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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

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 (Aelterman 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 (Aelterman 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. Aelterman et al. (2006) reported a three-fold 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 (Aelterman 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 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 visualize 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 0.6 mm mesh (Endecotts Ltd, UK) to remove large particles and stored at 4°C. MFC culture medium contained (per liter): NH_4Cl: 0.31 g; $NaH_2PO_4 \cdot H_2O$: 5.38 g; Na_2HPO_4: 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 g L^{-1} in batch operation, while the continuously operated MFCs were fed with medium containing sucrose at a concentration of 0.1 g L^{-1}. All media preparations were autoclaved at 121°C for 15 min, except for the vitamins, mineral and sucrose solutions that were filter-sterilized through a 0.2 μm pore size membrane (Nalgene SFCA membrane, USA).

129 **MFC configuration.** The single-chamber MFCs consisted of anode chambers (9 cm^3) and
130 cover plates made of Perspex, with stainless steel metal plates serving as a contact between the
131 cathode and the electrical circuit. The anode electrode contained a carbon fibre veil (PRF
132 Composite Materials, UK) with PVA (polyvinyl alcohol) binder, with a geometric area of 32
133 cm^2, which was placed inside the anode chamber and connected to an electrical circuit with an
134 insulated Ni/Cr wire (Advent Research Materials, UK) knitted across the multi-layered anode.
135 The air-breathing cathode consisted of Type A carbon cloth (9 cm^2, E-TEK) coated with 4 mg
136 cm^{-2} of Pt black catalyst with PTFE (polytetrafluoroethylene) binder. The platinum side of the
137 cathode was painted with 0.5 – 1.0 mg cm^{-2} of Nafion perfluorinated ion-exchange ionomer (5%
138 w/v dispersion in lower aliphatic alcohols and H_2O, Aldrich). A Nafion-115 proton exchange
139 membrane (20 cm^2, DuPont) was pretreated by sequential boiling for 1 h in: 6% w/v H_2O_2, H_2O,
140 0.5 M H_2SO_4 and H_2O and subsequently stored in deionised water in the dark before assembly
141 between the anode chamber and cathode.
142
143 **MFC operation.** Three replicate MFCs were started up by suspending anaerobic digester
144 sludge in sucrose-containing medium at a 10% volume ratio. The MFCs were operated in batch-
145 mode during the initial enrichment period (approximately 2 weeks). During that time the anodic
146 suspension was repetitively replaced (5 times) initially by mixing (1:9) anodic suspension with
147 fresh N_2-purged sucrose-containing medium, and then after one week by replacing the entire
148 volume of the anodic suspension with fresh medium. MFCs were operated in batch-mode until
149 repeatable cycles of voltage generation were observed. In continuous mode, medium was
150 supplied to MFCs at a flow rate of 0.18 mL min^{-1} and purged with N_2 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 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 (%) 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 mean ± SEM (standard error of the mean), where n is the number of samples during the corresponding time interval, measured in duplicate. Chemical oxygen demand (COD_{Cr}) was analysed according to a standard method SFS 5504 (SFS 5504 1988). Coulombic efficiency (CE) 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. Polarization curves were recorded with decreasing external resistances (700 kΩ – 500 Ω, for a maximum of 5 min for each resistance) and

174 measuring the decrease in voltage. The volumetric power density was calculated as $P = UI/V$,
175 where U is the measured voltage, I is the current and V is the liquid volume of the anode
176 chamber. Electrochemical impedance spectroscopy (EIS) was used to measure the ohmic internal
177 resistance of MFCs. Impedance spectra were recorded between the anode and cathode (two-
178 electrode mode) with a Solartron Analytical 1260 frequency response analyzer operating with a
179 Solartron Analytical 1287 potentiostat/galvanostat (Solartron Analytical, UK) in the frequency
180 range of 0.1 Hz – 1 MHz and with a sinusoidal perturbation of 10 mV amplitude under open
181 circuit voltage (Zhao et al. 2008).
182
183 **Microbial community analysis.** Total DNA was extracted directly from either a sample of
184 anode electrode (approximately 1 cm^2) or 1 mL of anode suspension using FastDNA Spin Kit for
185 Soil (MP Biomedicals, UK). For sampling of the anode electrode, each MFC was temporarily
186 disassembled in an aseptic environment, a 1 cm^2 anode sample cut out using a sterile scalpel, and
187 the MFC re-assembled. Prior to DNA extraction, the anode suspension samples were centrifuged
188 (10,000*g*, 5 min), washed three times with 1 mL PBS (phosphate-buffered saline; 8.0 g L^{-1} NaCl,
189 0.2 g L^{-1} KCl, 1.15 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} KH_2PO_4, pH 7.3; Oxoid, UK) and resuspended in
190 100 µL of nuclease-free water (Promega, UK).
191 The partial bacterial and archaeal 16S rRNA genes were amplified and DGGE performed as
192 previously reported (Kim et al. 2011), with a denaturing gradient ranging from 40% to 70%.
193 Single samples were used in this study, as high similarity was previously observed in duplicate
194 DGGE profiles.
195 The DGGE gels were analysed using image analysis software (Gel2k, v. 1.2.0.6; Norland
196 2004; 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 chimeras, 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 (MVSP, Multi-Variate 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 *Change (%) = 100 – 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 ± 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 kΩ at the beginning and 4 – 6 kΩ at the end of the experiment. Typical polarization 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 (Fig. 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 and 1c). The results are presented as average values (± SEM) of MFC replicates. The consumption of sucrose was high, being 94 ± 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 ± 4.5 Ω (n = 3, number of MFCs) when measured after inoculation with sludge, and 17 ± 6.3 Ω (n = 3) when measured with medium only. At the end of operation, the ohmic internal resistance was 15 ± 1.4 Ω (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 – 5, S4 – 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 zero) 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 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 and S4 – 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 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 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 characterize 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

311 Marzorati et al. (2008), *Rr* values between 10 and 30 correspond to medium range-weighted
312 richness.

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

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

324

325 **Discussion**

326 The aim of this study was to relate the performance of MFCs to the composition and dynamics of
327 the anodic bacterial communities. The reproducibility of sucrose-fed MFCs was studied in terms
328 of performance and development of microbial communities using replicate MFCs. Semi-
329 quantitative community analysis was based on band intensities of DGGE community profiles.
330 DGGE allowed the assessment of relative composition (proportion trends) of a microbial
331 community present in the amplicon pool.

332

333 **MFC performance.** Our study shows that the performance of the MFCs presents a degree of
334 reproducibility in terms of power density, sucrose consumption and effluent pH (Fig. 1). The
335 maximum power density obtained compare well with previous reports for sucrose-fed MFCs,
336 where power outputs of similar order of magnitude were observed (He et al. 2005; Kim et al.
337 2011).

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

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

353

354 **Bacterial community analysis.** The bacterial community composition and dynamics of the
355 anodic biofilm and suspended culture were studied in replicate MFCs by PCR-DGGE. The

356 species present were identified on the basis of the 16S rRNA sequencing and comparison with
357 their closest plausible relative. Cluster analysis of both the anodic biofilm and suspension
358 samples revealed that the bacterial communities evolved differently in the replicate MFCs. The
359 similarities between anodic biofilm communities of replicate MFCs were 33-46% at the end of
360 operation. This is low compared to the results by Aelterman et al. (2006), showing high
361 reproducibility (≥97%) of bacterial biofilm communities in an acetate-fed stack MFC consisting
362 of six individual MFCs after 200 days of operation. The bacterial communities of two
363 thermophilic acetate-fed MFCs were found to be similar (>89%) to each other after 100 days of
364 operation (Wrighton et al. 2008). The reproducibility of duplicate sucrose-fed tubular MFCs,
365 evaluated by cluster analysis and Jaccard's coefficient, showed 80-90% similarity in species
366 composition (Kim et al. 2011). The difference observed between our results and other MFC
367 studies could be attributed to differences in reactor design, operational conditions and whether
368 inoculum was acclimatized prior to the start-up of replicate reactors (Wrighton et al. 2008;
369 Kaewpipat and Grady 2002). The reproducibility of the community could also be affected by
370 reactor size. In the present study the volume of the anode chamber was one to two orders of
371 magnitude lower than in the above mentioned studies. The lack of reproducibility of bacterial
372 communities has been reported in other studies of biological wastewater treatment (Fernández et
373 al. 2000; Gentile et al. 2007; Kaewpipat and Grady 2002) and has been attributed to the chaotic
374 dynamics of bacterial communities, according to which a small change in conditions can cause
375 communities to diverge (Curtis and Sloan 2004; Kaewpipat and Grady 2002). It has been
376 suggested that chaotic behaviour of bacterial communities is associated particularly with small-
377 scale wastewater treatment systems, whereas large-scale biological treatment systems present
378 less dynamic behaviours (Smith et al. 2003; Curtis and Sloan 2004). HRT could be another

379 factor affecting the community composition and the predictability of biological processes with
380 continuous-flow and without recycle. Further studies on the effect of HRT on the anodic
381 community dynamics and performance of MFCs are warranted.

382 In this study, no firm correlation common to all replicate MFCs was found between the
383 relative proportion of any one bacterial species and the maximum power output (Table 2). The
384 observed shifts in the relative proportions of fermentative bacteria within the anodic biofilm of
385 each MFC suggests temporal changes among species with metabolic similarity, namely the
386 succession from *A. mobilis* to *C. indolis* in MFC-A, from *B. graminisolvens* and *D. mossii* to *C.*
387 *indolis* in MFC-B and from *B. graminisolvens* and *C. indolis* to *A. mobilis* and *Bacteroides* sp. in
388 MFC-C (Figs. 5a and S4) (Jeong et al. 2007; Lawson et al. 2002; Nishiyama et al. 2009).
389 Dynamic changes among fermentative bacteria were also detected in the anodic suspension
390 (Figs. 5b and S5). At the end of operation, *C. indolis* was the predominant fermentative
391 bacterium in the anodic biofilm of MFCs A and B, whereas *A. mobilis* dominated in MFC-C
392 (Table 1). The most abundant non-fermentative species were *D. vulgaris* and *C. denitrificans* in
393 MFC-A, *Rhodocyclus* sp., *C. denitrificans* and *O. antropi* in MFC-B and *A. cryaerophilus, C.*
394 *denitrificans* and *D. vulgaris* in MFC-C (Table 1), all of them able to use organic acids, and in
395 the case of *Rhodocyclus* sp. and *D. vulgaris*, also H_2, a product of sucrose fermentation
396 (Gumaelius et al. 2001; Smith et al. 2005; Vandamme et al. 1992; Zuo et al. 2008; Holt 1994). *C.*
397 *denitrificans* and *O. antropi* represent known exoelectrogenic species (Zuo et al. 2008; Xing et
398 al. 2010). The appearance or increase in relative proportion of *C. denitrificans* in the anodic
399 biofilm of all MFCs by day 28, accompanied by the disappearance of a species belonging to the
400 same genus but without denitrifying capability (Wauters et al. 2003) (Figs. 5a and S4), strongly
401 suggests 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-oxidizing, 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 suspension showed high similarity. As this continuous-flow system is fed with sterile medium, the community in 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 (≥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 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., *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. (See Supplementary information for details of composition dynamics of Archaea). The hypothesized importance of denitrifiers (*Comamonas denitrificans, Ochrobactrum 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 exopolysaccharides 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

447 air during biofilm sampling, which may have affected the low redox environment required by the
448 species. Also, *Geobacter* is not able to grow using sucrose as substrate, preferring volatile fatty
449 acids such as acetate. Other studies have reported the lack of dominance of *Geobacter* spp. in
450 MFCs (Rabaey et al. 2004; Aelterman et al. 2006).

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

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

462 The external resistance was same throughout the study, except during the measurement of
463 polarisation curves. In a separate study, we have operated an MFC where the cell voltage was
464 optimized throughout the experiment to control operation at maximum sustainable power output
465 (V = ½ OCP, according to (Ieropoulos et al. 2008). However, this optimization did not have
466 significant effect on maximum power output or community composition obtained (data not
467 shown). Other studies have used similar external resistance values compared to our study
468 amongst others (Katuri et al. 2011) and reported that power densities and community activity
469 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 characterized 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 − 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 characterized 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

493 reproducibility is essential to obtain meaningful conclusions in studies of microbial communities
494 in MFCs. No consistent correlation was found between the presence of anodic or suspended
495 bacterial species and the power density in the replicate MFCs. However, in all replicate MFCs
496 putative exoelectrogenic denitrifiers and sulphate-reducers accounted for approximately 24 % of
497 the bacterial biofilm community at the end of the study. Pareto-Lorenz curves of all MFCs over
498 time were suggestive of community structures where shifts between functionally similar species
499 occur, as observed within the dominant fermentative bacteria, and where stable MFC
500 performance is likely to be maintained in changing environmental conditions. In addition, the
501 anodic communities that developed in replicate MFCs produced similar levels of power although
502 the community composition would differ, suggesting that the anodic performance is not
503 determined by the phylogenetic identity of species. Previously in the field of biological fuel
504 cells, the cathodic bacterial community structure (richness and evenness) rather than the
505 phylogenetic affiliation has been correlated with cathodic performance (Wrighton et al. 2010).
506 Changes in the microbial community composition, where specific species with redox
507 systems best suited for using the anode at specific potential would outcompete other species and
508 increase in proportion in the anode surface, did not seem to be the means of microbial adaptation
509 in the present study. The bacterial communities developed into diverse consortia with functional
510 internal community structure in all MFC replicates. This finding further suggests that several
511 types of bacteria can adapt to generate electricity in MFCs. The universality of exoelectrogenic
512 ability would be supported by the large diversity of bacteria in MFCs reported in the literature,
513 the high number of species detected that are distantly related to any known cultured organisms
514 by their 16S rRNA sequence and the abundance of bacteria with different types of metabolism
515 that are found to be exoelectrogenic in pure culture. Further research is required to clarify the

516 means of microbial adaptation in MFCs in order to engineer systems able to achieve power
517 levels suitable for practical applications.

518

519

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752 **Figure 1.** Time course plots of (a) maximum power density of MFCs A (◆), B (■) and C (○) in
753 continuous-mode. The Nafion membrane and cathode were replaced in each MFC after
754 indication of fouling on days 41, 56 and 58 for MFCs A, B and C, respectively. The increase in
755 power density between the values marked with brackets was due to the replacement of Nafion
756 and cathode; (b) total carbohydrate concentration of influent (●) and effluent (○) of replicate
757 MFCs (data shown as mean ± SEM; n = 3 MFCs, averages of duplicate measurements); (c)
758 effluent pH of replicate MFCs in continuous-mode with linear regression (data shown as mean ±
759 SEM; n = 3 MFCs).
760
761 **Figure 2.** DGGE profile of bacterial communities of the anodic biofilm (a) and suspended
762 culture (b) analyzed by DGGE of PCR-amplified genes coding for 16S rRNA; Std: Standard
763 mix; Inoc: anaerobic sludge; *: chimeras. Numbers indicate bands excised for sequence analysis.
764 The 16S rRNA genes of bands marked with -‘- were reamplified by PCR and run on another
765 DGGE gel (Fig. S3) due to insufficient quality of sequences obtained. Same numbering is used
766 for identical sequences. Unlabelled lanes correspond to a replicate MFC sampled at different
767 time-points, and therefore not included in the analysis.
768
769 **Figure 3.** Cluster analyses of bacterial community profiles of anodic biofilm (a) and suspended
770 culture (b). Each node in the tree (indicated by reactor name and operation day) represents one
771 lane in DGGE gel. The trees were generated using Jaccard similarity coefficient and UPGMA
772 clustering algorithm.
773

774 **Figure 4.** Phylogenetic tree based on partial bacterial 16S rRNA gene sequences (named *MFC-*
775 *DGGE band nr*) of anodic biofilm and suspended culture of three replicate sucrose-fed MFCs.
776 Sequences found only in biofilm or suspension are marked with 'b' or 's', respectively. The tree
777 was constructed using a distance matrix and neighbour joining algorithm with 1,000
778 bootstrappings. The bootstrap values ≤ 99% are shown. The archaeon *Methanosarcina barkeri*
779 (AJ012094) was used as an out-group. The scale bar represents 10% sequence divergence.
780
781 **Figure 5.** Proportion trends of the most abundant bacterial species in the anodic biofilm (a) and
782 suspension (b) of MFC-C based on band intensities of DGGE community profiles and
783 identification of excised bands by sequencing and comparison to known 16S rRNA sequences in
784 Genbank.
785
786 **Figure 6.** Time course plots of the range-weighted richness (*Rr*) of (a) bacterial anodic biofilm
787 and (b) suspended culture communities of MFCs A (◆), B (■) and C (○).
788
789 **Figure 7.** Pareto-Lorenz distribution curves derived from DGGE patterns of the anodic biofilm
790 bacteria of MFCs A, B and C on days 14, 28, 41, 56, 70 and 91. Arrows indicate the range of
791 cumulative band intensities corresponding 20% of the bands.
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796 **Table 1.** Relative proportions of the most abundant (>5%) bacterial species found in the anodic
797 biofilm and suspension of MFCs A-C at the end of operation.

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

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

803

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

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808 **Figure 1**.

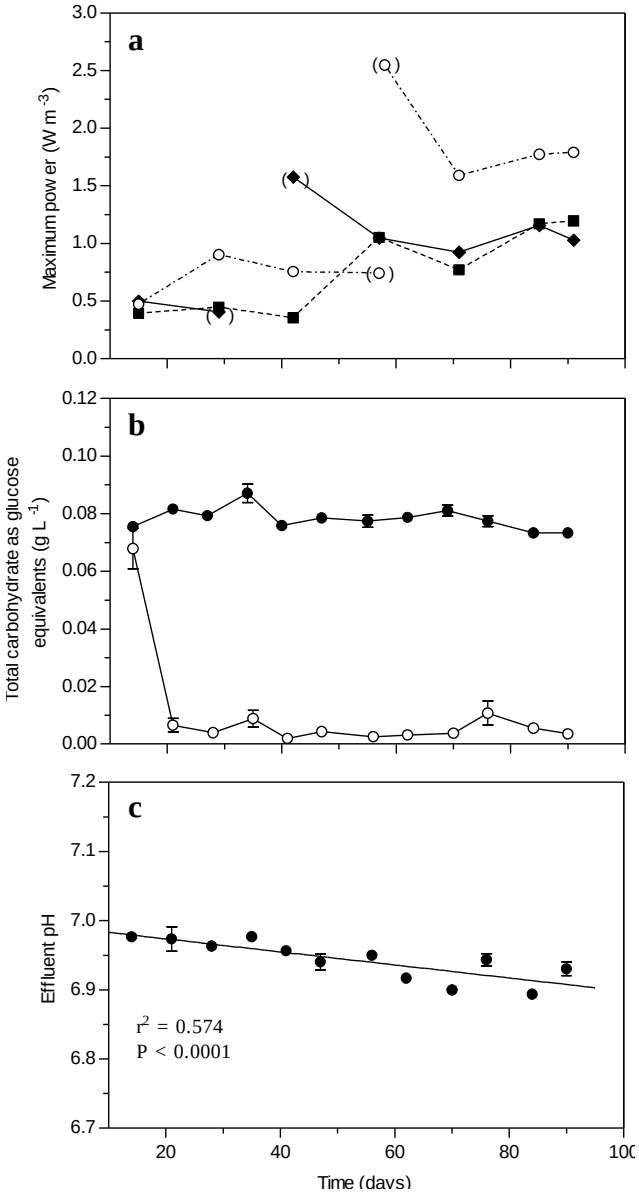

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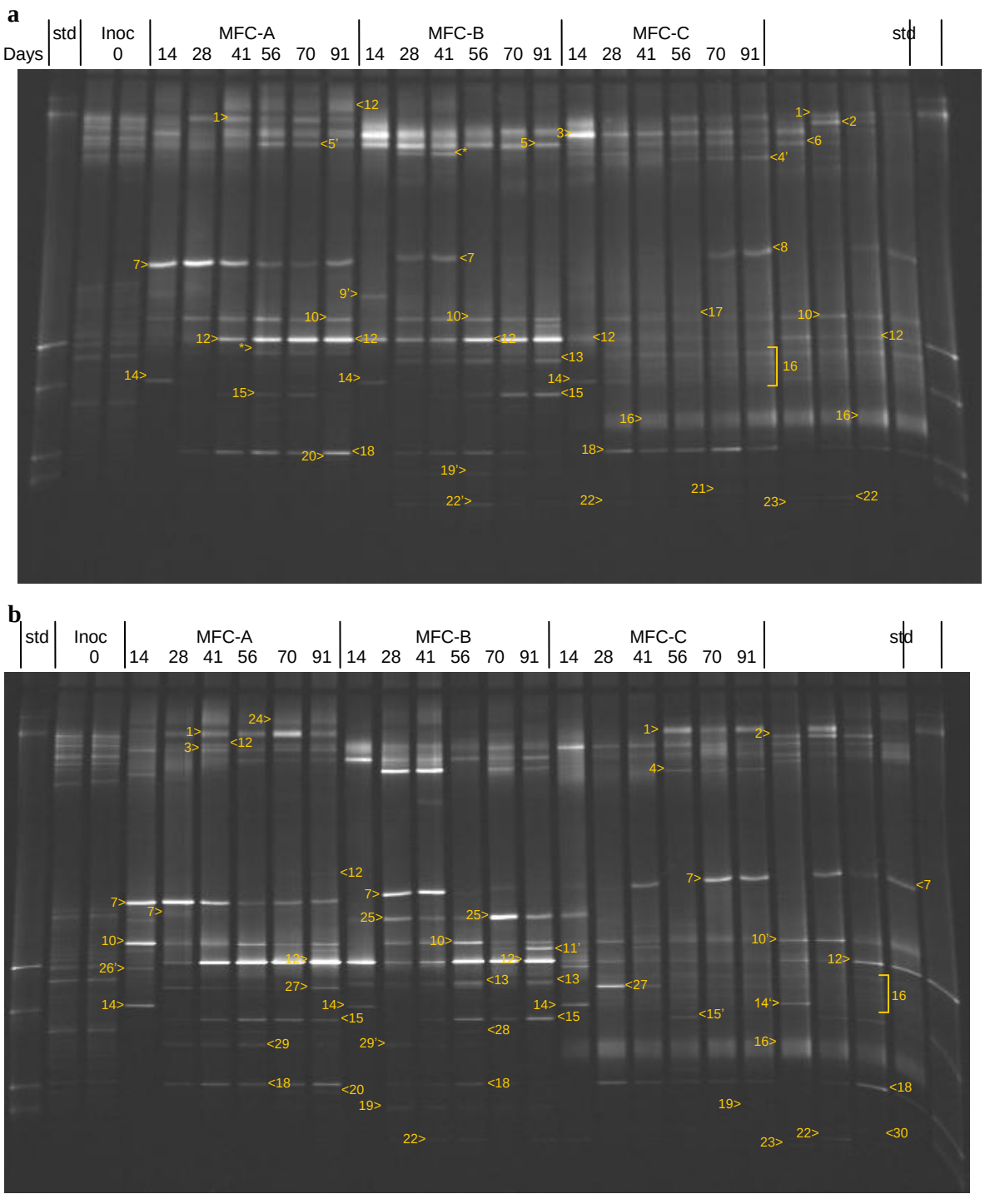
Figure 2.


a
Days
std
Inoc
0
MFC-A
14 28 41 56 70 91
MFC-B
14 28 41 56 70 91
MFC-C
14 28 41 56 70 91
std
b
std
Inoc
0
MFC-A
14 28 41 56 70 91
MFC-B
14 28 41 56 70 91
MFC-C
14 28 41 56 70 91
std

818

819 **Figure 3.**

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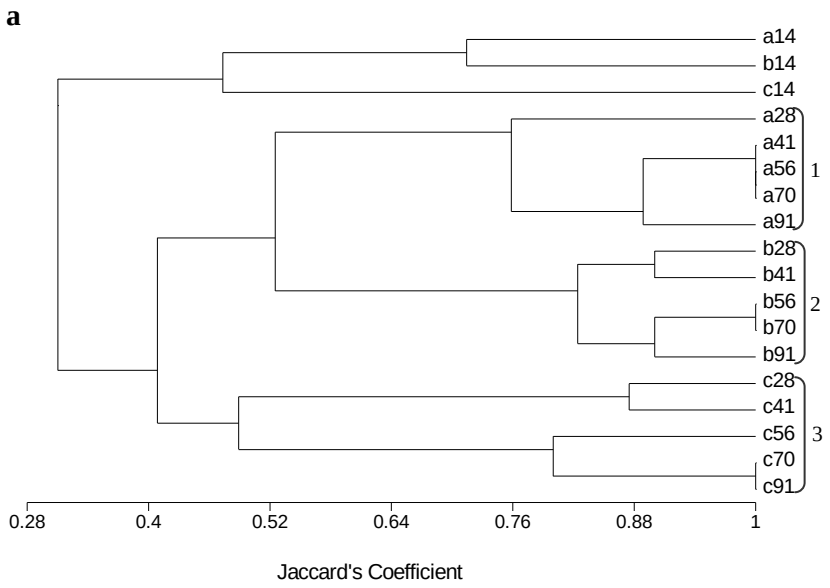

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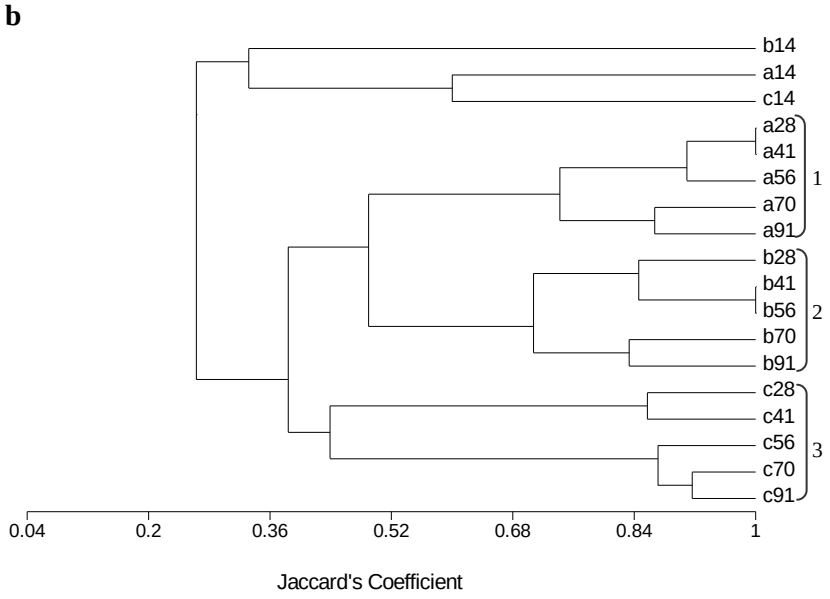

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827 **Figure 4.**

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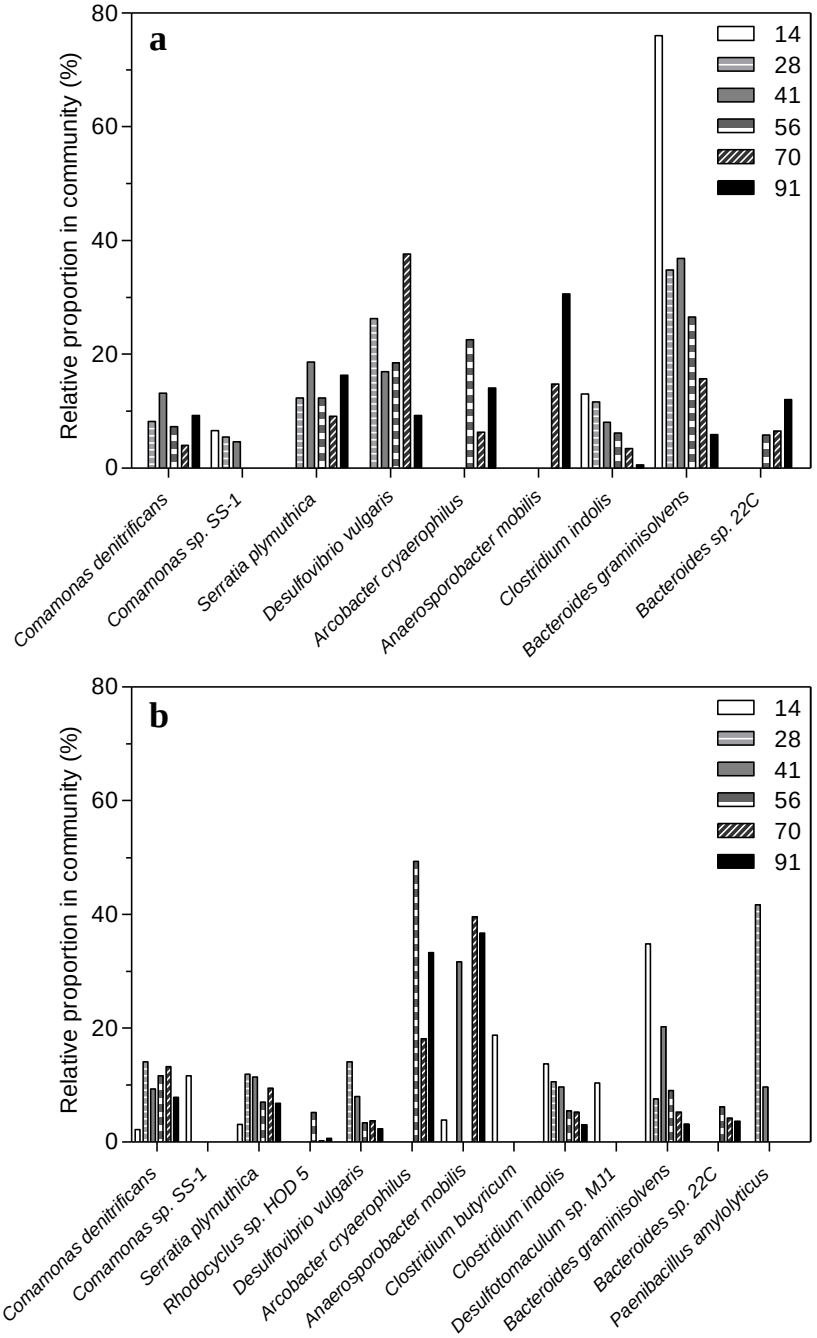
Figure 5.


a
b
Relative proportion in community (%)
0
20
40
60
80
14
28
41
56
70
91
Comamonas denitrificans
Comamonas sp. SS-1
Serratia plymuthica
Rhodocyclus sp. HOD 5
Desulfovibrio vulgaris
Arcobacter cryaerophilus
Anaerosporobacter mobilis
Clostridium butyricum
Clostridium indolis
Desulfotomaculum sp. MJ1
Bacteroides graminisolvens
Bacteroides sp. 22C
Paenibacillus amylolyticus

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839 **Figure 6.**

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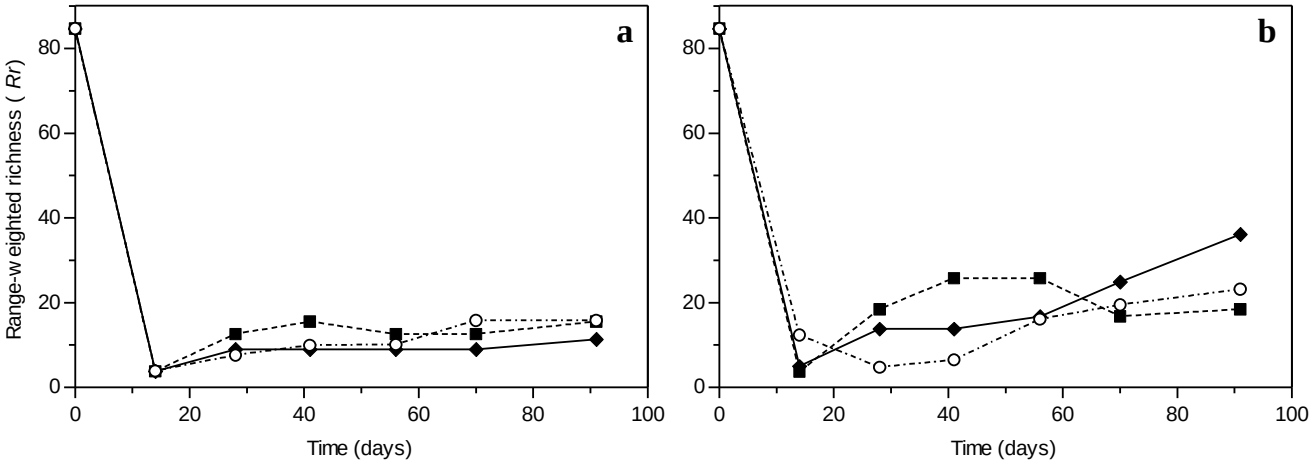

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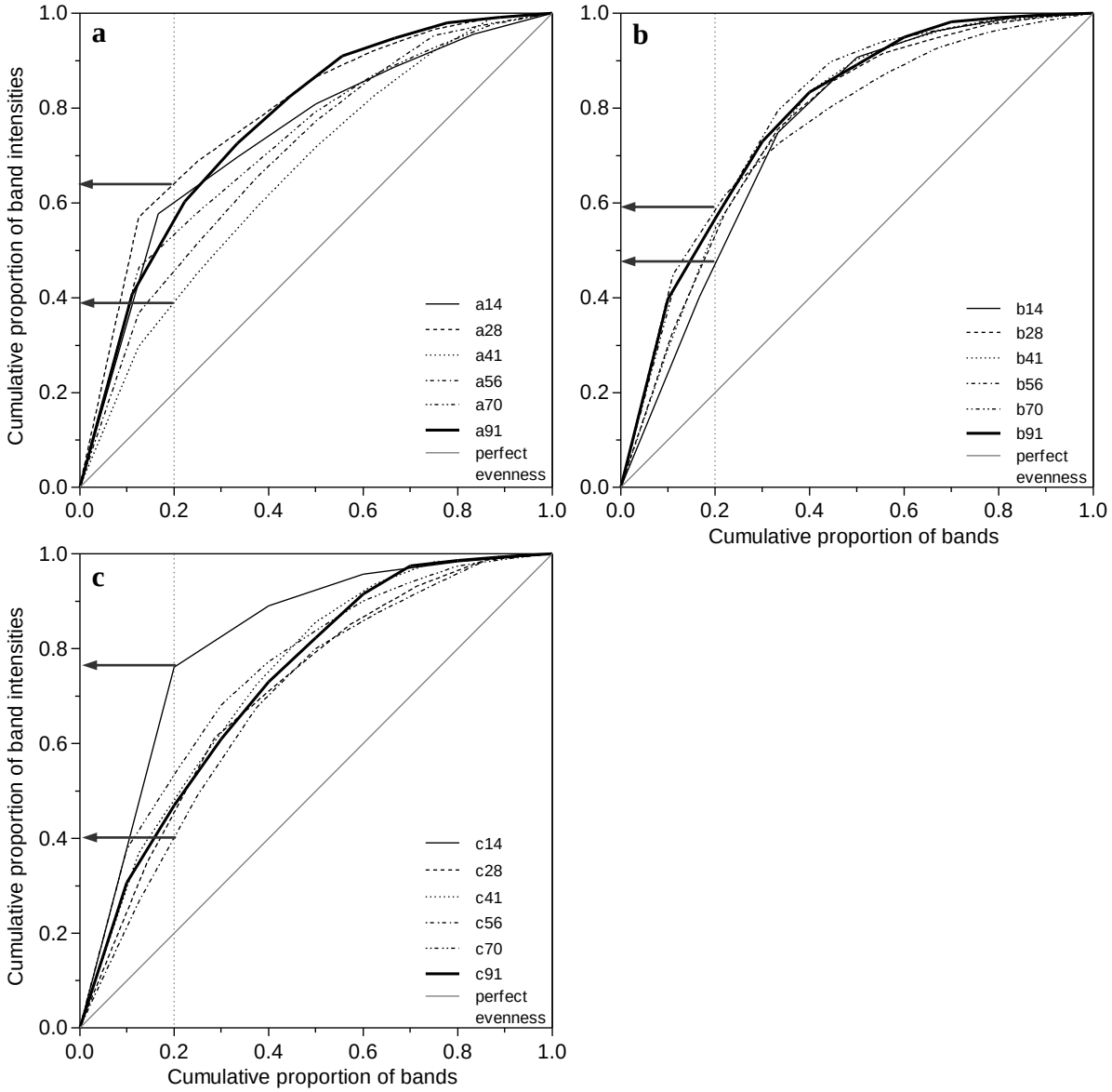
Figure 7.


a
b
c
Cumulative proportion of band intensities
Cumulative proportion of bands
a14
a28
a41
a56
a70
a91
perfect evenness
b14
b28
b41
b56
b70
b91
c14
c28
c41
c56
c70
c91
0.0
0.2
0.4
0.6
0.8
1.0