Microbial community analysis of anodes from sediment microbial fuel cells powered by rhizodeposits of living rice plants

Running title: Microbial communities on anodes from rice SMFCs

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By placing the anode of a sediment microbial fuel cell in the rhizosphere of a rice plant, root excreted rhizodeposits can be microbially oxidized with concomitant current generation. Here, various molecular techniques were used to characterize the composition of bacterial and archaeal communities on such anodes, as influenced by electrical circuitry, sediment matrix and plant presence. Closed circuit anodes in potting soil were enriched with Desulfobulbus-like species, Geobacteraceae and as yet uncultured Archaea representatives.
Living plants release substantial amounts of carbon in the soil as rhizodeposits, which are to a large extent transformed into the greenhouse gas methane in wetlands (21). It was recently demonstrated (8, 33) that the rhizodeposits can be harvested by plant microbial fuel cells (plant MFCs) and transformed into electricity. In its most straightforward form, a plant MFC is an adaptation of a sediment MFC (SMFC), which has an anode buried in (planted) sediment, allowing (microbial) oxidation of reduced compounds, and a cathode in the overlying water.

The roots and surrounding rhizosphere in a plant SMFC add an extra parameter to the as yet multifaceted SMFC system. In the present study, two molecular profiling techniques (DGGE and T-RFLP) will be applied to evaluate the effect of plant presence, support material, operation of the electrical circuit and anode depth on the bacterial and archaeal communities associated with rice SMFC anodes. Phylogenetic analysis will give further insight in their composition.

**Experimental setup and operation.** Several groups of rice planted SMFCs were set up, operated and electrochemically evaluated as previously described (8). Two series (A and B) of SMFCs were installed during subsequent summers as replication in time. Both series consisted of one group of reactors filled with vermiculite (exfoliated vermiculite, Sibli SA, Andenne, Belgium), and one with potting soil (Structural Professional type 1, M. Snebbout N.V., Kaprijke, Belgium) as support for plant growth. The potting soil was based on NPK enriched peat with a mean of 150 mg SO$_4^{2-}$ L$^{-1}$ and 20% organic substances. In the reactors of series A, two anodic carbon felts were placed at 6 and 14 cm (depth indicated as H and L) below the support surface (one anode at 6 cm in open circuit reactors). For the more extensive series B, three anodic carbon felts were placed at 5, 11 and 17 cm depth, (H, M and L). The reactors were inoculated with effluent from an acetate fed MFC (series A and B) and with a methanogenic culture (presettler of constructed wetland, Wontergem, Belgium) (only series A). At the end of the reactor runs, pH was 6.2 ± 0.6 for reactors with soil and 7.0 ± 0.5 for those with vermiculite.
Apart from the support type used (in reactor names indicated with V for vermiculite and S for (potting) soil) and the experimental period (a and b for series A and B), there were three types of reactors: 1) P-CC reactors, with plants and closed electrical circuit, allowing the harvest of electrical current, 2) NP-CC control reactors, without plants, but with closed circuit, 3) P-OC control reactors, with plants, but with open circuit (no electron harvest). Table S1 in Supplemental Material shows the overall reactor setup, nomenclature and biological replicates.

Electrochemical performance of series A and part of B was previously reported (8) and is summarized in Table S2. Vermiculite reactors only produced electricity in the presence of plants. The current output of soil reactors was 3 times higher with plants. Series B with vermiculite was not reported before. Plant growth as well as current output remained limited for this group (Table S2, factor 60 lower than series A), but was a factor 3 higher in the presence of plants.

**Molecular profiling techniques.** Anodes were removed and stored at -20°C once all plants per series had started to senesce, i.e. about 195 and 140 days after SMFC startup for series A and B respectively. For DGGE analysis, DNA from 2 g wet anode was extracted by standard methods (6). 16S rRNA gene fragments were amplified with primers P338f-GC and P518r for *Bacteria* (26), Ar3f and Ar9r, followed by Saf-GC and Parch 519r (nested PCR) (27) for *Archaea*

from series A and Arc915f and 1352ar-GC (non-nested PCR) (30) for *Archaea* from series B. PCR products were analyzed by DGGE with a denaturing gradient from 45 to 60% for *Bacteria* (8% acrylamide, 16h at 38V) (5) and 55 to 70% for *Archaea* (7% acrylamide, 30 min at 40 V, 16h at 70V) (30). Gel patterns were normalized with Bionumerics 5.1 (Applied Maths). For T-RFLP analysis, three parallel DNA extracts were made from ~0.5 g anode using bead-beating (25), DNA was mixed and T-RFLP analysis proceeded according to Egert et al. (10). Briefly, 16S rRNA genes were amplified using primers 5’ 6-carboxyfluorescein labeled Ba27f and Ba907r for *Bacteria* and Ar109f and Ar912r for *Archaea*. Amplicons (~100 ng) were digested with
restriction enzymes *MspI* for *Bacteria* and *TaqI* for *Archaea* (Promega). Electrophoresis was
performed on an ABI PRISM 3130 Genetic Analyzer (Applera Deutschland GmbH, Darmstadt).
Electropherograms were analyzed with GeneScan 4.0 (Applied Biosystems). Only peaks from 40
to 900 bp were considered; peak heights were standardized to the minimum (9). Cluster analysis
of DGGE and T-RFLP profiles was performed with Bionumerics 5.1 and based on the Pearson
correlation matrix and UPGMA algorithm, cluster cutoff on Point-Bisectional Correlation.

**Clone libraries and band excision.** For the clone libraries, 16S rRNA genes were
amplified using primers *Ba27f* and *Ba907r* for *Bacteria* and *Ar109f* and *Ar912r* for *Archaea*.
PCR fragments were cloned using the pGEM-T Vector System II (Promega). Sequence analysis
(ADIS, Max Planck Institute for Plant Breeding Research, Cologne, Germany) of randomly
selected clones resulted in a total of 133 sequences for *Bacteria* and 52 for *Archaea*. Clone
libraries were screened for chimera using the Bellerophon server (16) and Mallard software (3);
44 putative chimera for *Bacteria* and 2 for *Archaea* were verified by fractional treeing (23) and
excluded from further analysis. The diversity coverage (35) of the libraries was 84% and 88% for
*Bacteria* and *Archaea* respectively. Alternatively, bands were excised from DGGE gels (1). The
final product was amplified without GC clamp, purified (Qiagen PCR Purification Kit),
sequenced (IIT Biotech, Bielefeld, Germany) and checked using Chromas 2.33. The sequences
obtained through cloning and band excision were compared to GenBank sequences using BLAST
(2) (NCBI, October 2009). Phylogenetic analysis of the clone sequences was conducted with
ARB software (http://www.arb-home.de): the sequences were added to the database, aligned with
the Fast Aligner tool (v. corrected 01-'04, released 01-'05) and phylogenetic trees were
constructed by fast parsimony and neighbor joining with Jukes Cantor correction. All 16S rRNA
gene sequences obtained were deposited with GenBank under accession numbers GQ458057 to
GQ458194 (clone libraries) and GQ422145-GQ422149 (DGGE bands I to V).
Molecular fingerprints – Factors affecting the microbial communities. Clustering of the fingerprint analyses of series A, consisting of SMFCs with vermiculite and with soil, revealed that the type of support had a key influence on the composition of the bacterial (Fig. 1a, b) as well as the archaeal community (Fig. 1c, d). Both supports received the same inoculum mixture. However, whereas the anodes in exfoliated vermiculite were primarily influenced by the added inoculum (direct or indirect through rice transplantation), the anodes in soil were also influenced by endogenous micro-organisms. The difference in chemical (e.g. mineral status) and physical parameters further affected the residing microbial communities.

The presence of plants was of major importance. This was especially apparent for the bacterial communities found in reactors with the inert vermiculite, where the plants were the only source of organic compounds (Fig. 1a, b, dashed arrows in absence of plants (series A) and Fig. 2e, f (larger series B)). The plant effect was to a large extent also applicable for reactors with soil (Fig. 1a, b, 2a, b), but this was only clear from DGGE (Fig. 1a) and not from T-RFLP (Fig. 1b) for series A. Plants, releasing a range of organic compounds, considerably stimulate growth of soil micro-organisms (13). Moreover, several studies suggest that plants select for taxonomic and functional groups in the rhizosphere (32). For the archaeal communities (e.g. Fig. 2g, h), an effect of plants could also be observed, be it however less pronounced. For vermiculite series B, there was a high similarity between the archaeal DGGE (Fig. 2g) and T-RFLP (Fig. 2h) cluster, while the effect of plants in soil series B could only be noticed in the DGGE profiles (Fig. 2c).

Closing the electrical circuit, allowing a capture of electrons by the anode, resulted in a clear shift in the bacterial community of soil reactors (Fig. 1a, b, full arrows for open circuit and Fig. 2b), which was also observed in conventional non-planted SMFCs (15). The microbial community on anodes is considered responsible for the generation of electrical current (28) and hence fulfils a pivotal role in MFCs. For vermiculite, the shift was not as clear. It can be noted
that samples from reactor Vb-P-CC1, producing a negative current near sampling time, clustered
distinct (T-RFLP, Fig. 2f) or with samples from an open circuit reactor (DGGE, Fig. 2e). The
clustering furthermore demonstrated an effect of the electrical circuit on the Archaea. These were
less influenced than Bacteria. The effect was only apparent with the use of soil (Fig. 1d, 2c, d).
Reimers et al. (29) found that the diversity of bacterial communities increased with anode
depth. In the present research, the effect of anode depth was minor, it could only be noticed in
soil series A (T-RFLP, Bacteria, Fig. 1b). This lack of trend could be related to the interruption
of the typical redox gradient due to the dense root systems, unequally releasing oxygen and
organic substrates into the support matrix (13).
Overall, the dendrograms obtained through DGGE and T-RFLP were comparable. Some
effects were evident from both analyses (e.g. support, plants in vermiculite), while other were
only evident from one analysis (e.g. plants in soil). This shows the complementariness of the

techniques and allows to discern between the weight of the influencing factors.

Phylogenetic community analysis on Bacteria. Clone libraries were made for the
bacterial and archaeal communities residing on the anode of a current producing rice SMFC with
soil and are represented by phylogenetic trees in Fig. 3 and S2. The relative abundance of the
most important phylogenetic groups found in different operational conditions, based on the clone
libraries and T-RFLP profiles, is shown in Fig. 4. The most common bacterial groups on the
closed circuit anode with plants (Fig. 3, 4a) were those of the Desulfobulbus cluster (56% of all
clones) and Geobacteraceae (16%). δ-Proteobacteria made up a total of 75% of all bacterial
clones. Furthermore, Desulfobulbaceae and Geobacteraceae were not detected on non-current
producing anodes (T-RFLP, Fig. 4a). The enrichment of these groups upon current generation
was also shown through excision of DGGE-bands: bands III, IV and V (see Fig. 1) were stronger
on current producing anodes and showed respectively 92, 96.5 and 90% sequence identity with
the *Geobacter* (bands III and IV) and *Desulfobulbus* (band V) related clones. *δ*-Proteobacteria and more specifically *Geobacteraceae* have often been found enriched on closed circuit anodes (15, 19). The latter are known for anaerobic respiration of organic compounds such as acetate with concomitant reduction of insoluble Fe(III), often replaceable by a solid electrode (4). *Desulfobulbus* (and/or *Desulfocapsa*) species have also been found enriched on anodes (15, 29).

The sulfate reducing *Desulfobulbus propionicus* was found able to oxidize organic compounds (but not acetate) with electrode reduction (14), but its role was also suggested to be linked to the ability to oxidize $S^0$ to sulfate with the electrode as electron acceptor and/or the ability to disproportionate $S^0$ to sulfate and sulfide (31). The *Desulfobulbus* related sequences found here might represent a new species as they were only 89% similar with *Desulfobulbus propionicus*.

Other affiliations of importance on a current producing anode were *Chlorobi* (8% of all clones), *Chloroflexi* (6%) and *Bacteroidetes* (3%). *Chloroflexi* have been found enriched on the anode of a cellulose-fed MFC (18), but their current relevance for a closed circuit anode was not clear when comparing T-RFLP profiles. The bacterial species found here do not correspond with those found important in an earlier research regarding rice SMFCs (20) (*Natronocella*, *Beijerinckia*, *Rhizobiales*) The latter however employed a rice paddy field without inocula.

Based on the T-RFLP profiles (Fig. 4a), the non-current producing anode was dominated by uncultured *Bacteroidetes*. More phylogenetic groups could be detected in the absence of plants, involving a *Nitrospira* – related species (DGGE band I).

**Phylogenetic community analysis on Archaea.** Almost half (47%) of the archaeal clone sequences derived from the closed circuit anode (Fig. 4b, S2) were most closely related to uncultured *Archaea*, and not to any of the known methanogenic lineages (11) or novel rice cluster lineages (24). These sequences clustered in two groups, holding 35 and 12% of total clones. The archaeal clones that could be assigned (Fig. 4b, S2) belonged to a few methanogenic groups, with
as most dominant *Methanobacteriaceae* (20% of all clones), *Methanosarcinaceae* (18%) and *Methanosaetaceae* (10%). These are also found important in genuine rice paddy soil (12).

When comparing T-RFLP fingerprints for closed and open circuit, a shift in archaeal community could be observed (Fig. 4b). Current production led to a (variable, but up to fourfold) increase in the relative abundance of the uncultured *Archaea*. Within the methanogens, there was a fourfold increase for *Methanobacterium* (*CH*₄ production from H₂ and CO₂ and/or formate) and a threefold decrease for the strictly acetotrophic *Methanosaetaceae*. There was a small increase (from 20% to 27%) for the generalist *Methanosarcina* (*CH*₄ production from H₂ and CO₂, acetate and/or C₁-compounds). These changes might reflect an increased importance of hydrogenotrophic compared to acetotrophic methanogenesis combined with possible growth promotion of a group of (uncultured) *Archaea* upon current generation. The research regarding archaeal anodic communities is largely unexplored so far. Ishii et al. (17) did find less (methanogenic) *Euryarchaeota* and suppressed methanogenesis in closed circuit compared to open circuit. To verify the specific effect of plant SMFCs on the methanogenic rate and pathways and on the overall metabolism in rhizospheres, follow up experiments, involving for example ^13^CO₂, are appropriate.

This research showed that despite the strong effect of support type and plants, an effect of the electrical circuit could also be observed, both on bacterial and archaeal communities. These findings can accordingly guide future practical and fundamental work regarding plant MFCs.
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FIGURE LEGENDS

FIGURE 1 Clustering with Pearson correlation (in %) of bacterial and archaeal 16S rRNA gene profiles of anodes of reactor series A with potting soil (Sa) and vermiculite (Va) as support material. a) Bacterial DGGE profiles b) Bacterial T-RFLP profiles c) Archaeal DGGE profiles d) Archaeal T-RFLP profiles. Dashed branches refer to cluster cutoff. Significant effects of the absence of plants are marked by dashed arrows, while open circuits are marked by full arrows. Excised DGGE bands I to V are marked and information regarding closest affiliation (with accession number and reference) and similarity are given for panel a). P is with plants, NP is without plants, CC is closed circuit, OC is open circuit, H is high and L is low anode position.

FIGURE 2 Clustering with Pearson correlation (in %) of bacterial and archaeal 16S rRNA gene profiles of anodes of reactor series B with potting soil (Sb) (panels a to d) and vermiculite (Vb) (panels e to h) as support material. DGGE and T-RFLP profiles are given for Bacteria and Archaea. P is with plants, NP is without plants, CC is closed circuit, OC is open circuit, H is high, M is middle and L is low anode position. The actual DGGE profiles are shown in Figure S1 in Supplemental Material.

FIGURE 3 Phylogenetic tree of δ-Proteobacteria 16S rRNA gene sequences from clones retrieved from the upper anode of a sediment MFC planted with rice, with potting soil as anodic support layer and operated with a closed electrical circuit (series A – Sa-P-CC1-H). The bar indicates 5% sequence divergence. Bootstrap values higher than 50% (for 1000 iterations) are shown at the nodes of the trees. Clones are shown in bold. Numbers in brackets represent the
silico T-RF in base pairs. Accession numbers for grouped clones can be found in Supplemental Material. The phylogenetic tree for the archaeal clone library can be found in Figure S2.

FIGURE 4 Comparison of relative abundance of phylogenetic groups on anodes in closed circuit (CC) and open circuit (OC) with (P) and without (NP) rice plants. a) Bacteria and b) Archaea. The analyzed samples originated from potting soil series A, i.e; Sa-P-CC1-H (n=1) for the first column, Sa-P-CC1-H and Sa-P-CC2-L (n=2) for the second Archaea column (supplemented with Sa-P-CC1-L and Sa-P-CC2-H (n=4) for the second Bacteria column), Sa-NP-CC-H and Sa-NP-CC-L (n=2) for the third column and Sa-P-OC-H (n=1) for the fourth column, with n the number of anode replicates. For columns with n > 1, the averages and standard deviations are mentioned on the figure. The phylogenetic affiliations given are the closest relatives. For the T-RFLP profiles, these are obtained through comparison with the in silico T-RFs from the corresponding clone library. “Others” contains all groups with an abundance of < 6% in case of Bacteria, comprising unc. OD1, Sphingobacteria, Desulfomonile, (unc.) Spirochaeta, unc. δ-Proteobacteria, Desulfovibrio, unc. Planctomycetes, unc. OP11 and unknown T-RFs and < 2% in case of Archaea, comprising unknown T-RFs. Unc. = Uncultured. Unknown T-RF (fragment length in bp) = affiliation of the fragment could not be deduced.
unc. Nitrospira sp. (EU043588, (34)), 100%
unc. Nitrospira-like bact. clone from rice paddy soil (EF613810), 100%
unc. (ε-Proteo)bacteria, 94%
Geobacter metallireducens GS-15 (CP000148), 96%
Geobacter hydrogenophilus (U46860, (7)), 87%
unc. Desulfobulbaceae bact. clone (EF613400, (22)), 92%