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Monophyletic group of unclassified γ-*Proteobacteria* dominates in mixed culture biofilm of high-performing oxygen reducing biocathode

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ABSTRACT

Several mixed microbial communities have been reported to show robust bioelectrocatalysis of oxygen reduction over time at applicable operation conditions. However, clarification of electron transfer mechanism(s) and identification of essential micro-organisms have not been realised. Therefore, the objective of this study was to shape oxygen reducing biocathodes with different microbial communities by means of surface modification using the electrochemical reduction of two different diazonium salts in order to discuss the relation of microbial composition and performance. The resulting oxygen reducing mixed culture biocathodes had complex bacterial biofilms variable in size and shape as observed by confocal and electron microscopy. Sequence analysis of ribosomal 16S rDNA revealed a putative correlation between the abundance of certain microbiota and biocathode performance. The best performing biocathode developed on the unmodified graphite electrode and reached a high current density for oxygen reducing biocathodes at neutral pH (0.9 A/m^2). This correlated with the highest domination (60.7%) of a monophyletic group of unclassified γ-*Proteobacteria*. These results corroborate earlier reports by other groups, however, higher current densities and higher presence of these unclassified bacteria were observed in this work. Therefore, members of this group are likely key-players for highly performing oxygen reducing biocathodes.

1. Introduction

Microbial fuel cells (MFCs) are emerging technologies which treat wastewater and generate electricity [1]. Besides, MFCs can be fuelled by anoxic sediments or by the rhizodeposits of living plants which e.g., can power sensors at remote locations [2–4]. A MFC usually consists of two compartments separated by a membrane [5]. Electrochemically active bacteria oxidise organic matter in anaerobic conditions and deliver electrons to the anode which flow via a load to the cathode. At the cathode a reduction reaction must take place. Here, oxygen reduction is attractive as it has a high redox potential, oxygen is widely available, and the reaction can be catalysed by a self-maintaining bacterial biofilm attached to a carbon electrode [6,7]. The open access of micro-organisms to the electrode and the availability of nutrients from the environment are two boundary conditions which allow operation of mixed culture oxygen reducing biocathodes. Performance of oxygen reducing biocathodes is determined by a wide range of parameters which include: cathode (over)potential, type of biocatalyst (enzyme, pure culture or mixed culture) [8], mass transfer of reactants and products, availability of essential nutrients, buffer capacity, temperature, electrode configuration and materials [6,9–13]. While biological oxygen reduction is a common physiological trait, i.e., micro-organisms employ oxygen as the terminal electron acceptor in their metabolism, the mechanism(s) of extracellular electron transfer coupled to microbial oxygen reduction and the natural occurrence of this type of physiology is still to be revealed [14,15]. In several studies, species from oxygen reducing biocathodes have been isolated and identified [15–20]. However, in most cases the catalytic effect (i.e., a high current density and/or lowering the cathodic over potential) was poor and/or the biofilm was poorly developed. So far, high performing oxygen reducing biocathodes reached current densities up to 0.6 A/m^2 [6]. Effective use of electrode surface and high-potential cathodic catalysis is essential for MFCs in energy generating applications.

Recently, it was shown that some modifications of the anode electrode surface resulted in a power output at most two times higher than an unmodified anode. Fluorescence in situ hybridization (FISH) revealed that the nature of anode modification and/or degree of the modification affected the biofilm density and composition [21]. To what

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extent the improved power output was due to differentiation in the biofilm and/or to the modifier, which e.g., could affect charge transfer resistance, remains to be clarified. Notably, this study revealed that the modified electrode outperformed the unmodified electrode and that these surface modifications can be used to create selection pressure on an initial microbial inoculum while aiming to achieve optimized mixed culture bio-electrodes. The technique of surface modification using electrochemical reduction of aryl diazonium salts is versatile for covalent modification of graphitic carbon electrodes. This technique allows grafting of a wide range of groups with different chemical and physical properties at different thicknesses on the electrode for usage as anode and cathode [22].

The objective of the present study was to shape different microbial communities at oxygen reducing cathodes by means of surface modification to discuss the relation of microbial composition and performance. Modified cathode surfaces led to the development of different microbial community biofilms and biocathodes with distinct performances. Microbial analysis allowed discussion of the complexity, spatial and microbial composition of the biofilm. Interestingly, the highest current density (i.e., biocatalytic effect) was reached with the biocathode that was not modified and that displayed the highest microbial selectivity. The amplicon pyrosequencing and denaturing gradient gel electrophoresis (DGGE) analysis of the 16S rRNA gene showed that this enriched catalytic biofilm contained a microbial species that has not been cultured so far.

2. Materials and methods

2.1. Electrochemical set-up and operation

Five bioelectrochemical setups were used [9]. Each set-up consisted of an anode and a cathode made of non-porous graphite plates (geometric surface area: 22 cm^2) separated by a cation exchange membrane (re-inforced membrane Fumasep-FKB, Germany). The catholyte and anolyte used have been described previously [9]. Both catholyte and anolyte were recirculated at a rate of 12 L h^{-1}. The biocathode was inoculated with fresh aerobic sludge (Wastewater treatment plant Bennekom, The Netherlands) and was started under air saturated conditions (oxygen concentration ~7.5 mg/L) using air pumps. The cathode potential was fixed at +0.150 V vs. Ag/AgCl using a potentiostat (IVIUM, The Netherlands). The pH was controlled at 7.0 ± 0.5 as previously described [8]. Water evaporated from the catholyte was replenished using demineralised water. Unless stated otherwise, all potentials are reported versus the chloride saturated Ag/AgCl reference electrode (+197 mV vs. Standard Hydrogen Electrode). The reference electrode was connected to a Luggin-Haber capillary which was positioned in the centre of the flow through cathode compartment. Distance of capillary tip to the cathode electrode was 4 mm.

2.2. Electrochemical surface modification

Four graphite plates (Müller & Rössner GmbH & Co, Germany) have been modified using the electrochemical reduction of two diazonium salts; 4-nitro benzene diazonium and 4-decyl aryl diazonium. Aryl diazonium salts were generated in situ in acid media (0.1 M HCl, 100 mL) containing 10 mM of the starting aryl amine and followed by addition of 20 mM sodium nitrite [23]. This solution was then directly used as the electrolyte for the modification procedure of the graphite working electrode by electrochemical reduction of the aryl diazonium salts using a potentiostat (Autolab PGSTAT302N). A three electrode cell configuration was used with a KCl saturated calomel electrode (SCE) as the reference electrode and a graphite electrode as the counter electrode. The four graphite electrodes were modified by the reduction of the two different aryl diazonium salts, in each case using 20 or 0.5 cycles from 0.4 to −0.2 V vs. SCE at a scan rate of 0.1 V/s. Nomenclature of the different electrodes used in this study and the way they have been modified are given in Table 1. Contact angle measurements (Table 1) were carried out before and after modification (Krüss, Easydrop DSA).

2.3. Scanning electron microscopy

On day 40 of the experiment, biocathode parts were removed using a chisel and hammer. Scanning electron microscopy (SEM) samples were fixed overnight with glutaraldehyde (2.5%) in 0.1 M phosphate buffer pH = 7 at room temperature. Samples were washed using phosphate buffer and successively immersed in different aqueous solutions with increasing ethanol content (60, 70, 80, 90%), then with absolute ethanol, and critical-point dried. Samples were finally coated with Au/Pd before SEM observation.

2.4. DNA extraction, PCR-DGGE

On day 40 of the experiment, biofilms were scraped off the cathodes and dissolved in filter sterilized PBS solution. Total DNA was extracted from 2 mL of liquid according to Boon et al. [24]. DNA was subsequently purified (Wizard genomic DNA purification kit, Promega) according to the manufacturer's instructions. The 16S rRNA gene was amplified by PCR with primers 338F-GC and 518R for denaturing gradient gel electrophoresis (DGGE) [25] with 5 min 94 °C initial denaturation, 30 cycles 1 min 95 °C denaturation; 1 min 53 °C annealing; 2 min 72 °C elongation and a final elongation step of 10 min at 72 °C. Separation of fragments was done with an 8% polyacrylamide gel and a denaturing gradient of 40–60% for 16 h at 120 V on an INGENY phorU2X2 system (Goes, The Netherlands). DNA was stained for 20 min with 16 μL SYBR Green in 300 mL 1× TAE buffer. Images were obtained with a UV-illuminator and a digital camera. Obtained profiles were clustered using BioNumerics software (v5.1, Applied Maths, Belgium). Theoretical ecological parameters Range weighted richness (Rr; a measure of habitability of an environment) and Community organization (Co; an approximate of functional diversity) were attributed according to the microbial resource management (MRM) toolbox [26].

2.5. 454 amplicon sequencing

The same DNA extracts isolated for DGGE were used for 454 sequencing analysis. If not otherwise mentioned, all the steps were performed according to the current Roche 454 sequencing protocol for amplicons. To generate the amplicon library for bacteria, specific primers were selected: 926-F (5′-AAACTYAAAKGAATTGACGG-3′, *Escherichia coli* position 907–926) [27], and 630-R (5′-CAKAAAGGAGGTGATCC-3′, *E. coli* position 1528–1544) [28]. These primers were extended as amplicon fusion primers with respective A and B adapters, key sequence and multiplex identifiers (MID) as recommended and tested for their optimal annealing temperature (50 °C), cycle number (30) and amount of

Table 1
Given names of cathodes and summary of modifications.

Given name	N-20	N-0.5	D-20	D-0.5	Unmodified
Aryl diazonium salt	4-Nitro benzene diazonium		4-Decyl aryl diazonium		
Degree of modification (number of applied cycles)	20	0.5	20	0.5	0
Contact angle (±3°)	58°	66°	96°	79°	70°

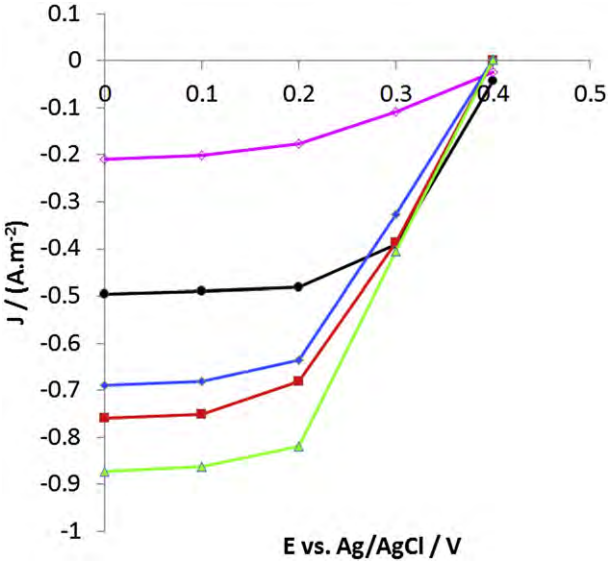

Fig. 1. Cathodic current density by surface (un)modified oxygen reducing biocathodes during polarisation (10 min equilibriation time) after 20 days of operation. Green = unmodified; red = D-20; blue = D-0.5; black = N-20; magenta = N-0.5.

template DNA (20 ng) to produce approximately 10^{12} molecules as mentioned earlier [29]. The PCR products were purified with AMPure Beads (Agencourt, Beckman Coulter, Krefeld, Germany) and pooled in equimolar amounts.

Emulsion PCR (emPCR), emulsion breaking of DNA-enriched beads and sequencing run of the amplicon pools were performed on a second-generation pyrosequencer (454 GS FLX Titanium, Roche) using Titanium reagents and Titanium procedures as recommended by the developer following protocols for bidirectional amplicon sequencing. Quality filtering of the pyrosequencing reads was performed using the automatic amplicon pipeline of the GS Run Processor (Roche) to remove failed and low quality reads from raw data and to remove adaptor sequences.

Newbler assembler v 2.3 (Roche) was used to batch sequences based on MID-identifiers and to combine corresponding sequences derived from forward and reverse reads with a similarity of 98% and an overlap of 200 bases for the bacterial sequences.

The alignment and phylogenetic analyses were performed using the ARB 5.1 software package [30]. After phylogenetic allocation of the sequences down to the family or genus level, the number of reads belonging to the same phylogenetic groups were summed up and listed as a percentage of the total sequencing reads obtained from each sample. Simpson (1-D) and Shannon (H) indices, as well as rarefaction curves were calculated using PAST [31] based on genus affiliations.

The assembled unique sequences with their phylogenetic allocation were deposited in GenBank. During the submission process the consensus sequences were inspected for chimera and putative chimera were removed from the dataset, which resulted in 750 unique sequences individually accessible under the GenBank numbers KJ599874–KJ600623 or as a set using the PopSet database (http://www.ncbi.nlm.nih.gov/).

2.6. Fluorescence in situ hybridization (FISH)

The same samples as used for the DNA extraction were used for fluorescence in situ hybridization (FISH). The samples were fixed for 2 h at room temperature [32] with 4% paraformaldehyde for Gram-negative bacteria. Hybridisation with fluorochrome (Cy5)-labelled oligonucleotide probes was carried out following the protocols described by [33] and [34] with modifications for solid samples. In brief, samples were hybridized in bottle caps not on slides with the appropriate volume of hybridization solution to submerge the samples completely. With sterile forceps they were then dipped in washing solution to remove the adhering hybridization buffer, followed by a washing step in 2 mL fresh washing buffer. For the microscopic observations, the FISH stained graphite pieces were embedded in Citifluor (Citifluor Ltd., Canterbury, UK) and observed with a confocal laser scanning microscope (CLSM).

The 23S rRNA targeted probe GAM-42a was labelled with Cy5 and always used with its unlabelled competitor BET-42a-Oligo [33]. FISH labelled samples were then stained for 10 min in the dark with the DNA binding dye Syto Orange 80 (Molecular Probes, Invitrogen, Carlsbad, USA) at a concentration of 5 μM in 10 mM Tris, 1 mM EDTA (pH 8.0) and subsequently washed twice with ultrapure water.

The fluorescently labelled cells were detected using a Zeiss LSM510. An argon ion laser supplied 488-nm illumination and a helium–neon laser provided 543 nm, which were both used for Syto orange excitation. A second helium–neon laser at 633 nm was used for Cy5. For each hybridisation probe GAM-42a labelled with Cy5 (blue) was combined with a Syto orange DNA staining (red and green). While FISH using probe GAM-42a targets only γ-*Proteobacteria* with high ribosomal content, which are those with active protein biosynthesis, the DNA binding staining Syto orange labels all bacteria. The binding of both fluorophores resulted in a bright yellow to white staining of the target cells.

3. Results

3.1. Oxygen reducing biocathodes performed best using unmodified electrodes

Oxygen reducing biocathodes developed on all electrodes. Before inoculation, background current densities at 0.15 V were lower than 10 mA/m^2. After a start-up time of 20 days, biocathodes reached stable catalytic currents ranging from ca. 200 to 900 mA/m^2 (Figs. 1 and 2). Control experiments in which N_2 gas was supplied to the developed biocathodes resulted in minimal current densities of ~10 mA/m^2 for all biocathodes i.e., a loss of >95% of activity. Moreover, control experiments in which no inoculum was added but air sparging was present also resulted in background current densities of ca. 10 mA/m^2 up to −0.1 V (data not shown). Both lines of evidence indicate that the microorganisms on the cathode were responsable for drawing a current while using O_2 as an electron acceptor. The unmodified electrode performed better compared to all other modified graphite electrodes, regardless of the surface modification (Fig. 1). The performance of the

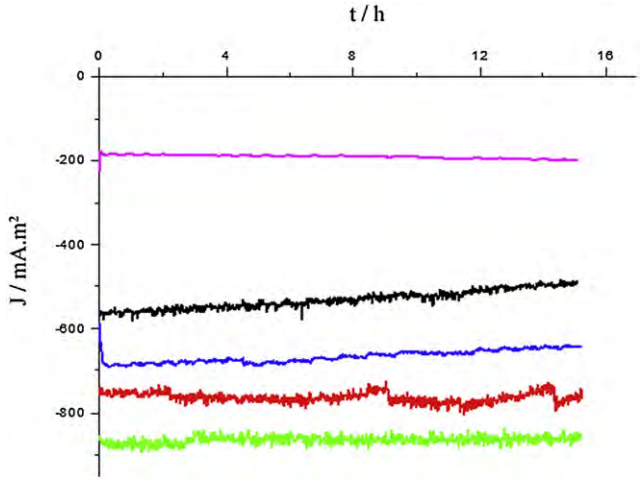

Fig. 2. Representative data of stable catalytic cathodic current densities at different oxygen reducing biocathodes operated at +0.150 V vs. Ag/AgCl after 20 days of operation. Green = unmodified; red = D-20; blue = D-0.5; black = N-20; magenta = N-0.5.

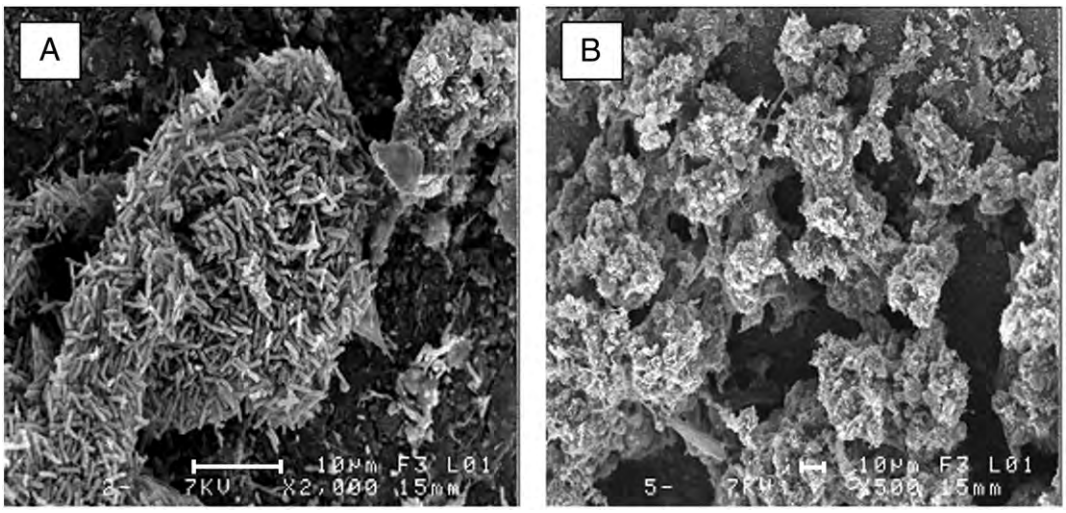

Fig. 3. Representative SEM pictures of oxygen reducing biofilms at biocathodes (A) a granule like cluster of microorganisms at the N-20 biocathode; (B) biofilm structure and coverage of the N-20 biocathode. Scale bar represents 10 μm.

unmodified biocathode was not similar to experiments applying the same inoculum source and experimental set-up and operation [6,9]. These earlier studies reached an average current range of 244 ± 53 mA/m^2 between 10 and 60 operation days. Evidently, in our study, the comparable unmodified biocathode reached a 3 times higher current density. This can be explained by a variation of the inoculum composition due to changing operational conditions of the wastewater treatment plant (WWTP) from which both inocula originated. It is well known that variable operating conditions at WWTPs result in shifting microbial populations [35,36].

3.2. Biofilms containing mostly γ-Proteobacteria developed on the electrodes

As expected from their catalytic performance, SEM analysis revealed that biofilms were formed at the unmodified as well as at the modified electrodes (Fig. 3). The biofilm (approx. 20 μm estimated from SEM) with a high density of bacteria on the electrode is likely related to the high current density. SEM observations of an oxygen reducing biocathode in earlier work showed only sparse microbial coverage [19]. In case the catalysis of oxygen reducing biocathodes is limited by the amount and activity of electrochemically active bacteria, a well-established biofilm is needed to improve the catalytic performance.

Confocal laser scanning microscopy of all five biocathode samples indicated variable biofilm thickness and coverage depending on the surface modification. From these micrographs the weakest biofilm in terms of estimated cell density and thickness (<5 μm) could be detected on the N-0.5 electrodes (Fig. 4A and Table 1 for electrode nomenclature), followed by the N-20 (about 5 μm thick biofilms) and the D-20 and D-0.5 (both about 10 μm thick biofilms). The largest biofilm with an average thickness of 15–20 μm and a maximum of up to 30 μm was detected on the unmodified graphite plate. FISH staining was positive for an active *γ-Proteobacteria* subpopulation increasingly dominating with greater biofilm thickness (Fig. 4B), which also could explain the higher performance of the biocathodes.

3.3. Microbial communities showed a clear distinction between the inoculum, the recirculation liquid and the enriched communities in the biofilms

Initial screening of the cathodic microbial communities by means of DGGE showed a marked distinction between the inoculum and the enriched microbial communities after 40 days of operation. All communities were clearly different (i.e., lower richness (Rr) and a higher organization (Co) [26]) compared to the inoculum but common bands could be detected among all communities (Fig. 5). A closer examination revealed that all well performing cathode biofilms were dominated by one common band (arrow head, Fig. 5). These communities had a low richness (max. 33) and a low evenness (min. 48). The cathodic biofilm of N-0.5 deviated from all other biofilms and clustered with all liquid samples. This indicates that neither selection nor differentiation took place during development of this community over 40 days.

Interestingly, after the unmodified electrode, hydrophobic modifications (i.e., cathodes modified with 4-decyl aryl diazonium: D-20 and D-0.5) of the cathode surface produced the highest current densities. This difference was however not directly observed in the DGGE of the biofilm. All biofilm communities, except for that developed on N-0.5 (low degree modified hydrophilic cathode), showed a similar microbial community composition. This indicates that although the same microorganisms were present, the reasons for the performance differences need to be sought in a varying biofilm thickness and coverage associated with differing quantities of bacteria from the various groups on the electrodes. Directly related to this, the electrode modifications might also influence the performance by impacting biofilm attachment to the electrode and/or associated electron transfer kinetics.

3.4. The best performing biocathode (0.9 A/m^2) reached the highest domination (60.7%) of a monophyletic group of γ-Proteobacteria

Rarefaction curves clearly levelled off, indicating sufficient depth of the sequencing analysis (supplementary fig. S1). Analysing the sequencing data, the dominance of a yet unclassified group of bacteria belonging to the *γ-Proteobacteria* was most striking. It was found to be highly abundant in the biofilm samples from the 4 best performing cathodes (N-20, D-0.5, D-20, unmodified) with a relative abundance of up to 60.7% detected for the best biocathode (unmodified) which reached a current density of 0.9 A/m^2. In the biofilms on this unmodified electrode also Simpson and Shannon indices were lowest compared to all other samples. Accordingly, the overall highest Simpson and Shannon index values for all analysed samples were found for the worst performing biocathode N-05, where also only minor amounts of the unclassified *γ-Proteobacteria* group could be detected (Table 2). The dominant band in the DGGE analysis in the biofilm samples from the well performing cathodes (Fig. 5) also reflected such a dominating group and therefore supported the 454 sequencing findings.

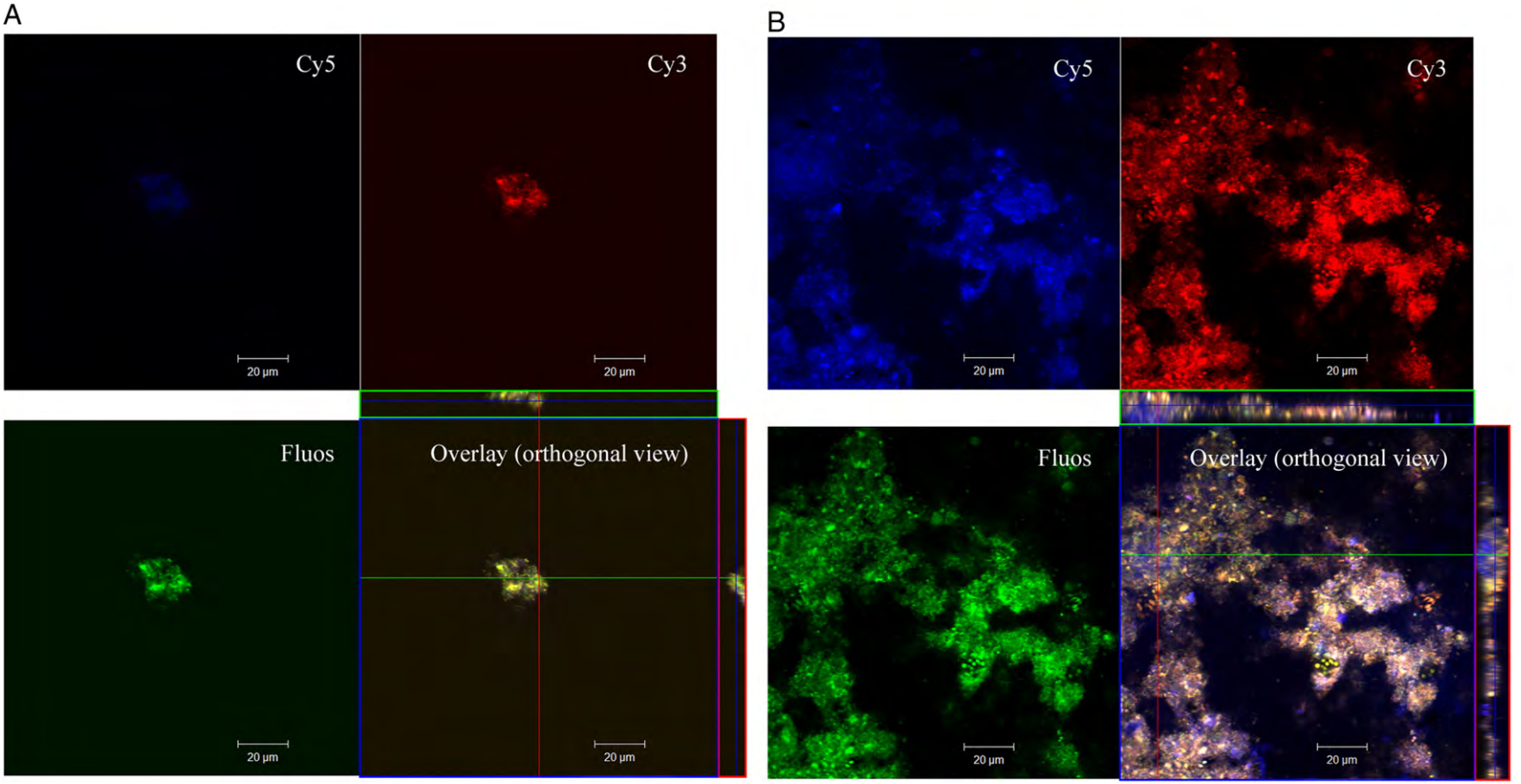

Fig. 4. Representative confocal laser scanning micrographs of graphite samples taken from the N-0.5 (A) and unmodified (B) biocathodes. FISH probe GAM-42a labelled in Cy5, detecting most members of the class γ-*Proteobacteria*, is depicted in blue (top left picture), Syto orange DNA staining in red and green (top right and bottom left pictures). The overlay (bottom right picture) is shown as an orthogonal view created from a *z*-stack of *xy*-scans. The top view, framed in blue, shows one picture from the middle of this *z*-stack. The red and green lines represent vertical optical cuts through the stack, which results in the side view images framed in red and green, respectively. In these side views the blue line marks the vertical position, where the top view image is located within the *z*-stack. The presence of fluorescent signals in all three channels (green, red, blue) identify active γ-*Proteobacteria*, while only two fluorescent colours (green and red, resulting in orange or yellow after overlay) mark all other bacteria or the inactive γ-*Proteobacteria*.

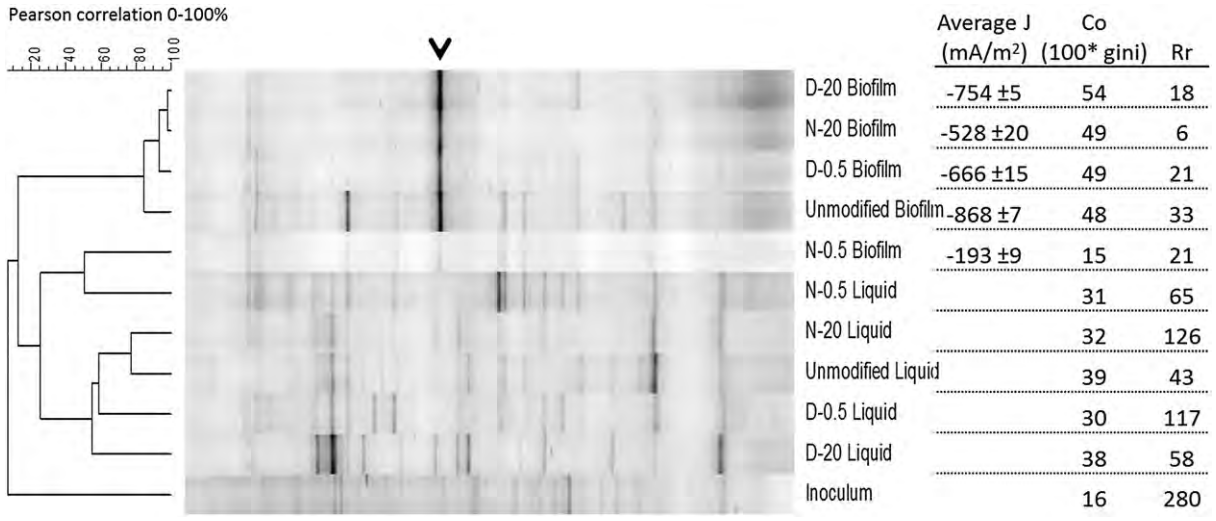

Fig. 5. Clustering of DGGE patterns of the biofilm and planktonic microbial community based on the Pearson correlation coefficient. For further explanation of band numbers see text. Microbial resource management parameters (Co; Community organization and Rr; Richness) have been calculated according to Read et al. 24.

Furthermore, there were several abundant genera (>5%) detected in the biofilm samples from the cathodes, mainly *Brachymonas* (*β-Proteobacteria*, *Comamonadaceae*), *Anaerosinus* (*Firmicutes*, *Veillonellaceae*), and *Azospira* (*β-Proteobacteria*, *Rhodocyclaceae*), but none of these were present on all biocathode samples or solely restricted to the samples of the well performing ones (see GenBank/PopSet database for details).

In the liquid catholyte samples the genus *Brachymonas* was found to be present in large numbers in all five samples as well as in the inoculum, being the most abundant in three of them (N-20, D-20, and D-0.5). The sequencing reads showed the highest similarity to *Brachymonas denitrificans*. Members of this genus are characterised as aerobic chemoorganotrophs with a strictly respiratory type of metabolism using oxygen as the terminal electron acceptor [37,38].

4. Discussion

As all sequencing results in this study were obtained from one single sampling time-point without parallels, all abundance values must be interpreted with due care. But even with this necessary caution the detected *γ-Proteobacteria* can be considered as strikingly dominant in

Table 2
Table of the 454 sequencing results with phylum and family affiliations given in percent of total reads per sample with inoculum, catholyte and biofilm samples in comparison. Values are based on one single sampling. Simpson and Shannon diversity indices based on genus affiliations are given in the bottom rows. The dominant bacterial families are marked in light grey for inoculum including catholyte, and as medium grey for biofilms. The biofilm samples containing these families with an abundance of over 10% are additionally printed in white on dark grey.

Phylum	Family	Inoc.[1]	Catholyte			Biofilm					
			N–20	D–20	unm[2]	D–0.5	N–0.5	N–20	D–20	unm[2]	D–0.5	N–0.5
		%	%	%	%	%	%	%	%	%	%	%
Acidobacteria	IncertaeSedis	1.1	4.6	1.9	3.1	6.9	11.3	3.6	3.6	11.1	8.5	4.6
Actinobacteria	*Micrococcaceae*	0	1.3	0	0.8	8.6	3.2	0	0	0	0	0
Actinobacteria	*Nocardiaceae*	0	3.3	10.7	5.0	4.6	10.3	0.6	0.4	0	3.2	2.0
Chloroflexi	*Anaerolineaceae*	3.4	0.9	0	4.4	0.7	24.6	0.5	0.4	8.6	0.3	6.8
Firmicutes	*Lachnospiraceae*	1.1	8.7	0	6.2	6.1	0	5.2	6.2	0.1	3.7	15.0
Firmicutes	*Ruminococcaceae*	0.4	8.4	0	3.6	4.4	0	4.4	4.2	0	2.6	8.7
Firmicutes	*Veillonellaceae*	0.4	8.7	0	8.0	5.4	0	6.3	7.6	0	4.2	16.3
Proteobacteria α	*Bradyrhizobiaceae*	0	0.3	4.4	0.6	1.7	0.7	0.2	0.3	0.3	1.9	0.6
Proteobacteria α	IncertaeSedis	0	1.3	0.3	1.0	0.9	0.7	0.6	0.6	0	0.2	1.5
Proteobacteria α	*Rhodobiaceae*	0.9	2.7	6.2	4.3	1.3	0.6	1.0	0	0	2.2	0.8
Proteobacteria β	*Alcaligenaceae*	6.4	0	0	0.3	0	0	0.4	0	0	0	0.7
Proteobacteria β	*Comamonadaceae*	41.0	20.5	52.4	13.7	21.9	16.4	7.9	5.9	1.9	24.6	5.3
Proteobacteria β	*Rhodocyclaceae*	10.7	10.5	5.3	11.1	11.3	3.6	9.1	10.4	2.5	10.1	9.0
Proteobacteria γ	unclassified	0.4	0	0.8	0	0.4	0	46.8	47.2	60.7	24.0	2.0
Proteobacteria γ	*Xanthomonadacea*	1.3	4.5	6.3	3.6	3.8	7.2	1.9	1.8	2.2	3.6	1.8
	Simpson (1–D)	0.90	0.95	0.89	0.96	0.96	0.88	0.75	0.74	0.57	0.90	0.96
	Shannon (H)	3.11	3.48	2.65	3.47	3.69	2.64	2.44	2.35	1.49	3.02	3.68

[1] inoculum
[2] unmodified

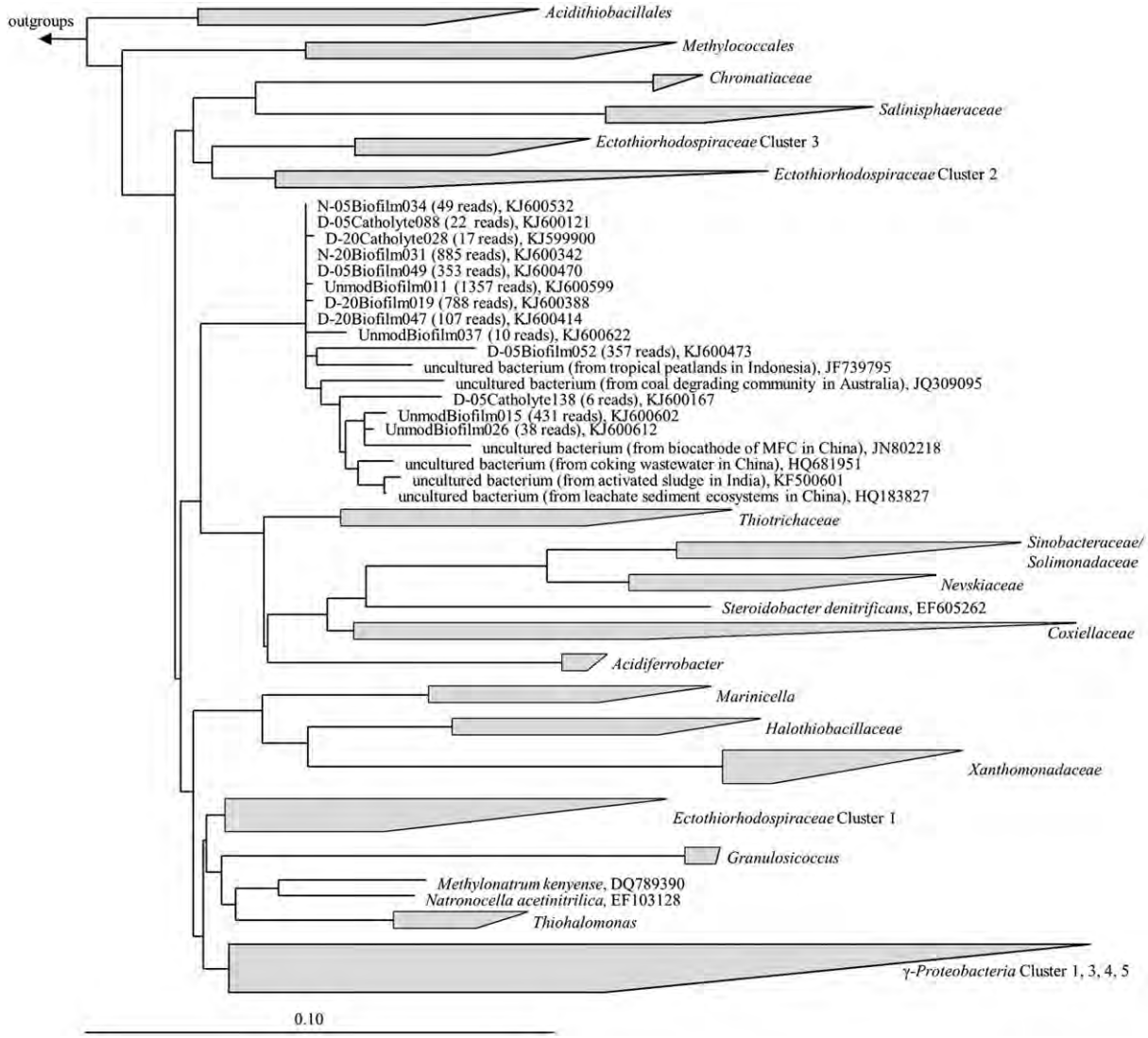

Fig. 6. Phylogenetic allocation of the dominant sequences with the ARB software package. The dendrogram showing cluster 2 of the γ-*Proteobacteria* and outgroups is based on neighbour joining tree calculation. Bar indicates 10% sequence divergence.

four out of five analysed samples. Furthermore, the found sequences in the biofilm show highest similarity to several sequences from diverse geographical origins and habitats, including a coal degrading community in Australia (JQ309095), as well as leachate sediments [39], coking wastewater [40] and also a biocathode community in China [41] (Fig. 6).

All these sequences have in common that they were derived from uncultivated bacteria forming a distinct monophyletic group within cluster 2 of the γ-*Proteobacteria*. Within this group sequence similarities all range above 96.9%, which means they most probably belong to one genus (>95%) and might even represent one single species (>97%). To the next phylogenetic relative within the γ-*Proteobacteria*, *Alkalilimnicola ehrlichii* (NR_074775.1), the highest similarity is 94.3% which is already below genus level (supplementary table S1). Therefore this group of 16S sequences might very well represent a novel but so far completely uncultivated bacterial family. However, the taxonomy of cluster 2 within the γ-*Proteobacteria* is problematic and under current change and discussion up to order level (*Xanthomonadales*) [42], which makes a correct phylogenetic assignment of newly found 16S sequences very difficult. Therefore we decided to label the found sequences as belonging to unclassified γ-*Proteobacteria* until a more permanent taxonomy has been settled.

Some bacterial genera that have already been described to be connected with oxygen reducing biofilms, like *Enterobacter*, *Pseudomonas* [17], or *Acidovorax*, *Acinetobacter* [19] as well as *Desulfobulbus* [43], were present only in minor amounts in some but not all samples. Other genera, e.g., *Roseobacter*, *Halomonas*, *Silicibacter*, *Sulfitobacter* [44], which have been detected previously in electro-active biofilms formed in seawater, were not found at all in the studied samples, which might be also explained by the absence of these species in the initial inoculum coming from a wastewater treatment plant.

The putative relationship between the high current densities (to our best knowledge, the highest current densities for flat-plate oxygen biocathodes documented so far) and high percentages of colonization by the found unclassified γ-*Proteobacteria* indicates a microorganism adapted to become dominant in our system. Likely, these microorganisms gain their energy from oxygen reduction while accepting electrons from the cathode. Since a full electron and carbon balance was not elucidated, we cannot clarify whether the indicative dominant species was autotrophic or not. The formed biofilm was estimated to be rather thick (20 μm) and appeared to be porous (Figs. 3 and 4), enabling oxygen diffusion and possibly advection within the biofilm. Biocathode research postulated that diffusion of oxygen into biofilms could limit performance assuming biofilm thicknesses of beyond 56 μm [9]. Since the biofilms in this work were thinner, it is expected that oxygen could reach bacterial cells on the cathode electrode surface. Nevertheless, denitrification was thermodynamically feasible. Thus, there might be

anoxic areas within the biofilm which could as well allow denitrification processes [45] fuelled by the nitrate transferred with the inoculated nitrifying sludge and possibly produced during nitrification of NH_4Cl from the supplied catholyte. At a current density of 400 mA/m^2 it would take 16.6 days to reduce all nitrate theoretical producible. An interplay with the nitrifying *B. denitrificans* or other nitrifying species found in the catholyte, which is clearly oxic, could provide additional nitrate for anaerobic denitrification in the anoxic zones of the biofilm. However, our performed experiments lasted for 40 days and showed higher current densities >400 mA/m^2 which supports the development of an actual oxygen reducing biocathode. To confirm whether oxygen reduction indeed occurred, the catholyte was flushed with N_2 gas to create anoxic conditions. This resulted in a current drop to a minor current density of less than 10 mA/m^2 (data not shown). This demonstrates that the performance of the bioelectrochemical cell was principally a result of bioelectrocatalysed oxygen reduction. Still, bioelectrocatalytic denitrification may play a small role.

Interestingly, in the work of Xia et al. [41] very similar 16S-rDNA sequences have been obtained from oxygen reducing biocathodes operated at three different set electrode potentials. However, these sequences have been clearly misassigned to *Thiorhodospira*, a genus which is only very distantly related to the found sequences (90.9% similarity to the type species of the genus, *Thiorhodospira sibirica*, AJ006530). In the biofilms of the biocathodes described by Xia et al. [41] up to 13% of the sequences could be identified as belonging to the same group of bacteria as in this work. Furthermore, increasing current density correlated with higher abundance of these sequences, although the dominance was not as pronounced as in the present system and *Bacteroidetes* were in that case more numerous. The current density in the system of Xia et al. [41] was lower than 370 mA/m^2 (calculated from polarisation curve Fig. 2, day 106 at 3.7 mA) compared to 900 mA/m^2 in our best biocathode. As in our lower performing biocathodes also considerably less of the specific γ-proteobacterial sequences could be found, it is tempting to speculate that the lower current density detected by Xia et al. [41] was also due to the lower abundance of these unclassified *γ-Proteobacteria*. Variations in the set potential +150 mV vs. Ag/AgCl in our study compared to +60 mV vs. SCE (approx. +17 mV vs. Ag/AgCl), −100 mV vs. SCE (approx. −67 mV vs. Ag/AgCl), inoculum (aerobic sludge compared to aerobic sludge plus previously enriched biocathode consortium) as well as the set-up and chemical surface properties of the graphite might account for the observed differences in colonization density. Wang et al. [46] and Zhang et al. [47] revealed that *Proteobacteria* were most abundant in a mixed culture oxygen reducing biocathode biofilms. These results support the likely biocatalytic role of species belonging to the genus of *Proteobacteria*. A very recent study by Rimboud et al. [48] described a similar phenomenon as was found in the present study, that the worst performing biocathodes were showing the highest bacterial diversities in their biofilm, and unclassified *γ-Proteobacteria* were claimed to be significant components of the analysed biocathodes. The use of ammonium as a nitrogen source in this work and Rimboud's electrolyte can explain the relative high abundance of *γ-Proteobacteria* since they are well-known ammonium oxidizers. The role of ammonium (and possibly oxidised N-compounds) with respect to electron transfer pathways and its role in selection pressure in oxygen reducing biocathodes needs to be further investigated.

Since in our study the same inoculum was used for each experiment, the surface modification seems to play a role in the differing bioelectrochemical performance. The given experimental results indicate that the presence of the modifier on the electrode surface had a detrimental influence on the development and performance of oxygen reducing biocathodes. It is to be stressed that although the modified electrodes all performed worse than the unmodified one, all were biocatalytic, and all modification types and extents clearly affected biocatalysis. In a previous study, dealing with the reduction of aryl diazonium salts at the anodic side of a MFC, we have shown that the type and degree of electrode modification had a critical influence on bioanode performance [21]. Indeed, the use of a high degree of modification may decrease the electron transfer rate between the modified surface and the biofilm. For this reason, two degrees of modification were applied in the present work. Practically, one way to obtain different degrees of modification consists of changing the number of recurrent cyclic voltammetry sweeps applied. As described in the methods section two degrees of modification were chosen for this study (20 or 0.5 cycles from 0.4 to −0.2 V). However, and contrary to the work reported in reference [21], the amount of modifier has not been optimized in the present study. It is possible that more modifiers is needed at the electrode to outperform the unmodified one. We nevertheless know from work reported in reference [21] that too much modifier on the surface (above optimum) impedes fast electron transfer between the biofilm and the electrode, or even its connection altogether. However, the fact that chemical modification of the electrode surface affects biocatalysis may point to a possible direct electrical connection between the electroactive microbial catalyst and the electrode as in chemolithotrophic metabolism. The identification and study of the microbial biocatalyst is needed to confirm this hypothesis. To establish conclusions on the role of the modification further parallel and duplicated experiments are necessary and will be carried out.

In many cases biofilm development of wastewater and soil isolates was shown to be increased on hydrophilic surfaces [49–51], but, in contrast, unmodified and to a lesser extent more hydrophobic surfaces were preferred by the dominant bacterial strains in this study resulting in thicker biofilms. In the work by Petrackova et al. [51] *Streptomycetes* were also found to colonize hydrophobic surfaces but this resulted in an altered protein expression. A similar phenomenon might also play a role in the selectivity of the surfaces used in this work for the specific group of *γ-Proteobacteria*, which were initially inoculated in equal amounts by using the same sludge. Especially hydrophilic surfaces do not seem to be ideal for the enrichment of these bacteria. This could be due to an influence of the surface modification on the expression of proteins involved in the electrochemical competence of this strain. Alternatively or additionally, the initial attachment of the bacteria might be hampered by hydrophilic surfaces as bacteria only tend to attach to hydrophilic surfaces if their surface energy is higher than in the suspending medium [52,53]. However, in order to verify this, an isolation of the found strain would be necessary, which was to date not successful, probably due to its very specific growth requirements.

5. Conclusions

In conclusion, we found a putative relationship between the performance of biocathodes and the abundance of bacteria belonging to a monophyletic group of unclassified *γ-Proteobacteria*. In the best performing biocathode with a current density of 0.9 A/m^2 60.7% of all found 16S rDNA sequences belonged to this bacterial group. According to the inoculation and operational conditions it is possible that the dominant species in the well performing cathodes is gaining its energy from oxygen reduction via electron transfer from the cathode. This could determine its competitive advantage in this specialized habitat. Additionally, depending on oxygen levels within the biofilms, denitrification supported by nitrification processes in an oxygen rich surrounding catholyte seems to play a minor role. Unmodified and to a lesser extent also the more hydrophobic graphite surfaces, appeared to favour biofilm development and enrichment of electrochemically active species. Most importantly, all modifications of the electrode surface negatively impacted the biocatalytic performance which would be consistent with a direct interaction between the cathode and the biofilm and with a chemolithotrophic metabolism of the microbial catalyst. Probably due to their specific cultivation demands and selective natural habitats the found cluster within the *γ-Proteobacteria* has not been well studied so far. Our findings highlight that oxygen reducing biocathodes can be an important habitat for organismic discoveries. In addition, the detailed

understanding of the microbiology and physiology of these catalytic biofilms together with the characterisation of the microbial–cathode interface may be unravelled in part by means of surface modifications. Finally, mastering the development of high current density, stable oxygen reducing biocathodes is a prerequisite for operating completely biological MFCs with microbial catalysis both at anode and cathode.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bioelechem.2015.04.004.

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