambers. Fig. 2 shows bacterial population changes of effluent and current generation during one cleaning cycle (8 days in this case). After cleaning, suspended cell numbers measured in OD_{600} and CFU initially dropped, then rose following an exponential curve whereas current production did not seem to be affected by the cleaning. Therefore suspended cells, which consisted of daughter cells from the anode electrode biofilm, daughter cells from the anode chamber wall biofilm, and planktonic cells did not have a great effect on electricity generation of the MFCs used in this work. This also suggests that the anodic biofilm on the electrode was relatively stable.

Voltage change measured every 5 min during the cleaning to remove build-up, which lasted for about 30 min, also supported this finding (Fig. 3). When the anolyte including suspended bacteria was removed by cleaning, the voltage output returned rapidly within 20–35 min to the levels observed before removing the planktonic bacteria.

It is generally accepted that two types of anodic electron transfer mechanisms exist in MFCs; direct electron transfer (DET) and mediated electron transfer (MET) [34,36]. Unlike DET that requires physical contact of bacterial cells to an anode electrode or other cells, MET can take place from a longer distance with the help of mobile electron shuttling compounds. In this case, even non-anodophiles existing within and/or outside of the anodic biofilm, can contribute to electricity generation of MFCs by producing endogenous redox mediators through their metabolic pathways.

Unlike batch fed MFCs with relatively large anolyte volume and small size anodes, this system had a small anolyte volume (5.75 mL) and fast feedstock supply rate for the reactor (1.89 mL/h; HRT = 3 h). Thus the proportion of suspended cells to anode attached cells was expected to be relatively small. Suspended cells could therefore either have a positive or a negative effect on current generation. If they produce extracellular mediators, this aids anodophiles to transfer electrons from the substrate to the anode. Alternatively some could break substrates down into smaller molecules that can be used by anodophiles to produce electricity, which is unlikely in the case of acetate since it is already a simple structured substrate. On the other hand, their consumption of substrate could result in deficiency of available substrate for anodophiles, which is again unlikely in this work, since the COD of feedstocks was relatively high. If the two scenarios co-exist, then the two opposite effects offset each other. In any case, these results indicated the absence of soluble electron mediators from the suspended cells or their negligible contribution to current generation. Overall it is safe to assume that the cells within the anodic biofilm matrix — rather than suspended bacteria or electron mediators produced by the bacteria — were the main contributors for the power generation in this system.

3.2. Power response to substrate change

Fig. 4 shows the power output profile of the two groups at each stage. At the first stage, group A fed with sodium acetate and group B fed with casein reached steady states 20–25 days after inoculation. Group A showed higher power generation (80–87 μW) than group B (20–29 μW) and this performance lasted for 3 weeks until the two substrates were swapped. Since casein requires digestive hydrolysis, the

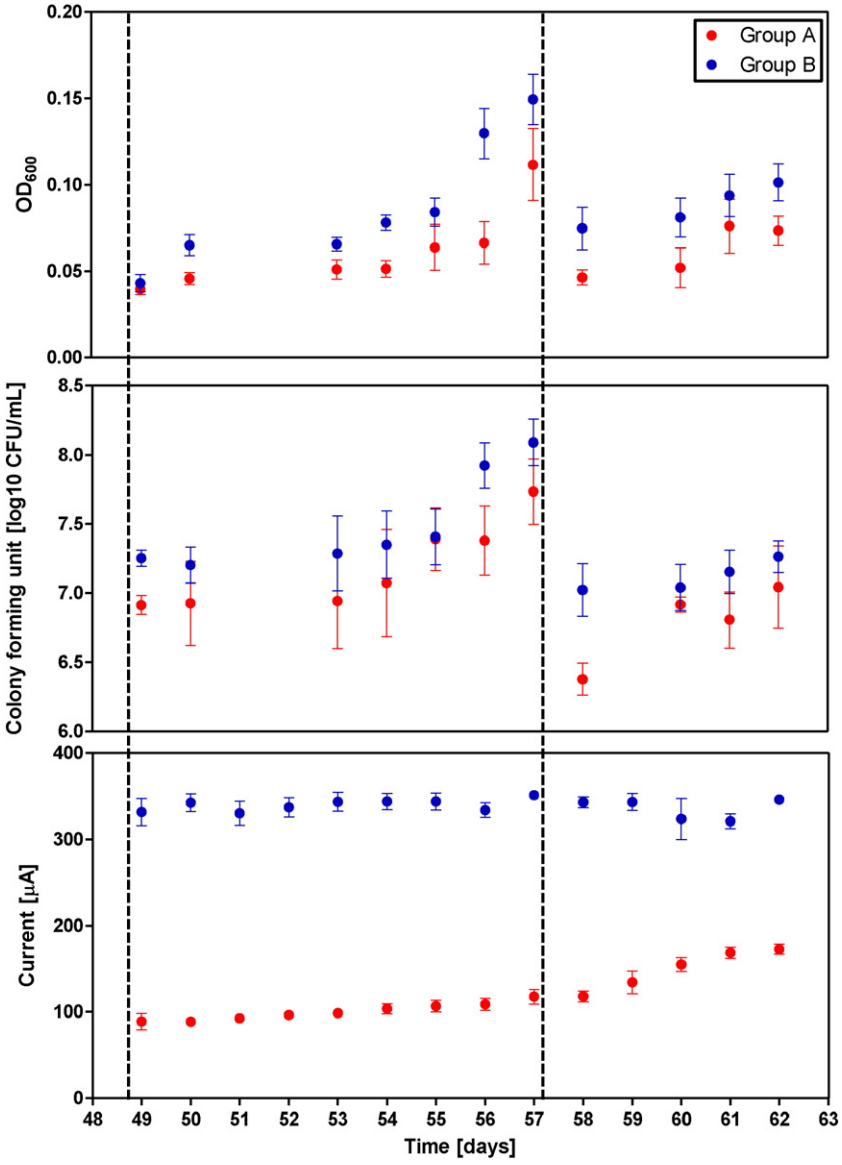

Fig. 2. OD_{600}, CFU of group A (acetate enriched group) and group B (casein enriched group) perfusate as bacterial population indicators and current generation of the two groups over the same period (n = 3). The dotted lines indicate when anodic chamber cleaning was carried out.

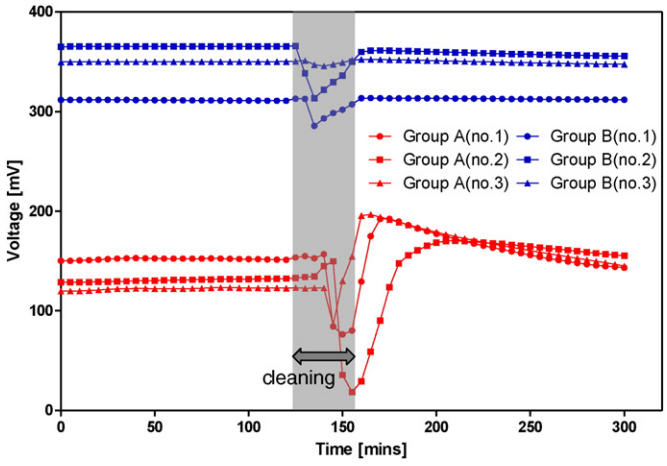

Fig. 3. Voltage change during anodic chamber cleaning and after. The grey area is when anodic chambers were cleaned. Data presented are from the 3 MFCs of each group separately.

low performance of casein fed MFCs could imply its consumption by non-electroactive species such as methanogens. Moreover, for most of microorganisms, it would be harder to utilise casein due to its complex molecular structure and composition, which needs to be broken down into monomers first. When the two groups of MFCs were supplied with different feedstocks, group B responded to the substrate change and reached the new steady state more quickly than group A, probably due to the readily biodegradable nature of acetate. Also group B produced higher power than group A when fed with acetate. This could be anticipated since similar responses were observed by others, where MFCs enriched with a more complex structured carbon source, showed better substrate versatility than when a simpler structured carbon source was provided [3]. The power output of group A steadily increased and reached a new steady state in the 3rd week after the first substrate swap. The slow power increase indicated that the group A biofilm needed to acclimate to casein by producing peptidolytic enzymes or encouraging other types of proteolytic bacteria. At the second steady state, Group A reached a similar power output level to that of group B when fed with casein, and this signified that group A was able to utilise casein to a similar extent as group B. Hence it was demonstrated that in a continuous flow system, MFCs could adapt to achieve the same level of

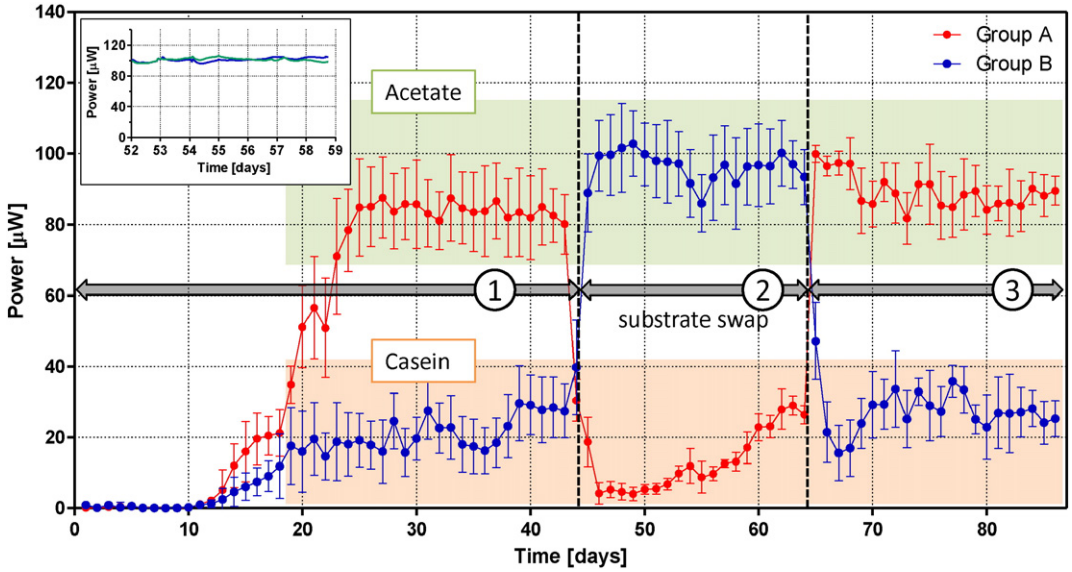

Fig. 4. Temporal profile of power generation from both groups at each experimental stage. Numbers in circles indicate the stages and stage 2 is when the two initial substrates were swapped. Data presented as average values of 6 cells of each group. Inset graph: Example of steady state power production from two MFCs of group B over a week (from day 52 to day 59).

utilisation as more complex carbon source enriched MFCs with time, even though they were adapted to metabolise a relatively simple carbon source from the maturing period. This suggests that the metabolic activity of anodic biofilm microbial communities changes dynamically with changes in nutrient conditions.

Another meaningful observation was the stable performance of the anodic biofilm in terms of power production (inset graph, Fig 4). As previously mentioned, continuous flow of substrates facilitates steady states and when steady states were reached, power output was very stable (100.9 ± 2.3 μW and 101.2 ± 2.2 μW for a week) until feedstock conditions were changed.

Three weeks later, when the two substrates were swapped again, the power output from both groups went back to their previous level within a week, which shows the resilience of the anodic biofilm. Again the group switched to acetate from casein responded faster than the other group, but this time group B reached a stable power production very close to the previous level in a much shorter period compared to the previous times for both group B and A. This result implies that the microbial community of the anodic biofilm 'acquired' the ability of utilising different carbon sources through exposure to different substrates.

Unlike single species, which switch their metabolic pathways by producing different enzymes under different feedstock conditions, a more complex response is expected when a large community of diverse microorganisms is involved. In the case where the main carbon source in the feedstock is changed, a single bacterial cell would switch its metabolic pathway by activating different enzymes if at all possible. Some cells may be inactive if they lack suitable enzymes for metabolising the given feedstock. In this case, other species that can utilise the given feedstock will dominate and in a microbial community, these shifts can take place simultaneously. However, from a higher level of complexity, the anodic biofilm community seemed to be able to acclimate to a new environment with a new substrate and this acclimation can be accelerated by exposure to diverse carbon sources.

The statistical results support the above findings. The ordinary two-way ANOVA test results indicate that 'substrate' accounted for 96.77% of the total variance ($P < 0.0001$), and 'group' accounted for 0.39% ($P < 0.0001$). The ANOVA test demonstrates that the level of power output produced by any of the two groups was highly dependent on the type of substrate, which shows great potential for accurate MFC biosensors. The unpaired t-tests carried out for two groups fed with the same substrate indicate that, i) there was an extremely significant effect ($P < 0.0001$) of the initial substrate on power output when either group A or B was fed with acetate (mean ± SEM of group A: 89.0 ± 0.6 μW, n = 1440; mean ± SEM of group B: 96.8 ± 0.4 μW, n = 1440; difference between means: 7.8 ± 0.7 μW), and ii) there was a moderately significant effect ($P = 0.0034$) of the initial substrate on power output when fed with casein (mean ± SEM of group A: 25.2 ± 0.2 μW, n = 1440; mean ± SEM of group B: 26.0 μW ± 0.2, n = 1440; difference between means: 0.7 μW ± 0.2). Therefore exposure to a specific substrate (initial substrate in this case) has to be taken into account when implementing MFCs as biosensors since it could affect significantly on power output.

Substrates had a significant effect on the MFC performance, not only in terms of power output but also in terms of internal resistance, which affected P_{MAX} as depicted in Fig. 5. During the 6th week of this experiment, before the first substrate swap, P_{MAX} of an MFC belonging to group A was 215.8 μW then it dropped to 67.7 μW, when the main carbon source in the feedstock was changed from acetate to casein. At the same time, R_{INT} increased to 3 kΩ from 500 Ω. This new performance level returned to the previous level, after switching back to its initial carbon source (acetate) during the 10th week. In the case of group B, the opposite behaviour was observed. P_{MAX} rose from 137.5 μW to 242.6 μW, whilst R_{INT} dropped from 1 kΩ to 500 Ω with the first substrate change. As seen in the temporal profile of power generation (Fig 4), power curves clearly presented that the power performance of group A improved each week whereas there was no significant change in group B during the second stage (7–9th week).

At the end of this line of work, a 3rd and a 4th substrate, glutamine and glucose (both equivalent to COD 1125 ± 31), were supplied to the two groups for 3 days in order to investigate how both groups would respond to a different substrate that was not previously supplied. There was no significant difference between the two groups when they were fed with glutamine (130.9 ± 4.6 μW for group A and 122.5 ± 6.8 μW for group B), however group A showed higher output (78.5 ± 12.4 μW) than group B (56.3 ± 3.8 μW) when glucose was provided. Although the reason for the different substrate specificity is unclear, group A appeared to have a higher affinity to glucose. Considering that both groups showed similar level of diverse substrate utilisation (section C), it is unlikely that this difference was caused by the presence of more complex microbial community consisting of diverse anodophiles or their syntrophic bacteria in group A. On the other hand, the molecular structure of glucose is closer to that of acetate than casein, thus it might have been preferred more by group A for the relatively short time of 3 days.

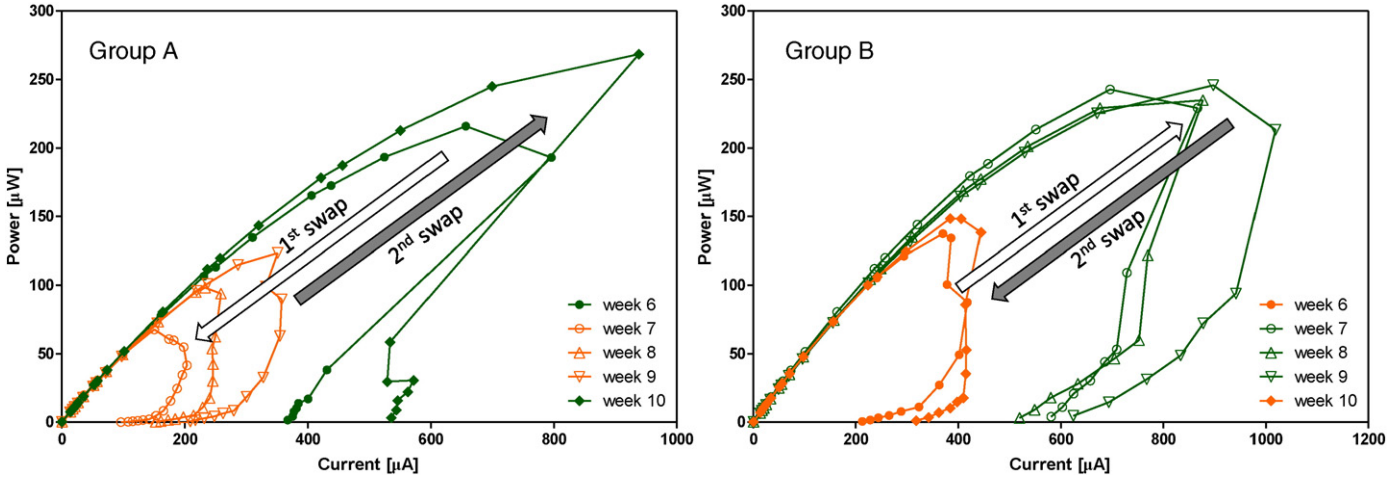

Fig. 5. Power curves of two individual MFCs of both groups between the 6th and 10th week. The white and grey arrows indicate the power curve change after the first and second substrate swap respectively. A single MFC from each group was chosen instead of average values of multi MFCs for the sake of clarity.

Other studies investigating possible application of MFCs as a BOD or COD biosensor demonstrated a linear correlation between MFC electrical output and BOD/COD values of water samples with BOD up to 350 ppm or COD up to 500 ppm [7]. Although the current study had different conditions such as continuous feeding mode and relatively high COD concentration, different substrates resulted in different levels of power output, which verifies the importance of anodic biofilm metabolic activity. In general, field samples are a mixture of various types of organic matter. Thus it is likely that even the same COD value of field samples can result in different output levels from an MFC sensor depending on the particular composition of organic compounds in samples. In order to avoid this kind of error, anodic biofilms need to be fully adapted to the nature of samples before being implemented, since a different microbial community will have a different substrate affinity, as shown in this study.

3.3. Community level physiological profiling

CLPP, introduced by Garland and Mills [12], was originally developed for identifying pure cultures of bacteria based on their metabolic properties. This technique has also been used for heterotrophic microbial communities especially for soil bacteria from different habitats [2,8,10,26] since the profile of substrate utilisation provides information on the microbial metabolic capabilities and hence on the functional diversity of a microbial community.

Initially it was expected that there would be a noticeable difference between the two groups, in terms of the number of substrates used (substrate utilisation pattern) after the first stage, once the anodic biofilm fully matured under different feedstock conditions. However they both exhibited the ability to utilise a wide range of substrates throughout the study due to the microbial diversity in the inoculum. After 120 h of incubation, all the wells except E8 (itaconic acid) turned purple, which indicates that over 90 substrates on the Biolog AN plate could be used by the microbial community of both anodic biofilms.

Fig. 6A shows the Shannon–Wiener index (SI) of the anodic biofilm samples at different stages of this study. CLPP is sensitive to inoculum density [26,38] thus it was suggested to compare different data sets with approximately the same AWCD [11]. AWCD values of group A and group B for the same sampling point of SI (24 h incubation) were 0.51 ± 0.10 and 0.50 ± 0.06 respectively, which were thought to be valid for statistical analyses. Samples were measured twice at the first stage (stages 1–1 and 1–2 shown in the graph) with 3-week intervals, in order to verify the steady states of the anodic biofilm. The result shows that metabolic functional diversity of both groups increased as the work progressed, which indicates a wider range of substrates could be utilised in later stages. Meanwhile, the SI of the group B anodic biofilm was slightly higher than that of group A throughout. This could be explained by the complex mixed consortium of diverse anodophiles and their syntrophic bacteria as a result of the production of various amino acids during the degradation of casein, which resulted in a wider substrate specificity of the group B biofilm than group A. This wider substrate specificity (but lower electricity generating performance of the group B) also supports the possibility of more antagonistic metabolic pathways existing in group B than group A. However, the difference between group B and A was not significant, and the SI values of both groups were very close to each other at the 3rd stage. Although it is not possible to see the anodic biofilm microbial community composition like with other molecular assay based methods, such as denaturing/temperature gradient gel electrophoresis (DGGE/TGGE), CLPP allows full anodic biofilm viewing as a whole, and it showed that potential metabolic diversity had been enhanced. The results demonstrate the

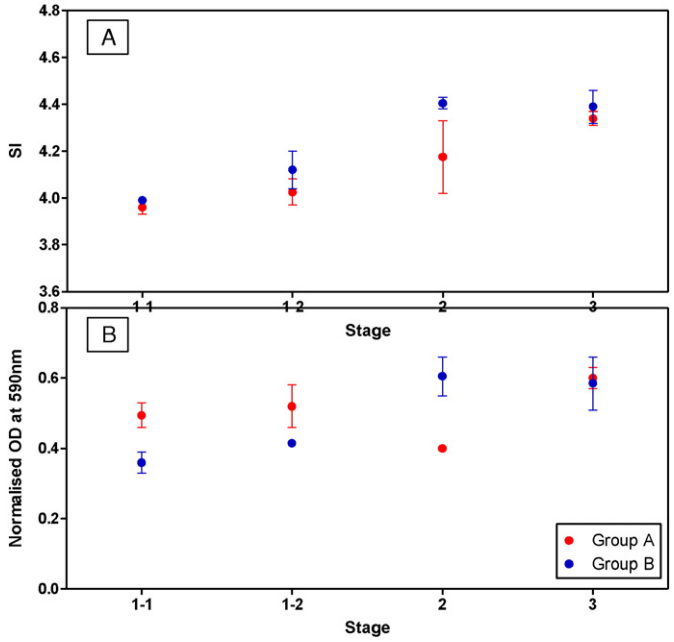

Fig. 6. Biolog data analysis at different stages (A) Shannon–Wiener index (SI) of anodic biofilm samples (n = 2). (B) normalised OD values of the Biolog AN plate well containing acetic acid (well no. E2) (n = 2).

possibility of manipulation of physicochemical conditions, e.g. exposure to different feedstocks to encourage biofilm adaptation.

Another noteworthy finding is that the affinity to certain substrates could change even after the anodic biofilm has fully established. Normalised OD values of the Biolog AN plate well, which contained acetic acid, changed at each stage as shown in Fig. 6B. During the biofilm maturation period (stage 1) group A showed higher OD than group B, which signifies higher affinity to acetic acid as a sole carbon source. This was expected, since the anodic biofilm of group A was initially enriched with acetate. At stage 2 when the two main carbon sources were swapped, the substrate affinity of group B to acetic acid increased whereas the affinity of group A declined, which resulted in a reverse pattern to the previous stage. This pattern change happened once again when the substrates were switched back to their original substrate conditions.

4. Conclusions

In this study, MFC anode biofilms were exposed to different feedstock conditions (acetate and casein based) at each stage of experimental work, in order to investigate changes in anodic biofilm dynamics in terms of power generating performance and metabolic activity. The MFCs in continuous feeding mode showed a stable power output once new steady states had been reached at all stages. Also the similar level of output when fed with the same feedstock demonstrated the reliability of MFCs as a biosensor. The choice of using these substrates revealed the different dynamics of the microbial communities adjusting to the two distinctly different substrates, which can be very useful in a biosensor application context, where the MFC biosensing system is expected to be exposed to a wide range of different substrates of variant complexity. CLPP results showed that the metabolic functional diversity of anodic biofilms increased through exposure to different feedstock conditions. Stability and reliability of the anodic biofilm presented in this study are worthy of further investigation for practical implementation of the MFC technology.

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References

[1] X.C. Abrevaya, N.J. Sacco, M.C. Bonetto, A. Hilding-Ohlsson, E. Cortón, Analytical applications of microbial fuel cells. Part II: toxicity, microbial activity and quantification, single analyte detection and other uses, Biosens. Bioelectron. 63 (2015) 591–601, http://dx.doi.org/10.1016/j.bios.2014.04.053.
[2] D.a. Bossio, M.S. Girvan, L. Verchot, J. Bullimore, T. Borelli, A. Albrecht, K.M. Scow, A.S. Ball, J.N. Pretty, A.M. Osborn, Soil microbial community response to land use change in an agricultural landscape of western Kenya, Microb. Ecol. 49 (2005) 50–62, http://dx.doi.org/10.1007/s00248-003-0209-6.
[3] K.-J. Chae, M.-J. Choi, J.-W. Lee, K.-Y. Kim, I.S. Kim, Effect of different substrates on the performance, bacterial diversity, and bacterial viability in microbial fuel cells, Bioresour. Technol. 100 (2009) 3518–3525, http://dx.doi.org/10.1016/j.biortech.2009.02.065.
[4] I.S. Chang, J.K. Jang, G.C. Gil, M. Kim, H.J. Kim, B.W. Cho, B.H. Kim, Continuous determination of biochemical oxygen demand using microbial fuel cell type biosensor, Biosens. Bioelectron. 19 (2004) 607–613, http://dx.doi.org/10.1016/S0956-5663(03)00272-0.
[5] S.F. D'Souza, Microbial biosensors, Biosens. Bioelectron. 16 (2001) 337–353, http://dx.doi.org/10.1016/S0956-5663(01)00125-7.
[6] C. Dai, S. Choi, Technology and applications of microbial biosensor, Open J. Appl. Biosens. 2 (2013) 83–93, http://dx.doi.org/10.4236/ojab.2013.23011.
[7] M. Di Lorenzo, T.P. Curtis, I.M. Head, K. Scott, A single-chamber microbial fuel cell as a biosensor for wastewaters, Water Res. 43 (2009) 3145–3154, http://dx.doi.org/10.1016/j.watres.2009.01.005.
[8] S. El Fantroussi, L. Verschuere, W. Verstraete, E.M. Top, Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles, Appl. Environ. Microbiol. 65 (1999) 982–988.
[9] Y. Feng, W.F. Harper, Biosensing with microbial fuel cells and artificial neural networks: laboratory and field investigations, J. Environ. Manag. 130 (2013) 369–374, http://dx.doi.org/10.1016/j.jenvman.2013.09.011.
[10] M. Frąc, K. Oszust, J. Lipiec, Community level physiological profiles (CLPP), characterization and microbial activity of soil amended with dairy sewage sludge, Sensors 12 (2012) 3253–3268, http://dx.doi.org/10.3390/s120303253.
[11] J.L. Garland, Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization, Soil Biol. Biochem. 28 (1996) 213–221, http://dx.doi.org/10.1016/0038-0717(95)00112-3.
[12] J.L. Garland, A.L. Mills, Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization, Appl. Environ. Microbiol. 57 (1991) 2351–2359.
[13] J. Greenman, A. Gálvez, L. Giusti, I. Ieropoulos, Electricity from landfill leachate using microbial fuel cells: comparison with a biological aerated filter, Enzym. Microb. Technol. 44 (2009) 112–119, http://dx.doi.org/10.1016/j.enzmictec.2008.09.012.
[14] P.T. Ha, B. Tae, I.S. Chang, Performance and bacterial consortium of microbial fuel cell fed with formate, Energy Fuel 22 (2008) 164–168, http://dx.doi.org/10.1021/ef700294x.
[15] Y. Han, C. Yu, H. Liu, A microbial fuel cell as power supply for implantable medical devices, Biosens. Bioelectron. 25 (2010) 2156–2160, http://dx.doi.org/10.1016/j.bios.2010.02.014.
[16] M.-C. Hsieh, Y.-C. Chung, Measurement of biochemical oxygen demand from different wastewater samples using a mediator-less microbial fuel cell biosensor, Environ. Technol. 35 (2014) 2204–2211, http://dx.doi.org/10.1080/09593330.2014.898700.
[17] L. Huang, X. Chai, S. Cheng, G. Chen, Evaluation of carbon-based materials in tubular biocathode microbial fuel cells in terms of hexavalent chromium reduction and electricity generation, Chem. Eng. J. 166 (2011) 652–661, http://dx.doi.org/10.1016/j.cej.2010.11.042.
[18] I.A. Ieropoulos, P. Ledezma, A. Stinchcombe, G. Papaharalabos, C. Melhuish, J. Greenman, Waste to real energy: the first MFC powered mobile phone, Phys. Chem. Chem. Phys. 15 (2013) 15312–15316, http://dx.doi.org/10.1039/c3cp52889h.
[19] I. Ieropoulos, J. Greenman, C. Melhuish, Urine utilisation by microbial fuel cells; energy fuel for the future, Phys. Chem. Chem. Phys. 14 (2012) 94–98, http://dx.doi.org/10.1039/c1cp23213d.
[20] S. Jung, J.M. Regan, Comparison of anode bacterial communities and performance in microbial fuel cells with different electron donors, Appl. Microbiol. Biotechnol. 77 (2007) 393–402, http://dx.doi.org/10.1007/s00253-007-1162-y.
[21] A. Kaur, J.R. Kim, I. Michie, R.M. Dinsdale, A.J. Guwy, G.C. Premier, Microbial fuel cell type biosensor for specific volatile fatty acids using acclimated bacterial communities, Biosens. Bioelectron. 47 (2013) 50–55, http://dx.doi.org/10.1016/j.bios.2013.02.033.
[22] G.T. Kim, G. Webster, J.W.T. Wimpenny, B.H. Kim, H.J. Kim, A.J. Weightman, Bacterial community structure, compartmentalization and activity in a microbial fuel cell, J. Appl. Microbiol. 101 (2006) 698–710, http://dx.doi.org/10.1111/j.1365-2672.2006.02923.x.
[23] J.R. Kim, G.C. Premier, F.R. Hawkes, J. Rodríguez, R.M. Dinsdale, A.J. Guwy, Modular tubular microbial fuel cells for energy recovery during sucrose wastewater treatment at low organic loading rate, Bioresour. Technol. 101 (2010) 1190–1198, http://dx.doi.org/10.1016/j.biortech.2009.09.023.
[24] M. Kim, S.M. Youn, S.H. Shin, J.G. Jang, S.H. Han, M.S. Hyun, G.M. Gadd, H.J. Kim, Practical field application of a novel BOD monitoring system, J. Environ. Monit. 5 (2003) 640–643.
[25] C. Knight, K. Cavanagh, C. Munnings, T. Moore, K.Y. Cheng, A.H. Kaksonen, Application of Microbial Fuel Cells to Power Sensor Networks for Ecological Monitoring, in: S.C. Mukhopadhyay, J.-A. Jiang (Eds.), Wireless Sensor Networks and Ecological Monitoring, Smart Sensors, Measurement and Instrumentation, Springer Berlin Heidelberg, Berlin, Heidelberg 2013, pp. 151–178, http://dx.doi.org/10.1007/978-3-642-36365-8.
[26] A. Konopka, L. Oliver, R.F. Turko Jr., The use of carbon substrate utilization patterns in environmental and ecological microbiology, Microb. Ecol. 35 (1998) 103–115, http://dx.doi.org/10.1007/s002489900065.
[27] P. Ledezma, J. Greenman, I. Ieropoulos, Maximising electricity production by controlling the biofilm specific growth rate in microbial fuel cells, Bioresour. Technol. 118 (2012) 615–618, http://dx.doi.org/10.1016/j.biortech.2012.05.054.
[28] S.A. Lee, Y. Choi, S. Jung, S. Kim, Effect of initial carbon sources on the electrochemical detection of glucose by gluconobacter oxydans, Bioelectrochemistry 57 (2002) 173–178, http://dx.doi.org/10.1016/S1567-5394(02)00115-9.
[29] H. Liu, H. Hu, J. Chignell, Y. Fan, Microbial electrolysis: novel technology for hydrogen production from biomass, Biofuels 1 (2010) 129–142.
[30] Z. Liu, J. Liu, S. Zhang, Z. Su, Study of operational performance and electrical response on mediator-less microbial fuel cells fed with carbon- and protein-rich substrates, Biochem. Eng. J. 45 (2009) 185–191, http://dx.doi.org/10.1016/j.bej.2009.03.011.
[31] C. Melhuish, I. Ieropoulos, J. Greenman, I. Horsfield, Energetically autonomous robots: food for thought, Auton. Robot. 21 (2006) 187–198, http://dx.doi.org/10.1007/s10514-006-6574-5.
[32] H. Motulsky, Intuitive Biostatistics: a Nonmathematical Guide to Statistical Thinking, Oxford University Press, 2010.
[33] D. Natarajan, T. Van Nguyen, Three-dimensional effects of liquid water flooding in the cathode of a PEM fuel cell, J. Power Sources 115 (2003) 66–80, http://dx.doi.org/10.1016/S0378-7753(02)00624-9.
[34] K. Rabaey, J. Rodríguez, L.L. Blackall, J. Keller, P. Gross, D. Batstone, W. Verstraete, K.H. Nealson, Microbial ecology meets electrochemistry: electricity-driven and driving communities, ISME J. 1 (2007) 9–18, http://dx.doi.org/10.1038/ismej.2007.4.
[35] K.R. Rogers, Recent advances in biosensor techniques for environmental monitoring, Anal. Chim. Acta 568 (2006) 222–231, http://dx.doi.org/10.1016/j.aca.2005.12.067.

[36] U. Schröder, Anodic electron transfer mechanisms in microbial fuel cells and their energy efficiency, Phys. Chem. Chem. Phys. 9 (2007) 2619–2629, http://dx.doi.org/10.1039/b703627m.
[37] L. Su, W. Jia, C. Hou, Y. Lei, Microbial biosensors: a review, Biosens. Bioelectron. 26 (2011) 1788–1799, http://dx.doi.org/10.1016/j.bios.2010.09.005.
[38] C. Viti, L. Giovannetti, Characterization of cultivable heterotrophic bacterial communities in Cr-polluted and unpolluted soils using Biolog and ARDRA approaches, Appl. Soil Ecol. 28 (2005) 101–112, http://dx.doi.org/10.1016/j.apsoil.2004.07.008.
[39] X. Wang, N. Gao, Q. Zhou, Concentration responses of toxicity sensor with Shewanella oneidensis MR-1 growing in bioelectrochemical systems, Biosens. Bioelectron. 43 (2013) 264–267, http://dx.doi.org/10.1016/j.bios.2012.12.029.
[40] S. Wilkinson, "Gastrobots" — benefits and challenges of microbial fuel cells in food powered robot applications, Auton. Robot. 9 (2000) 99–111, http://dx.doi.org/10.1023/A:1008984516499.
[41] X. Xu, Y. Ying, Microbial biosensors for environmental monitoring and food analysis, Food Rev. Int. 27 (2011) 300–329, http://dx.doi.org/10.1080/87559129.2011.563393.
[42] J. You, J. Greenman, C. Melhuish, I. Ieropoulos, Electricity generation and struvite recovery from human urine using microbial fuel cells, J. Chem. Technol. Biotechnol (2015)http://dx.doi.org/10.1002/jctb.4617 (n/a–n/a).
[43] J. You, C. Santoro, J. Greenman, C. Melhuish, P. Cristiani, B. Li, I. Ieropoulos, Microporous layer (MPL)-based anode for microbial fuel cells, Int. J. Hydrog. Energy 39 (2014) 21811–21818, http://dx.doi.org/10.1016/j.ijhydene.2014.07.136.
[44] L. Yu, W. Chen, M. Qin, G. Ren, Experimental research on water management in proton exchange membrane fuel cells, J. Power Sources 189 (2009) 882–887, http://dx.doi.org/10.1016/j.jpowsour.2009.01.037.
[45] J. Zak, M. Willig, D. Moorhead, H. Wildman, Functional diversity of microbial communities: a quantitative approach, Soil Biol. Biochem. 26 (1994) 1101–1108, http://dx.doi.org/10.1016/0038-0717(94)90131-7.
[46] F. Zhang, G. Chen, M.a. Hickner, B.E. Logan, Novel anti-flooding poly(dimethylsiloxane) (PDMS) catalyst binder for microbial fuel cell cathodes, J. Power Sources 218 (2012) 100–105, http://dx.doi.org/10.1016/j.jpowsour.2012.06.088.
[47] Y. Zhang, I. Angelidaki, A simple and rapid method for monitoring dissolved oxygen in water with a submersible microbial fuel cell (SBMFC), Biosens. Bioelectron. 38 (2012) 189–194, http://dx.doi.org/10.1016/j.bios.2012.05.032.
[48] Y. Zhang, B. Min, L. Huang, I. Angelidaki, Electricity generation and microbial community response to substrate changes in microbial fuel cell, Bioresour. Technol. 102 (2011) 1166–1173, http://dx.doi.org/10.1016/j.biortech.2010.09.044.