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Dynamic changes in the microbial community composition in microbial fuel cells fed with sucrose

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Abstract The performance and dynamics of the bacterial communities in the biofilm and suspended culture in the anode chamber of sucrose-fed microbial fuel cells (MFCs) were studied by using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified partial 16S rRNA genes followed by species identification by sequencing. The power density of MFCs was correlated to the relative proportions of species obtained from DGGE analysis in order to detect bacterial species or taxonomic classes with important functional role in electricity production. Although replicate MFCs showed similarity in performance, cluster analysis of DGGE profiles revealed differences in the evolution of bacterial communities between replicate MFCs. No correlation was found between the proportion trends of specific species and the enhancement of power output. However, in all MFCs,

putative exoelectrogenic denitrifiers and sulphate-reducers accounted for approximately 24% of the bacterial biofilm community at the end of the study. Pareto–Lorenz evenness distribution curves extracted from the DGGE patterns obtained from time course samples indicated community structures where shifts between functionally similar species occur, as observed within the predominant fermentative bacteria. These results suggest the presence of functional redundancy within the anodic communities, a probable indication that stable MFC performance can be maintained in changing environmental conditions. The capability of bacteria to adapt to electricity generation might be present among a wide range of bacteria.

Keywords Microbial fuel cell (MFC) · Electricity generation · PCR-DGGE · Microbial community dynamics

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Introduction

The study of bioelectrochemical systems (BESs) for the production of electricity, biogases, desalinated water or valuable chemicals has gained increased popularity in the recent years (Pham et al. 2009; Rabaey and Rozendal 2010; Mehanna et al. 2010). The most studied BES is the microbial fuel cell (MFC) where microorganisms convert complex organic substrates in wastewater into electricity. The improvement of the performance of MFCs requires an understanding of the evolution and function of the microbial communities in these systems, which could result in improved reactor designs for processes such as renewable electricity generation or wastewater treatment, as well as for methods to monitor and control these bioreactors (Briones and Raskin 2003; Gentile et al. 2007; Röling et al. 2000; Wittebolle et al. 2009b).

The bacterial populations found in the anode of MFCs inoculated with natural communities are very diverse (Aeltermann et al. 2008a; Logan 2009), and species belonging to *Proteobacteria*, *Firmicutes* and *Acidobacteria* phyla all have been shown to display electrogenic activity in MFCs (Bond and Lovley 2003; Chaudhuri and Lovley 2003; Bretschger et al. 2007; Fedorovich et al. 2009; Xing et al. 2008). However, no typical electricity-producing consortium has yet been observed to develop. Although qualitative temporal changes in the composition of microbial communities in MFCs have been reported (Aeltermann et al. 2006; Rabaey et al. 2004; Wang et al. 2009; Xing et al. 2010; Zhang et al. 2009), thus far the power output of mixed-community MFCs has not been shown to correlate with the abundance of any specific species. The isolates obtained by Rabaey et al. (2004) from glucose-fed batch-mode MFC generated electricity via self-produced mediators such as pyocyanin by *Pseudomonas aeruginosa*. Therefore, the increase of the power output over time in the mixed-community MFC was thought to result from increasing abundance of these mediator-producing bacteria and/or their enhanced self-mediating capability, but these hypotheses could not be confirmed. Aeltermann et al. (2006) reported a threefold increase in maximum power density of acetate-fed continuous-mode MFCs, which was attributed to the concomitant shift from a *Proteobacteria*-dominated community (with minor proportions of *Firmicutes* and *Actinobacteria*) to a community dominated by *Brevibacillus* sp., a member of the *Firmicutes*. In a later study, metabolites produced by *Pseudomonas* sp. were shown to enable current generation by *Brevibacillus* sp. (Pham et al. 2008).

Quantitative MFC community studies have thus far only been reported by White et al. (2009), who investigated the dynamics of the microbial community over one batch cycle in a plankton-fed MFC operated at fixed cell voltage, targeting specific bacterial classes (γ - and ϵ -*Proteobacteria*) or genus (*Geobacter*, *Arcobacter* and *Flavobacterium*–*Cytophaga*–*Bacteroides* [FCB]). At the onset of power production, an increase in the relative abundance of γ -*Proteobacteria* was observed, with a later succession from γ -*Proteobacteria* to *Geobacter* and then to FCB phylotypes, suggesting the capability of different phylotypes to compete for resources, including the anode (White et al. 2009).

Previous studies of microbial community dynamics of MFCs have either been done in batch-operated MFCs (Jung and Regan 2007; Rabaey et al. 2004; Wang et al. 2009; White et al. 2009; Zhang et al. 2009) or have included only a few time points in continuously fed MFCs (Aeltermann et al. 2006; Borole et al. 2009). Most of these studies also relied on a single MFC for extracting conclusions about community development under specific operating conditions, which may not be enough considering the high degree of variability observed, e.g. in the communities of

replicate wastewater treatment bioreactors (Fernández et al. 2000; Gentile et al. 2007; Kaewpipat and Grady 2002). We have recently reported that similar dynamic changes in the bacterial community composition are obtained in duplicate tubular longitudinal MFCs (Kim et al. 2011)

In this work, the performance and semi-quantitative bacterial community dynamics of biofilm and suspended culture in the anode of replicate MFCs are studied by using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified partial 16S rRNA genes followed by species identification by sequencing. The power density of MFCs is correlated to the relative proportion trends of species obtained from DGGE analysis in order to detect species that may have an important functional role in electricity production. Range-weighted richness (Rr) and Pareto–Lorenz evenness distribution curves are extracted from DGGE patterns in order to evaluate the diversity and to visualise species abundance ratios over time in both anodic biofilm and suspended communities.

Materials and methods

Microorganisms and media

Anaerobic digester sludge collected from a biosolids mesophilic digester (Cog Moors Sewage Treatment Works, Cardiff, UK) was used to inoculate the MFCs. The sludge was sieved through a 0.6-mm mesh (Endecotts Ltd., UK) to remove large particles and stored at 4°C. MFC culture medium contained (per litre): NH₄Cl, 0.31 g; NaH₂PO₄·H₂O, 5.38 g; Na₂HPO₄, 8.66 g; KCl, 0.13 g (pH 7.0) (Kim et al. 2007), supplemented with trace mineral (12.5 mL) and vitamin (12.5 mL) solutions (Lovley et al. 1984). The concentration of sucrose in the medium was 5 gL⁻¹ in batch operation, while the continuously operated MFCs were fed with medium containing sucrose at a concentration of 0.1 gL⁻¹. All media preparations were autoclaved at 121°C for 15 min, except for the vitamins, mineral and sucrose solutions that were filter-sterilised through a 0.2- μ m pore size membrane (Nalgene SFCA membrane, USA).

MFC configuration

The single-chamber MFCs consisted of anode chambers (9 cm³) and cover plates made of Perspex, with stainless steel metal plates serving as a contact between the cathode and the electrical circuit. The anode electrode contained a carbon fibre veil (PRF Composite Materials, UK) with polyvinyl alcohol binder, with a geometric area of 32 cm², which was placed inside the anode chamber and connected to an electrical circuit with an insulated Ni/Cr wire (Advent

Research Materials, UK) knitted across the multi-layered anode. The air-breathing cathode consisted of type A carbon cloth (9 cm², E-TEK) coated with 4 mg cm⁻² of Pt black catalyst with polytetrafluoroethylene binder. The platinum side of the cathode was painted with 0.5–1.0 mg cm⁻² of Nafion perfluorinated ion-exchange ionomer (5% w/v dispersion in lower aliphatic alcohols and H₂O, Aldrich). A Nafion-115 proton-exchange membrane (20 cm², DuPont) was pre-treated by sequential boiling for 1 h in 6% w/v H₂O₂, H₂O, 0.5 M H₂SO₄ and H₂O and subsequently stored in deionised water in the dark before assembly between the anode chamber and cathode.

MFC operation

Three replicate MFCs were started up by suspending anaerobic digester sludge in sucrose-containing medium at a 10% volume ratio. The MFCs were operated in batch-mode during the initial enrichment period (approximately 2 weeks). During that time, the anodic suspension was repetitively replaced (five times) initially by mixing (1:9) anodic suspension with fresh N₂-purged sucrose-containing medium and then after 1 week by replacing the entire volume of the anodic suspension with fresh medium. MFCs were operated in batch-mode until repeatable cycles of voltage generation were observed. In continuous-mode, medium was supplied to MFCs at a flow rate of 0.18 mL min⁻¹ and purged with N₂ gas. The MFCs were operated at room temperature (21–22°C) and sampled for chemical and microbial community analysis. A control MFC operated in open circuit is described in the [Supplemental material](#).

Chemical analyses

The total carbohydrate concentration in the influent and effluent of MFCs was measured using a colorimetric phenol/sulphuric acid method (Dubois et al. 1956). Samples were filtered through a 0.45-μm filter and the filtered solution (200 μL) mixed with 200 μL of phenol (5% w/v) and 1 mL of concentrated sulphuric acid and vortexed immediately. Samples were let to stand for 15 min and the absorbance measured at 490 nm with an Ultrospec 2000 UV/visible spectrophotometer (Pharmacia Biotech, Sweden). A standard curve was prepared using glucose (0–100 mg L⁻¹) and the results expressed as glucose equivalents. All measurements were done in duplicate. The total carbohydrate consumption (in percent) was calculated as $C(\%) = (a_i - a_e) / a_i \times 100$, where a_i and a_e are the concentrations of carbohydrate in the influent and in the effluent of the MFC, respectively. The carbohydrate consumption over a specific time period is presented as the mean ± standard error of the mean (SEM), where n is the number of samples during the corresponding time interval,

measured in duplicate. Chemical oxygen demand (COD_{Cr}) was analysed according to the standard method SFS 5504 (SFS 5504 1988). Coulombic efficiency was calculated as previously described (Logan et al. 2006). The pH of the effluent medium was measured using a Mettler Toledo MP220 pH meter (Switzerland).

Electrochemical measurements

MFC voltage was monitored using an Arbin BT2000 battery tester (Arbin Instruments, USA) controlled with MITS Pro software (Arbin Instruments) across a fixed external resistance of 40 kΩ. Polarisation curves were recorded with decreasing external resistances (700 kΩ–500 Ω, for a maximum of 5 min for each resistance) and measuring the decrease in voltage. The volumetric power density was calculated as $P = UI/V$, where U is the measured voltage, I is the current and V is the liquid volume of the anode chamber. Electrochemical impedance spectroscopy was used to measure the ohmic internal resistance of MFCs. Impedance spectra were recorded between the anode and cathode (two-electrode mode) with a Solartron Analytical 1260 frequency response analyser operating with a Solartron Analytical 1287 potentiostat/galvanostat (Solartron Analytical, UK) in the frequency range of 0.1 Hz–1 MHz and with a sinusoidal perturbation of 10 mV amplitude under open circuit voltage (Zhao et al. 2008).

Microbial community analysis

Total DNA was extracted directly from either a sample of anode electrode (approximately 1 cm²) or 1 mL of anode suspension using FastDNA Spin Kit for Soil (MP Biomedicals, UK). For sampling of the anode electrode, each MFC was temporarily disassembled in an aseptic environment, a 1-cm² anode sample cut out using a sterile scalpel and the MFC re-assembled. Prior to DNA extraction, the anode suspension samples were centrifuged (10,000×g, 5 min), washed three times with 1 mL phosphate-buffered saline (8.0 g L⁻¹ NaCl, 0.2 g L⁻¹ KCl, 1.15 g L⁻¹ Na₂HPO₄, 0.2 g L⁻¹ KH₂PO₄, pH 7.3; Oxoid, UK) and resuspended in 100 μL of nuclease-free water (Promega, UK).

The partial bacterial and archaeal 16S rRNA genes were amplified and DGGE performed as previously reported (Kim et al. 2011), with a denaturing gradient ranging from 40% to 70%. Single samples were used in this study, as high similarity was previously observed in duplicate DGGE profiles.

The DGGE gels were analysed using image analysis software (Gel2k, v 1.2.0.6; <http://folk.uib.no/nimsn/gel2k/>) that estimates the relative position and area of peaks in a lane. The relative proportion of species in the communities can be inferred from the relative band intensities calculated by dividing the peak area of a band by the sum of peak areas

of all bands in a lane (excluding chimaeras, analysed as described below) (Koskinen et al. 2007; Fromin et al. 2002; Kim et al. 2011). Pearson correlation analysis between the relative proportions of bacterial species and the power density over time was performed using GraphPad Prism (v. 5.01, GraphPad Software, USA).

The DGGE profiles were compared by cluster analysis (Multivariate Statistical Package, v. 3.13b; Kovach Computing Services, UK), with Jaccard coefficients for the construction of similarity matrices and UPGMA clustering algorithm for the construction of dendrograms (El Fantroussi et al. 1999; Röling et al. 2000).

The range-weighted richness (Rr) was derived from the DGGE patterns of anodic biofilm and suspended culture communities over time and calculated as $Rr = N^2 \times D_g$, where N is the total number of bands in the pattern (lane) and D_g is the denaturing gradient between the first and the last band of the pattern (Marzorati et al. 2008). Microbial dynamics was analysed using moving window analysis and reported as average rate of change (Δ_t) between consecutive DGGE profiles according to $\text{Change (\%)} = 100 - \text{similarity (\%)}$ (Marzorati et al. 2008). The structure of the bacterial communities (species distribution) is graphically represented as Pareto–Lorenz evenness curves based on the DGGE profiles (Marzorati et al. 2008). For each lane, the bands were ranked from high to low according to their intensities and the results plotted as the cumulative proportion of band intensities (y -axis) vs. the cumulative proportion of bands (x -axis) (Marzorati et al. 2008). The curves were numerically interpreted by scoring the y -axis projection of their respective intercepts with the vertical 20% x -axis line. The results for each MFC were shown as the mean \pm SEM of scores at different time points.

Bands were excised from the DGGE gels and sequence and phylogenetic analysis of DNA fragments performed as previously reported (Kim et al. 2011). DNA of samples that could not be sequenced was re-amplified by PCR and the bands separated with a narrower denaturing gradient on a DGGE gel (Gafan and Spratt 2005; Green 2006).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences submitted to GenBank can be found under the accession numbers HM043254 to HM043289.

Results

MFC performance

The reproducibility of the performance of sucrose-fed single-chamber MFCs was investigated using three replicate MFCs.

The maximum power densities at the beginning of the continuous-mode operation of MFCs A, B and C were 0.50, 0.39 and 0.47 W m^{-3} , respectively, reaching 1.03, 1.20 and 1.79 W m^{-3} , respectively, at the end of the experiment (Fig. 1a). The peak power densities were reached within the ranges 18.5–25 $\text{k}\Omega$ at the beginning and 4–6 $\text{k}\Omega$ at the end

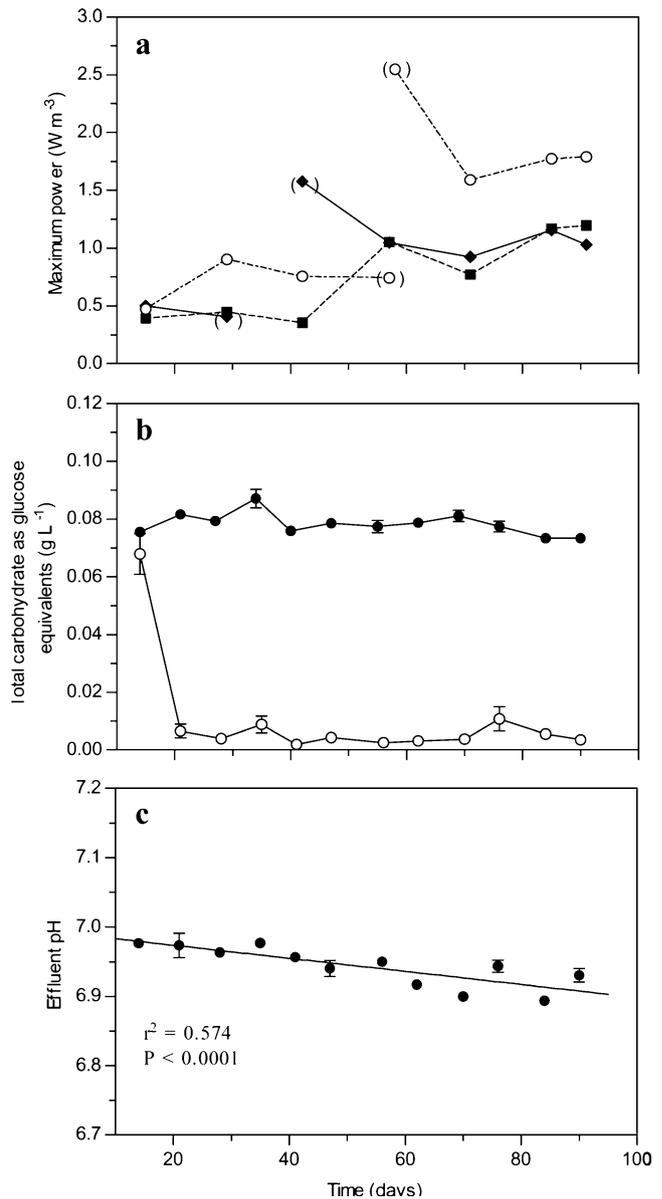


Fig. 1 Time course plots of **a** maximum power density of MFCs A (filled diamonds), B (filled squares) and C (empty circles) in continuous-mode. The Nafion membrane and cathode were replaced in each MFC after indication of fouling on days 41, 56 and 58 for MFCs A, B and C, respectively. The increase in power density between the values marked with brackets was due to the replacement of Nafion and cathode; **b** total carbohydrate concentration of influent (filled circles) and effluent (empty circles) of replicate MFCs (data shown as mean \pm SEM; $n=3$ MFCs, averages of duplicate measurements); **c** effluent pH of replicate MFCs in continuous-mode with linear regression (data shown as mean \pm SEM; $n=3$ MFCs)

of the experiment. Typical polarisation and power density curves are shown in Fig. S1. The maximum power density showed an increasing trend, coinciding with the increase in the DNA concentration of the anodic biofilm and suspended culture (Figs. 1a and S2).

The concentration of carbohydrates in the MFC effluents was monitored in order to evaluate the efficiency of sucrose removal from the artificial feed medium. The three replicate MFCs showed similar performances over time in sucrose consumption and effluent pH during continuous-mode operation (Fig. 1b, c). The results are presented as average values (\pm SEM) of MFC replicates. The consumption of sucrose was high, being $94\pm 1.0\%$ ($n=11$) from day 21 onwards (Fig. 1b). The coulombic efficiencies were approximately 4% at the end of the experiment in the replicate MFCs. The effluent pH decreased slightly over time from 6.98 ± 0.007 to 6.93 ± 0.01 (Fig. 1c).

Impedance spectroscopy was used to measure the ohmic internal resistance (electrode, membrane, interconnections and electrolyte resistances) of the MFCs in order to determine the extent of limitation of power output due to ohmic losses. The ohmic internal resistance for replicate MFCs at the start of operation was $29\pm 4.5\Omega$ ($n=3$, number of MFCs) when measured after inoculation with sludge and $17\pm 6.3\Omega$ ($n=3$) when measured with medium only. At the end of operation, the ohmic internal resistance was $15\pm 1.4\Omega$ ($n=3$).

Bacterial community analysis

The reproducibility of the bacterial community composition was evaluated using three replicate MFCs. The composition, dynamics and taxonomy of the bacterial communities in the anodic biofilm and in the suspended culture were analysed by PCR-DGGE of partial 16S rRNA genes and gene sequencing (Figs. 2, 3, 4, 5, S4, S5 and S6).

Despite its limitations, DGGE can be used to obtain details of the composition of microbial communities (Rittmann et al. 2008), by assuming that the relative proportions of the different species in the community can be estimated from the relative intensity of the bands (Koskinen et al. 2007; Zhang and Fang 2000). As shown by the differences between the band patterns of the initial samples (day 0) and those observed at other time points in all replicate MFCs, the composition of the community in the anaerobic sludge inoculum was very different to that present in the anodic biofilm or in the suspended culture (Fig. 2), reflecting the dynamics of the development of the microbial community during MFC operation.

Cluster analysis of bacterial community profiles

Cluster analysis of bacterial DGGE profiles of anodic biofilm and suspension were performed in order to detect

changes in communities over time and to compare the communities present in replicate MFCs. Both in the anodic biofilm and in the suspension, communities of replicate MFCs evolved differently from the first sampling point (day 14), which could be observed as separate clustering of samples of each MFC (clusters 1, 2 and 3) (Fig. 3). As an example, the similarities between anodic biofilm communities of replicate MFCs were 33–46% at the end of operation, based on Jaccard's similarity coefficient, which is defined as a number of bands shared between samples divided by the number of unique bands (Van Versevelde and Röling 2004). According to cluster analysis, both the biofilm and suspension changed over time within each MFC.

Bacterial community composition dynamics

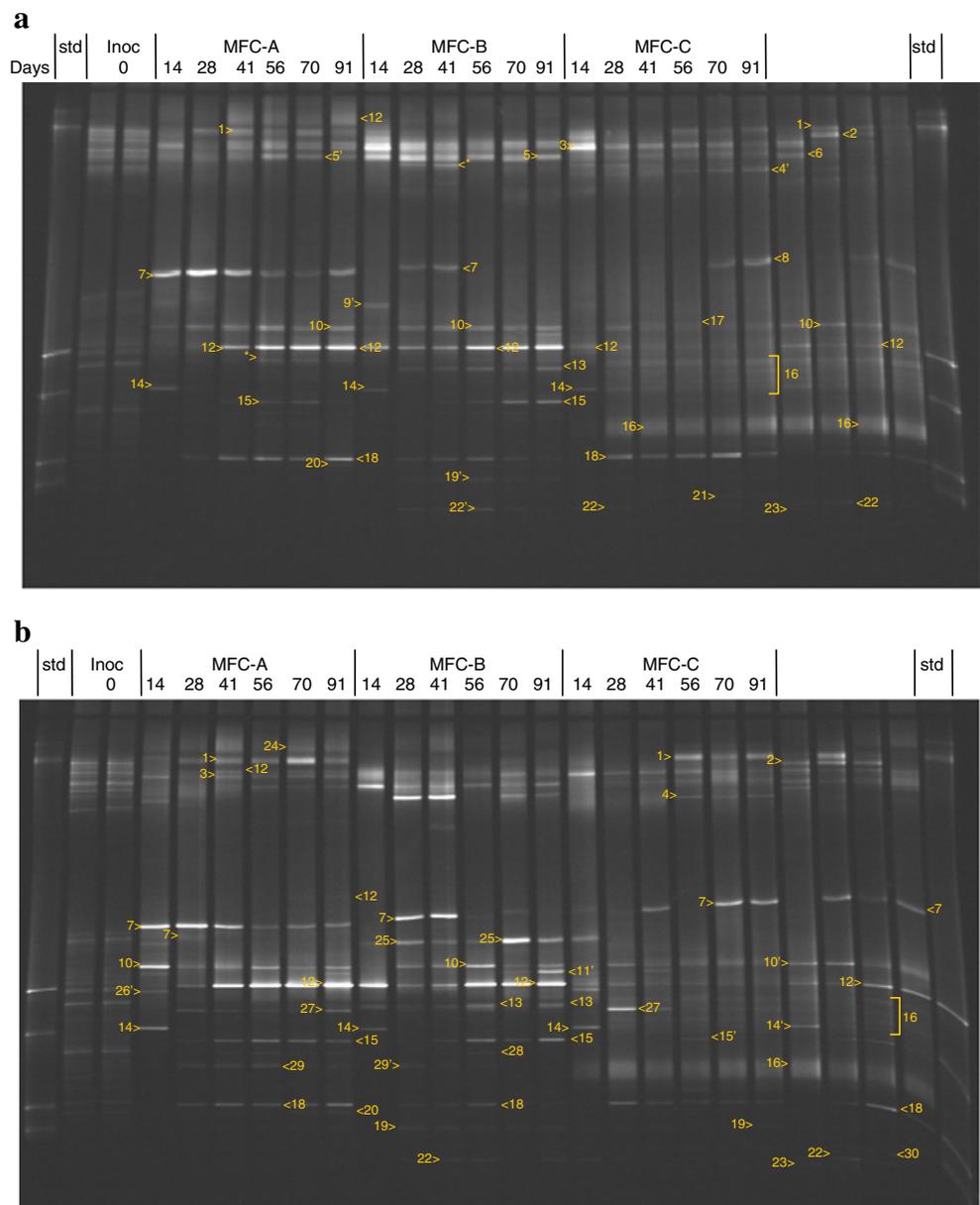
In order to link the power output of the MFCs with the relative proportion of a specific bacterial species or taxonomic group, the temporal change of bacterial species in the anodic biofilm or suspension was determined from the DGGE profiles. The DGGE bands were correlated with bacterial strains by comparing the DNA sequence of the 16S rRNA genes to known 16S rRNA sequences in the GenBank database.

Phylogenetic analysis revealed a diverse bacterial community both in the anodic biofilm and in the suspended culture, consisting mainly of the phyla *Firmicutes* and *Bacteroidetes* and different classes of the phylum *Proteobacteria* (Fig. 4). The relative proportions of species obtained from the DGGE profiles showed that the dominant bacterial species varied over time in the anodic biofilm and the suspension of all MFCs (Figs. 5, S4 and S5). Temporal development of bacterial communities differed between replicate MFCs, leading to variability in the composition of the most abundant bacterial species at the end of operation (Table 1).

Correlation analysis of bacterial species abundances with power density

Correlation analysis was applied in order to link changes in power output with the proportion trends of specific bacterial species in the anodic biofilm and in the suspension. The maximum power densities of samples taken at day 42 in MFC A, day 57 in MFC B and day 58 in MFC C were excluded from the Pearson correlation analysis to avoid the effect of the perturbation caused by the replacement of Nafion and cathode (Fig. 1a). From the results in Table 2, it can be seen that there is no consistent correlation between relative proportions of anodic and suspended bacterial species and power density in the replicate MFCs, and no clear trend of dependence of power output on any bacterial species common to all replicate MFCs could be deduced. The correlations found between

Fig. 2 DGGE profile of bacterial communities of the anodic biofilm (a) and suspended culture (b) analysed by DGGE of PCR-amplified genes coding for 16S rRNA; *Std* standard mix, *Inoc* anaerobic sludge, *asterisk* chimaeras. Numbers indicate bands excised for sequence analysis. The 16S rRNA genes of bands marked with *prime symbol* were re-amplified by PCR and run on another DGGE gel (Fig. S3) due to insufficient quality of sequences obtained. Same numbering is used for identical sequences. Unlabelled lanes correspond to a replicate MFC sampled at different time points and, therefore, not included in the analysis



species abundances and power density coincided with those found when calculated using current densities.

Bacterial community diversity, dynamics and internal community structure

The range-weighted richness index (Rr) can be used to estimate the diversity of microbial communities (Marzorati et al. 2008). The Rr was calculated for the DGGE pattern of each time point to characterise the diversity and shifting of the bacterial anodic biofilm and suspended communities in the replicate MFCs. Selective process promoted the initial disappearance of large number of species as observed in the

substantial decrease in the species richness from the Rr value of 85 in the anaerobic sludge inoculum to below 20 in the anodic biofilm (Fig. 6a). The species richness in the suspension was somewhat higher than in the biofilm, with Rr of 36, 18 and 23 at the end of operation of MFCs A, B and C, respectively (Fig. 6b). According to the classification of Marzorati et al. (2008), Rr values between 10 and 30 correspond to medium range-weighted richness.

Microbial dynamics was studied using moving window analysis (Marzorati et al. 2008). The anodic biofilm communities of MFCs A, B and C presented medium to high dynamics with an average rate of change (Δ_t) of 13 ± 5.5 , 10 ± 6.7 and 25 ± 6.4 , respectively.

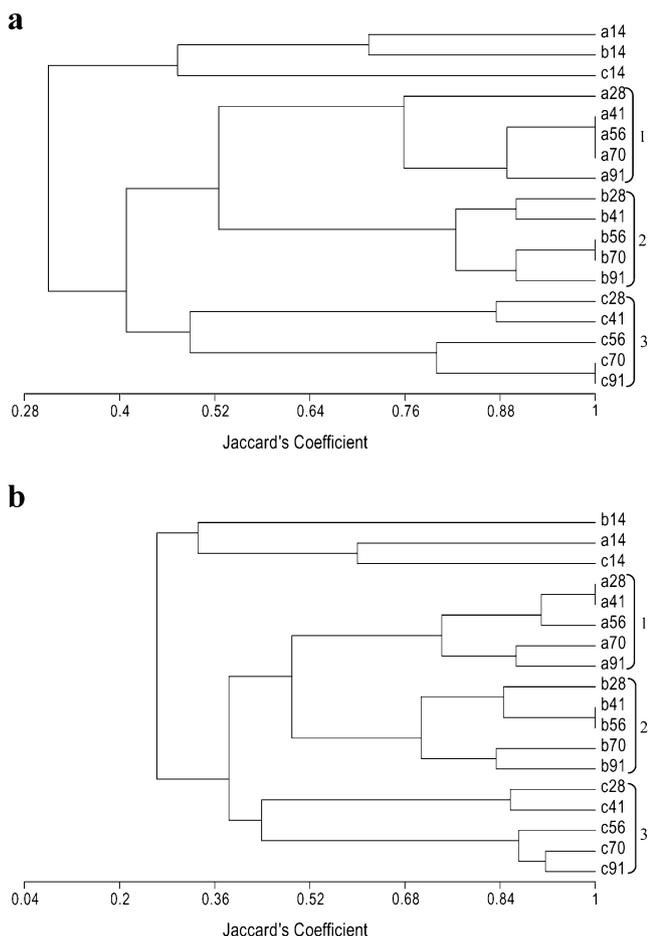


Fig. 3 Cluster analyses of bacterial community profiles of anodic biofilm (**a**) and suspended culture (**b**). Each node in the tree (indicated by reactor name and operation day) represents one lane in DGGE gel. The trees were generated using Jaccard similarity coefficient and UPGMA clustering algorithm

Pareto–Lorenz evenness curves (Lorenz 1905) were plotted over time for each MFC to visualise species abundance ratios in the biofilm communities. It was observed over time (n is the number of time points) that 20% of the bands for the anodic biofilm communities of MFCs A, B and C corresponded with $53 \pm 3.8\%$ ($n=6$), $54 \pm 1.6\%$ ($n=6$) and $51 \pm 5.2\%$ ($n=6$) of the cumulative species abundances, respectively (Fig. 7). Similar species distributions were found in the suspended cultures with 20% of the bands corresponding with $63 \pm 4.2\%$ ($n=6$), $57 \pm 3.5\%$ ($n=6$) and $55 \pm 5.0\%$ ($n=6$) of the cumulative species abundances in MFCs A, B and C, respectively (data not shown).

Discussion

The aim of this study was to relate the performance of MFCs to the composition and dynamics of the anodic bacterial communities. The reproducibility of sucrose-fed MFCs was

studied in terms of performance and development of microbial communities using replicate MFCs. Semi-quantitative community analysis was based on band intensities of DGGE community profiles. DGGE allowed the assessment of the relative composition (proportion trends) of a microbial community present in the amplicon pool.

MFC performance

Our study shows that the performance of the MFCs presents a degree of reproducibility in terms of power density, sucrose consumption and effluent pH (Fig. 1). The maximum power density obtained compare well with previous reports for sucrose-fed MFCs, where power outputs of similar order of magnitude were observed (He et al. 2005; Kim et al. 2011).

In general, the use of replicate MFCs is an exception rather than the norm, and only a few reports can be found in the literature where replicate MFCs are studied. Three glucose-fed MFCs operated in batch-mode showed a reproducible maximum power output of $40.3 \pm 3.9 \text{ mW m}^{-2}$ under controlled stirring rate and temperature (Jung and Regan 2007). Six acetate-fed MFCs showed high similarity in current generation ($74.7 \pm 5.8 \text{ mA}$) after 200 days of operation (Aelterman et al. 2006).

The ohmic internal resistance measured at the end of operation was low and very similar in all MFCs. Major factors affecting the power density in the MFCs of the present study were not associated with ohmic losses, but it can be speculated that the power was affected by the growth of fermentative organisms, leading to biomass, gas and other metabolite production. However, we deliberately used sucrose as a model fermentative substrate of practical wastewater treatment applications. In order to improve MFC performance, the use of metabolic products of fermentation (VFAs) for electricity generation by electrogens could be made more efficient by, for example, increasing the hydraulic retention time (HRT) and hydraulically connecting MFCs in series (Kim et al. 2011).

Bacterial community analysis

The bacterial community composition and dynamics of the anodic biofilm and suspended culture were studied in replicate MFCs by PCR-DGGE. The species present were identified on the basis of the 16S rRNA sequencing and comparison with their closest plausible relative. Cluster analysis of both the anodic biofilm and suspension samples revealed that the bacterial communities evolved differently in the replicate MFCs. The similarities between anodic biofilm communities of replicate MFCs were 33–46% at the end of operation. This is low compared to the results by Aelterman et al. (2006), showing high reproducibility ($\geq 97\%$) of bacterial biofilm communities in an acetate-fed

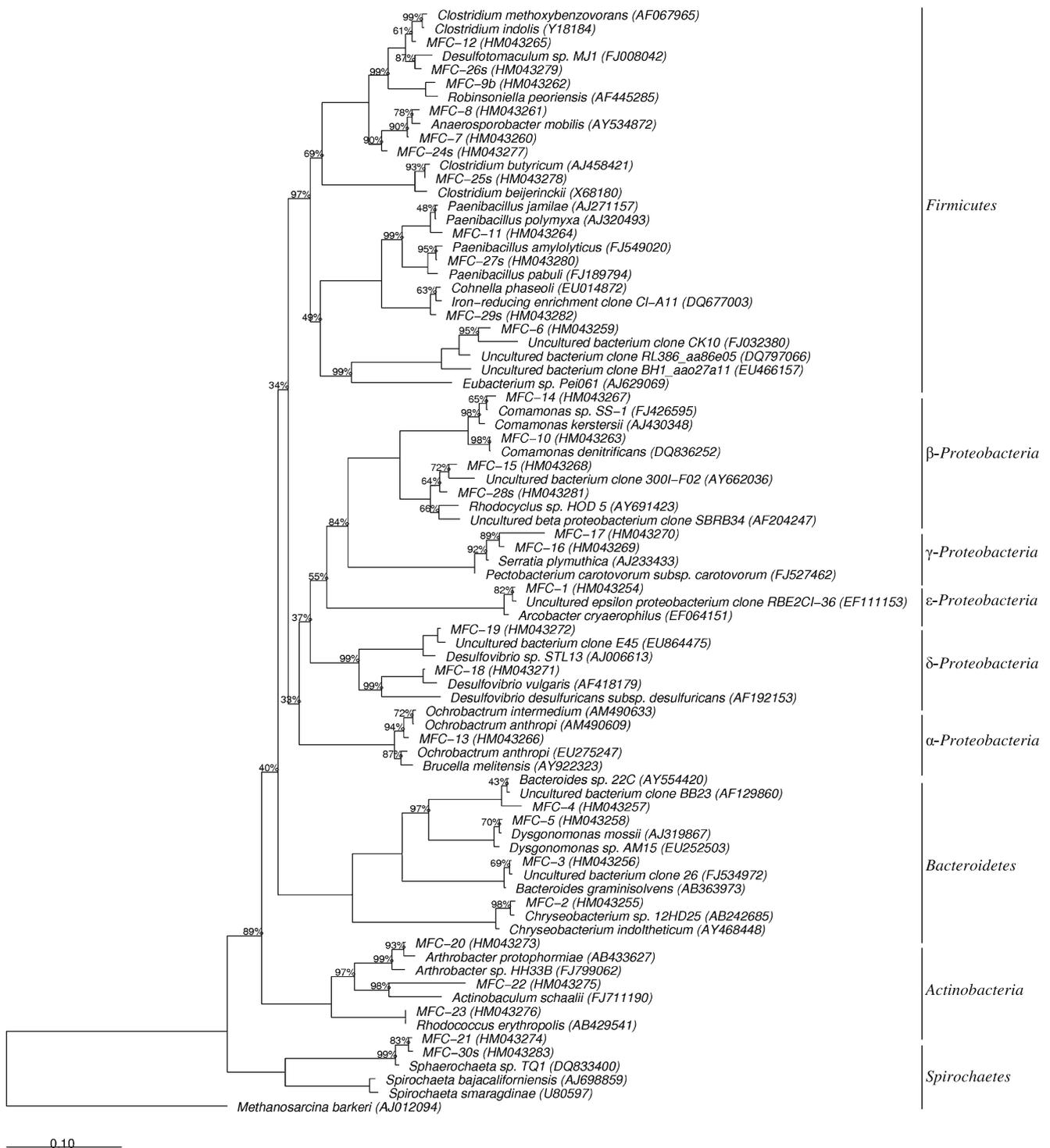


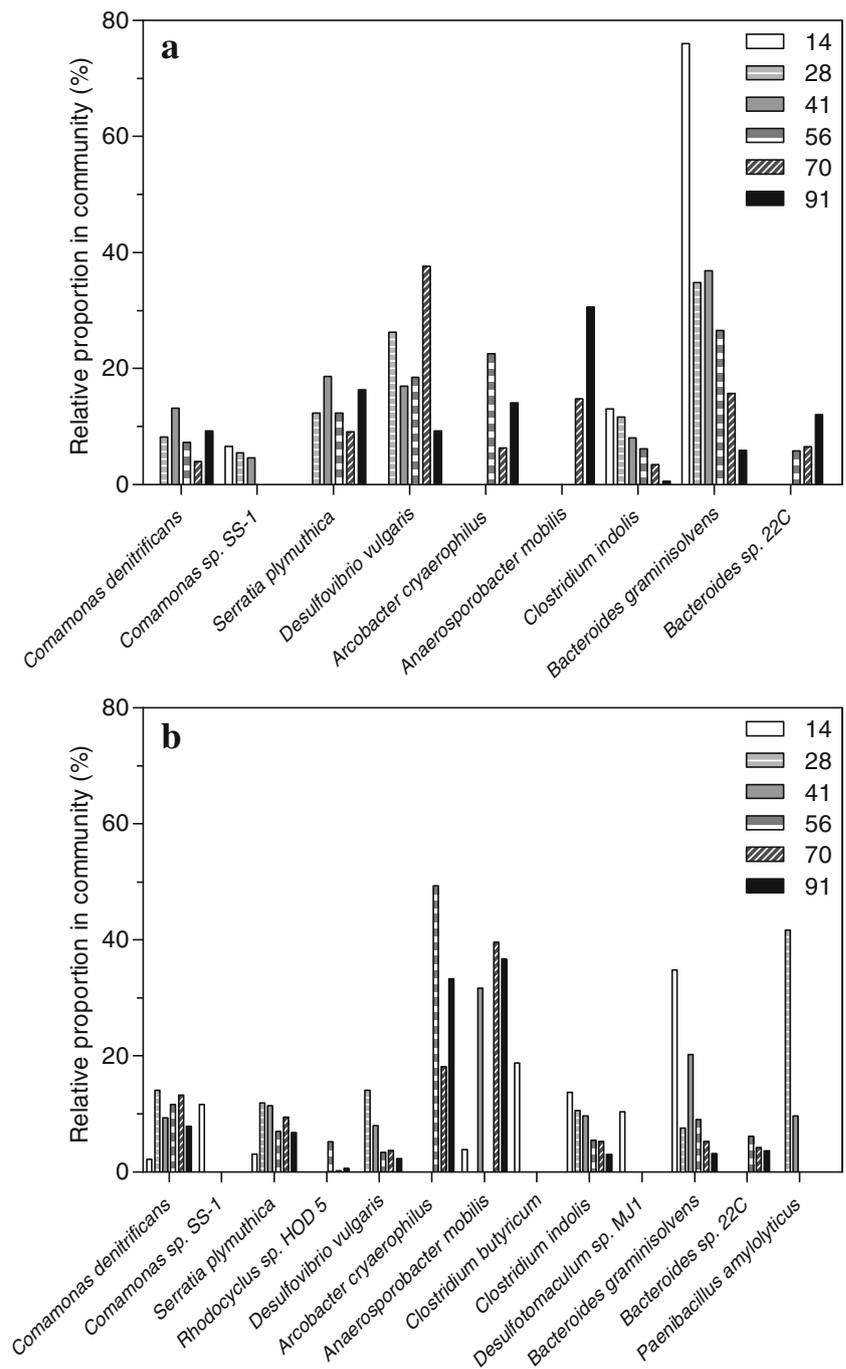
Fig. 4 Phylogenetic tree based on partial bacterial 16S rRNA gene sequences (named *MFC-DGGE band nr*) of anodic biofilm and suspended culture of three replicate sucrose-fed MFCs. Sequences found only in biofilm or suspension are marked with ‘b’ or ‘s’, respectively. The tree was constructed using a distance matrix and

neighbour-joining algorithm with 1,000 bootstrappings. The bootstrap values $\leq 99\%$ are shown. The archaeon *Methanosarcina barkeri* (AJ012094) was used as an outgroup. The scale bar represents 10% sequence divergence

stack MFC consisting of six individual MFCs after 200 days of operation. The bacterial communities of two thermophilic acetate-fed MFCs were found to be similar ($>89\%$) to

each other after 100 days of operation (Wrighton et al. 2008). The reproducibility of duplicate sucrose-fed tubular MFCs, evaluated by cluster analysis and Jaccard’s coeffi-

Fig. 5 Proportion trends of the most abundant bacterial species in the anodic biofilm (a) and suspension (b) of MFC C based on band intensities of DGGE community profiles and identification of excised bands by sequencing and comparison to known 16S rRNA sequences in GenBank



cient, showed 80–90% similarity in species composition (Kim et al. 2011). The difference observed between our results and other MFC studies could be attributed to differences in reactor design, operational conditions and whether inoculum was acclimatised prior to the start-up of replicate reactors (Wrighton et al. 2008; Kaewpipat and Grady 2002). The reproducibility of the community could also be affected by reactor size. In the present study, the volume of the anode chamber was 1 to 2 orders of magnitude lower than in the above-mentioned studies.

The lack of reproducibility of bacterial communities has been reported in other studies of biological wastewater treatment (Fernández et al. 2000; Gentile et al. 2007; Kaewpipat and Grady 2002) and has been attributed to the chaotic dynamics of bacterial communities, according to which a small change in conditions can cause communities to diverge (Curtis and Sloan 2004; Kaewpipat and Grady 2002). It has been suggested that the chaotic behaviour of bacterial communities is associated particularly with small-scale wastewater treatment systems, whereas large-scale

Table 1 Relative proportions of the most abundant (>5%) bacterial species found in the anodic biofilm and suspension of MFCs A-C at the end of operation

Species	% similarity in GenBank	Biofilm			Suspension		
		A	B	C	A	B	C
<i>Clostridium indolis</i>	99	41	40		46	33	
<i>Desulfovibrio vulgaris</i>	99	20		9	10		
<i>Bacteroides graminisolvens</i>	99	12	17	6	7		
<i>Anaerospobacter mobilis</i>	98–100	10		31	7		37
<i>Comamonas denitrificans</i>	100	9	6	9	4	13	8
<i>Dysgonomonas mossii</i>	100		16			6	
<i>Rhodocyclus</i> sp.	96		10			7	
<i>Ochrobactrum antropi</i>	100		6				
<i>Serratia plymuthica</i>	99			16			7
<i>Arcobacter cryaerophilus</i>	99			14	10		33
<i>Bacteroides</i> sp.	98			12			
<i>Clostridium butyricum</i>	100					12	
<i>Paenibacillus polymyxa</i>	99					20	

biological treatment systems present less dynamic behaviours (Smith et al. 2003; Curtis and Sloan 2004). HRT could be another factor affecting the community composition and the predictability of biological processes with continuous flow and without recycle. Further studies on the effect of HRT on the anodic community dynamics and performance of MFCs are warranted.

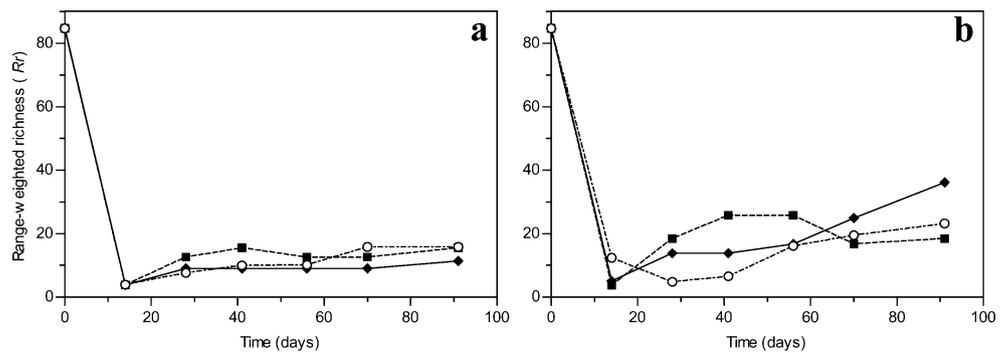
In this study, no firm correlation common to all replicate MFCs was found between the relative proportion of any one bacterial species and the maximum power output (Table 2). The observed shifts in the relative proportions of fermentative bacteria within the anodic biofilm of each MFC suggest temporal changes among species with metabolic similarity, namely, the succession from *Anaerospobacter mobilis* to *Clostridium indolis* in MFC A, from *Bacteroides graminisolvens* and *Dysgonomonas mossii* to *C. indolis* in MFC B and from *B. graminisolvens* and *C. indolis* to *A. mobilis* and *Bacteroides* sp. in MFC C (Figs. 5a and S4) (Jeong et al. 2007; Lawson et al. 2002; Nishiyama et al. 2009). Dynamic changes among fermentative

bacteria were also detected in the anodic suspension (Figs. 5b and S5). At the end of operation, *C. indolis* was the predominant fermentative bacterium in the anodic biofilm of MFCs A and B, whereas *A. mobilis* dominated in MFC C (Table 1). The most abundant non-fermentative species were *Desulfovibrio vulgaris* and *Comamonas denitrificans* in MFC A, *Rhodocyclus* sp., *C. denitrificans* and *Ochrobactrum antropi* in MFC B and *Arcobacter cryaerophilus*, *C. denitrificans* and *D. vulgaris* in MFC C (Table 1), all of them able to use organic acids, and in the case of *Rhodocyclus* sp. and *D. vulgaris*, also H₂, a product of sucrose fermentation (Gumaelius et al. 2001; Smith et al. 2005; Vandamme et al. 1992; Zuo et al. 2008; Holt 1994). *C. denitrificans* and *O. antropi* represent known exoelectrogenic species (Zuo et al. 2008; Xing et al. 2010). The appearance or increase in relative proportion of *C. denitrificans* in the anodic biofilm of all MFCs by day 28, accompanied by the disappearance of a species belonging to the same genus but without denitrifying capability (Wauters et al. 2003) (Figs. 5a and S4), strongly suggests

Table 2 Correlation analysis of relative proportions of anodic and suspended bacterial species with power density (r Pearson correlation coefficient, p probability in statistical significance testing)

MFC	Biofilm			MFC	Suspension		
	Species	r	p		Species	r	p
A	<i>C. indolis</i>	0.94	0.019	A	<i>C. indolis</i>	0.98	0.003
	<i>A. mobilis</i>	-0.97	0.006		<i>A. mobilis</i>	-0.95	0.014
B	<i>C. indolis</i>	0.89	0.046	B	<i>P. polymyxa</i>	0.99	0.001
	<i>Rhodocyclus</i> sp.	0.90	0.038		<i>Rhodocyclus</i> sp.	0.95	0.015
C	<i>C. indolis</i>	-0.87	0.023	C	<i>C. indolis</i>	-0.81	0.05
	<i>A. mobilis</i>	0.93	0.008				
	<i>Bacteroides</i> sp.	0.84	0.031				
	<i>B. graminisolvens</i>	-0.84	0.035				

Fig. 6 Time course plots of the range-weighted richness (R_r) of **a** bacterial anodic biofilm and **b** suspended culture communities of MFCs A (filled diamonds), B (filled squares) and C (empty circles)

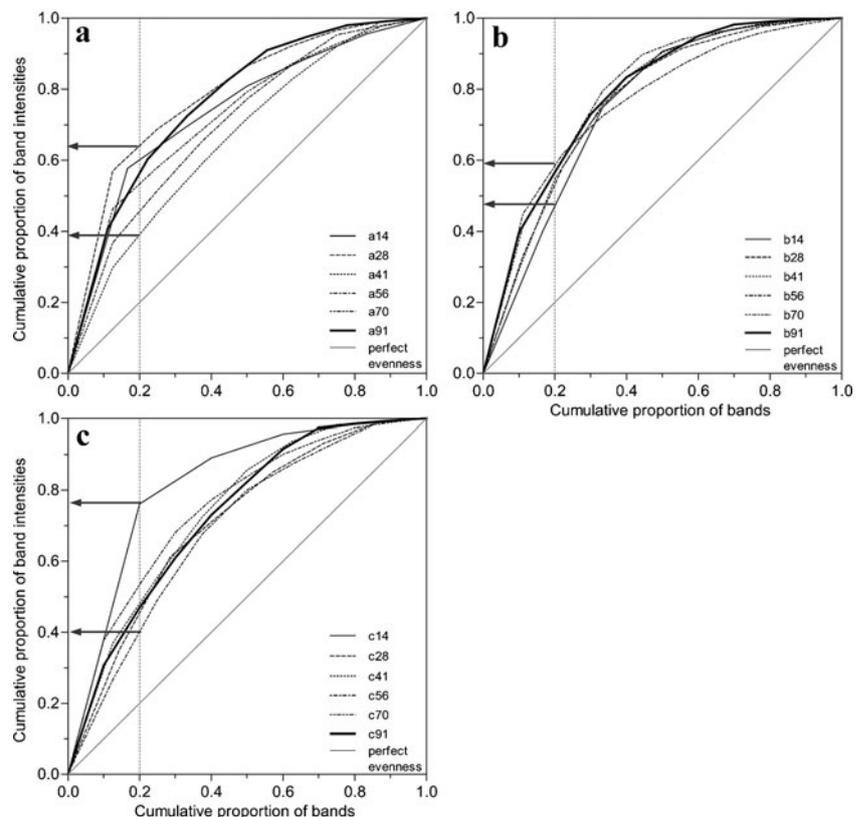


that bacteria possessing metabolic pathways for denitrification displace other bacteria from the anodic biofilm. The positive correlation of the proportion trend of *Rhodocyclus*-related species with power density in MFC B further demonstrated the important role of this H_2 -oxidising, denitrifying bacterium in electricity generation (Smith et al. 2005). Interestingly, *Rhodocyclus* sp. was not a member of the anodic community in the control MFC (for further details, see Supplemental material and Fig. S7).

In each MFC, the composition of the communities in the biofilm and in the suspension showed high similarity. As this continuous-flow system is fed with sterile medium, the community in the suspension is derived from the biofilm population.

The dynamic changes observed in the bacterial classes of biofilm and suspended bacterial communities reflect the changes observed in the relative proportions of species. At the end of MFC operation, *Clostridia* (belonging to the phylum *Firmicutes*) was the most abundant bacterial class in the anodic biofilm and the suspended communities in all replicate MFCs (Fig. S6). The next abundant bacterial classes ($\geq 10\%$) included *Bacteroidetes*, β -, γ -, δ - and ϵ -*Proteobacteria* and *Bacilli* and showed variations between biofilm and suspended communities and between replicate MFCs. Several studies have shown the dominance of either α -, β -, γ - or δ -*Proteobacteria* in glucose-fed MFCs (Chae et al. 2009; Choo et al. 2006; Chung and Okabe 2009; Jung and Regan 2007; Phung et al. 2004; Xing et al. 2009). In

Fig. 7 Pareto–Lorenz distribution curves derived from DGGE patterns of the anodic biofilm bacteria of MFCs A, B and C on days 14, 28, 41, 56, 70 and 91. Arrows indicate the range of cumulative band intensities corresponding to 20% of the bands



most of these studies, *Firmicutes* were also found to be important members (10–27%) of the bacterial community (Choo et al. 2006; Chung and Okabe 2009; Jung and Regan 2007; Xing et al. 2009). In accordance with our findings, *Firmicutes* dominated in the anode-attached community of a glucose- and lactate-fed MFC (Borole et al. 2009), a cellulose-fed MFC (Rismani-Yazdi et al. 2007) and acetate-fed MFCs (Aelterman et al. 2006; Wrighton et al. 2008; Xing et al. 2010).

The most significant temporal dynamic changes in the anodic biofilm occurred within fermentative bacteria. Shifts in the proportion trends of the putative exoelectrogens (*C. denitrificans*, *D. vulgaris*, *Rhodocyclus* sp. and *A. cryaerophilus*) were also observed in all replicate MFCs. An increase in the relative proportion of *C. denitrificans* by day 28 and temporal changes in proportions of either *D. vulgaris*, *Rhodocyclus* sp. or *A. cryaerophilus* after day 28 (Figs. 5a and S4) could possibly explain the increase in the maximum power density over time in each MFC. Based on the 16S rRNA gene sequences, fermenters were the largest metabolic group found in the biofilm of all MFCs, followed by denitrifiers, facultative anaerobes, sulphate-reducers and microaerophiles. The putative electricity-producing denitrifiers and sulphate-reducers represented approximately 24% of the total bacterial community at the end of MFC operation (for details of composition dynamics of Archaea, see [Supplementary information](#)). The hypothesised importance of denitrifiers (*C. denitrificans*, *O. antropi* and *Rhodocyclus* sp.) and sulphate-reducers (*Desulfovibrio desulfuricans*) could be supported by the ability of those species to secrete exopolysaccharides (EPS) or, in the case of *Desulfovibrio*, to express protein filaments; these capabilities may help in the attachment to surfaces and in biofilm formation. Similar observation could be made with regards to the nature of some dominant *Enterobacteriaceae* species in tubular MFCs (Kim et al. 2011). Several exoelectrogenic species that also produce EPS have been isolated and studied in MFCs (Fedorovich et al. 2009; Rabaey et al. 2004; Zuo et al. 2008; Yi et al. 2009).

Contrary to many other reports showing the dominance of *Geobacter* spp. in anodic communities (Jung and Regan 2007; Aelterman et al. 2008b; Xing et al. 2009), our results did not show the presence of this species in the MFC community. This may be explained by the diffusion of air from the air-breathing cathode to the anode, in addition to potential exposure to air during biofilm sampling, which may have affected the low-redox environment required by the species. Also, *Geobacter* is not able to grow using sucrose as substrate, preferring volatile fatty acids such as acetate. Other studies have reported the lack of dominance of *Geobacter* spp. in MFCs (Rabaey et al. 2004; Aelterman et al. 2006).

Further investigations on the dynamics of the anodic community structure and its metabolic functions are needed to detect the key contributors and their functions over time in electricity-generating communities, such as a combined metagenomics/transcriptomics approach involving random sequencing of the whole community and the measurement of metabolic products in MFC (Frias-Lopez et al. 2008; Urich et al. 2008).

The PCR-DGGE method used in this study for the determination of proportion trends of species is not purely quantitative (Muyzer and Smalla 1998; Green et al. 2009), and the results obtained from the molecular analysis provide only an indication of the degree of diversity in the anodic communities and should not be construed as an absolute measurement of diversity. However, it is believed that the approach provides valuable insight and allows trends in dynamic changes of anodic community composition to be identified.

The external resistance was same throughout the study, except during the measurement of polarisation curves. In a separate study, we have operated an MFC where the cell voltage was optimised throughout the experiment to control operation at maximum sustainable power output ($V=1/2$ OCP), according to Ieropoulos et al. (2008). However, this optimisation did not have significant effect on maximum power output or community composition obtained (data not shown). Other studies have used similar external resistance values compared to our study amongst others (Katuri et al. 2011) and reported that power densities and community activity were not affected by external resistance.

Diversity of the bacterial communities

The diversity and (especially) the functional redundancy of the community could be significant factors in determining the functional stability of a MFC system (Briones and Raskin 2003; Fernández et al. 2000). Anodic and suspended communities of all replicate MFCs could be characterised as presenting a medium diversity with a medium range-weighted richness (Marzorati et al. 2008). The internal structure of communities over time was assessed by constructing Pareto–Lorenz evenness distribution curves. Over the time course of the experiment, the anodic and suspended communities in the replicate MFCs presented high numbers of some species, while many others were available (in decreasing lower amounts) to proliferate and replace the dominant species. The Pareto–Lorenz evenness distribution curves suggest functional redundancy within the bacterial anodic communities, a speculation supported by the succession observed within the dominant fermentative species (Figs. 5, S4 and S5). Communities with such a structure can potentially preserve their functionality in sudden stress conditions (Marzorati et al. 2008; Wittebolle et al. 2009a), a

feature of potential benefit for the operation of MFCs used in processes subject to continuous environmental changes, such as wastewater treatment processes. Communities in acetate-fed MFCs have also been characterised as presenting medium to high range-weighted richness and similar internal community structure than that observed in this study, as analysed by Pareto–Lorenz curves (Aelterman et al. 2008b).

In conclusion, differences in the evolution of the anodic biofilm and the suspended communities were observed in three replicate sucrose-fed MFCs, demonstrating that the assessment of reproducibility is essential to obtain meaningful conclusions in studies of microbial communities in MFCs. No consistent correlation was found between the presence of anodic or suspended bacterial species and the power density in the replicate MFCs. However, in all replicate MFCs, putative exoelectrogenic denitrifiers and sulphate-reducers accounted for approximately 24% of the bacterial biofilm community at the end of the study. Pareto–Lorenz curves of all MFCs over time were suggestive of community structures where shifts between functionally similar species occur, as observed within the dominant fermentative bacteria, and where stable MFC performance is likely to be maintained in changing environmental conditions. In addition, the anodic communities that developed in replicate MFCs produced similar levels of power although the community composition would differ, suggesting that the anodic performance is not determined by the phylogenetic identity of species. Previously in the field of biological fuel cells, the cathodic bacterial community structure (richness and evenness) rather than the phylogenetic affiliation has been correlated with cathodic performance (Wrighton et al. 2010).

Changes in the microbial community composition, where specific species with redox systems best suited for using the anode at specific potential would outcompete other species and increase in proportion in the anode surface, did not seem to be the means of microbial adaptation in the present study. The bacterial communities developed into diverse consortia with functional internal community structure in all MFC replicates. This finding further suggests that several types of bacteria can adapt to generate electricity in MFCs. The universality of exoelectrogenic ability would be supported by the large diversity of bacteria in MFCs reported in the literature, the high number of species detected that are distantly related to any known cultured organisms by their 16S rRNA sequence and the abundance of bacteria with different types of metabolism that are found to be exoelectrogenic in pure culture. Further research is required to clarify the means of microbial adaptation in MFCs in order to engineer systems able to achieve power levels suitable for practical applications.

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