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Towards implementation of a benthic microbial fuel cell in lake Furnas (Azores): Phylogenetic affiliation and electrochemical activity of sediment bacteria

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ABSTRACT

This work was conducted to examine the composition and electrochemical activity of the bacterial community inhabiting lake Furnas sediments (Azores). Fingerprinting analysis of the bacterial 16S rRNA gene fragment was done by denaturing gradient gel electrophoresis. The sequences retrieved from lake Furnas sediments were affiliated to *Bacteroidetes/Chlorobi* group, *Chloroflexi, Alfa-*, *Delta-*, and *Gamma*-subclasses of *Proteobacteria, Cyanobacteria,* and *Gemmatimonadetes*. A cyclic voltammetric study was carried out with an enriched sediment bacterial suspension in a standard two chamber electrochemical cell using a carbon paper anode. Cyclic voltammograms (scan rate of 50 mV/s) showed the occurrence of oxidation–reduction reactions at the carbon anode surface. The benthic microbial fuel cell operated with lake Furnas sediments presented a low power density (1 mW/m^2) indicating that further work is required to optimise its power generation. These results suggested that sediment bacteria, probably from the *Delta- and Gamma*-subclasses of *Proteobacteria*, were electroactive under tested conditions.

1. Introduction

Recent advances in molecular microbial ecology suggested that sediments shelter a complex microbial consortium that thrive on several organic and inorganic electron donors and acceptors and where several processes (e.g. nitrification, denitrification, sulphate reduction, iron reduction) and metabolic pathways act in simultaneous [1–3]. Previous studies have shown that electric energy can be harvested from organic-rich aquatic sediments by electrochemically active microorganisms and a new application of the microbial fuel cell technology has emerged: the Benthic Microbial Fuel Cell (BMFC). A BMFC consists of an anode embedded in the anoxic sediment and a cathode suspended in the aerobic water column [4]. Bacteria in a BMFC mediate the transfer of electrons from carbon sources to the anode thus generating an electric current. The first BMFCs described in literature studies were associated to marine sediments due to the better ion conductivity between electrodes in saline environments [5,6]. Recently, sediments from rivers and lakes were also used to operate BMFCs [7]. The main application of BMFCs described in literature is long-term power sources for autonomous sensors and communication devices [4,5,8,9].

Among the electrochemically active microorganisms that can transfer electrons directly from the carbon source to an anode, without the need of electroactive intermediates [10], the most well known are *Shewanella putrefaciens*, a *Gamma-proteobacterium*, *Geobacter sulfurreducens*, *Geobacter metallireducens* and *Desulfuromonas acetoxidans*, all *Delta-proteobacteria*, and *Rhodoferax ferrireducens*, a *Beta-proteobacterium* [11,12]. As pointed out in several studies, microbial communities associated to BMFC anodes are enriched in *Delta-proteobacteria* [6,8,13,14]. The predominance of these bacteria is dependent on the environment: *Desulfuromonas* species are more abundant in marine sediments, while in freshwater sediments *Geobacter* species predominate [8].

The present work intended to describe the microbial diversity and to assess the electroactivity of the microbial community present in lake Furnas sediments. The diversity of sediment bacteria was ascertained by 16S rRNA analyses and sediment electroactivity was assessed by cyclic voltammetry. A microcosm experiment simulating a BMFC was carried out to evaluate electron transport from the degradation of organic matter to a carbon anode surface.

2. Materials and methods

2.1. Study area and sampling

Lake Furnas is a volcanic lake situated in S. Miguel, the major island of the archipelago of Azores (Portugal) that is located in the Atlantic

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Ocean, between the parallels 36°45´–39°43´of latitude North and meridians 24°45´–31°17´ of longitude west. Lake Furnas has a surface area of 12 km^2 and a maximum depth of 12 m. The temperature of the water ranges from 15 °C to 23 °C along the year. The sediment is fine and derived from volcanic pumice deposits. Morphometric and geochemical characteristics of the lake can be found in Ribeiro et al. [15]. Sediment cores were collected at the deepest point in lake Furnas with a gravitational Uwitec-corer (6 cm diameter, and 60 cm length) that penetrated about 30 cm in the soft sediments. Sediments with the overlying water were sealed in situ inside the core tubes, transported to the laboratory and stored at 4 °C.

2.2. DNA extraction and PCR amplification

A sediment core was cut into five slices which were used for DNA extraction: F1: 0–1 cm; F2: 1–2 cm; F3: 2.5–3.5 cm; F4: 5–6 cm; F5: 9–10 cm. DNA was extracted and purified from 0.7 g (wet weight) of sediment using the UltraClean Soil DNA kit (MoBio, Solana Beach, CA, USA) according to the manufacturer's instructions. Each 25 μL reaction mixture contained the following: 2.5 μL DNA template, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 0.04% (w/v) bovine serum albumin, 200 μM of each deoxynucleotide, 1.5 mM $MgCl_2$, 2.5 U/mL Taq DNA polymerase, and 0.5 μM of each forward and reverse primers. Primers 341F and 518R were used to amplify a variable region of the bacterial 16S rRNA gene [16]. A GC-rich clamp was added to the 5' end of 341F for DGGE. PCR reactions were performed with the MyCycler (Bio-Rad) using the program described by Muyzer et al. [16].

Partial 16S rRNA gene fragments were amplified by PCR using Geo564F and Geo840R primers directed toward conserved regions of the gene within the *Geobacteraceae* family [17]. The PCR mix was the same as that described above with the exception of the primers. PCR amplification was carried out by initial denaturation at 94 °C for 10 min, followed by 40 cycles at 94 °C for 30 s, 56.5 °C for 30 s, and 72 °C for 45 s. Reactions were finished by a final extension at 72 °C for 10 min. A positive control was provided by the amplification of a 16S rRNA gene fragment from *Geobacter bemidjiensis sp*. The PCR products were visualized by agarose gel electrophoresis 1% (w/v) with ethidium bromide staining.

2.3. DGGE, cloning and sequence analysis

Denaturing gradient gel electrophoresis (DGGE) was performed with the Dcode System (Bio-Rad) as described by Muyzer et al. [16]. Briefly, PCR products were separated on a 1.5 mm thick vertical gel containing 8% (w/v) polyacrylamide (37.5:1, acrylamide:bisacrylamide) and a linear gradient of the denaturants urea and formamide, increasing from 30% at the top of the gel to 60% at the bottom. The gel was loaded with 15 μL of PCR product and then electrophoresis was performed for 18 h at 75 V and 60 °C. The gel was stained for 1 h in 0.5 mg/mL ethidium bromide solution. Images were recorded with a Trans-illuminator Gel Doc 2000 (Bio-Rad).

To sequence the single DGGE bands, a small piece of gel from the middle of the target band was excised using a sterile scalpel and incubated in 50 μL sterile milli-Q purified water overnight at 4 °C. An aliquot of the DNA solution thus obtained was diluted 1:10 and used as template for a PCR reaction. The PCR product was separated in a DGGE gel to confirm that the target band has been amplified [18]. Subsequently, the target band was cut again and amplified with the primers 341F and 518R (no GC-clamp). The PCR product was inserted into the pCR2.1®-vector (TA Cloning® Kit, Invitrogen, Carlsbad, CA, USA). Seven clones per band were selected and the DNA was extracted, amplified and separated in a new DGGE gel to check again that the target clone has been amplified. Finally, the PCR product was purified with the Jetquick PCR Product Purification Spin Kit (Genomed, Löhne, Germany) and used as template in a sequencing reaction carried out with the M13R primer. A total of 23 bands were excised, cloned and sequenced. Each sequence resulting from a DGGE band was compared to sequences available in public databases using BLAST from the National Center of Biotechnology Information [19] and the phylum of the closest relative to the retrieved sequence from the sediment sample was ascertained.

2.4. Cyclic voltammetry

Sediment bacteria were enriched during 8 d in an anaerobic medium at 35 °C and pH 6.8 with the following composition per litre: 1.5 g NH_4Cl, 0.6 g Na_2HPO_4, 1.49 g NaH_2PO_4, 0.1 g KCl, 0.82 g $NaCH_3O_2$, 2.5 g $NaHCO_3$, 10 mL vitamins solution, 10 mL trace metals solution and 1 mL selenite–tungstate solution (adapted from DSMZ, medium 826).

Cyclic voltammetry was used to characterize the oxidation–reduction reactions at the surface of the working electrode in a standard two chambers electrochemical cell operated in batch at room temperature with both the enriched sediment bacterial suspension and the anaerobic medium (as a control) [20]. The electrochemical cell enclosed three electrodes connected to a potentiostat (Model 2051, Amel Instruments): working electrode (carbon paper — Toray), counter electrode (platinum: iridium, 90:10) and Saturated Calomel Electrode (SCE) as reference electrode. The current intensity response to potential, in an interval from −850 mV to 900 mV vs. SCE, was recorded directly from the potentiostat output at a scan rate of 50 mV/s. A dual channel voltage collection instrument was used in connection to a computer via a universal serial bus interface (USB, Intel) using a LabVIEW-based Acquisition System. To check the integrity of the electrochemical system and to verify the non-contamination of the system by chemical species, cyclic voltammetry with a carbon paper electrode was performed before each experiment in the presence of KCl 0.1 M at a scan rate of 50 mV/s. To ensure anaerobic conditions in the system both chambers were deaerated in the beginning of each experiment with pure argon (U Quality from Air Liquid) during 20 min. A study was carried out with different sweep scan rates (from 5 mV/s to 1000 mV/s) to assess electronic transfer processes associated to oxidation peaks in cyclic voltammograms. To determine the reversibility of the electronic transfer, the dependence of the peak potential on the sweep scan rate was evaluated; the peak potential being dependent on sweep scan rate for an irreversible process and independent in the case of a reversible process [21].

2.5. Microcosm experiment

The electroactivity of the lake Furnas sediment was determined in a microcosm experiment simulating a benthic microbial fuel cell (BMFC). A 1 L open reactor was filled with 400 g of sediment and 500 mL of lake water. Acetate was added to a final concentration of 20 mM. Carbon paper (Toray) electrodes with 30 cm^2 each were used as anode and cathode. The anode was buried in the sediment and the cathode was submerged in the water layer. The electrodes were connected via a 1 kΩ resistor and the voltage was recorded with a multimeter during 12 d. The current intensity (i) was calculated according to the Ohm´s law ($i = V/R$), where V is the voltage and R the resistance. The current density (I) was calculated as $I = i/A$, where A is the projected surface area of the anode electrode. The power density (P) was calculated as the product of current intensity and voltage divided by the projected surface area of the anode ($P = iV/A$). The polarization curve, describing voltage (V) as a function of current density (I), was recorded using a series of resistances in the range of 60 kΩ to 275 Ω. The internal resistance of the BMFC (R_{int}) was calculated from the slope of the polarization curve in the region dominated by Ohmic losses [22]. The open circuit voltage (OCV) was measured at infinite resistance.

3. Results and discussion

The bacterial community composition inhabiting lake Furnas sediments was studied by DGGE fingerprinting of a 16S rRNA gene

fragment. The DGGE gel depicted in Fig. 1 revealed that a total of 23 different bands were identified for sediment samples obtained at different depths. The DGGE patterns were quite similar between the sediment samples obtained at different depths.

Table 1 lists the closest relatives that match to 16S rRNA sequences derived from DGGE bands. The obtained sequences were generally related to uncultured bacterial sequences in the public databases and the majority of the closest relatives were isolated from lake, river and sea sediments, inland dune fields, grassland soils, wetlands and anaerobic reactors. Several sequences (6 clones) retrieved from the sediment could not be assigned to a specific phylum (designated as unclassified in Table 1). This result might be explained by the restricted number of bases amplified with the primers used in the PCR reaction (341F, 518R). The phylogenetic analysis also revealed

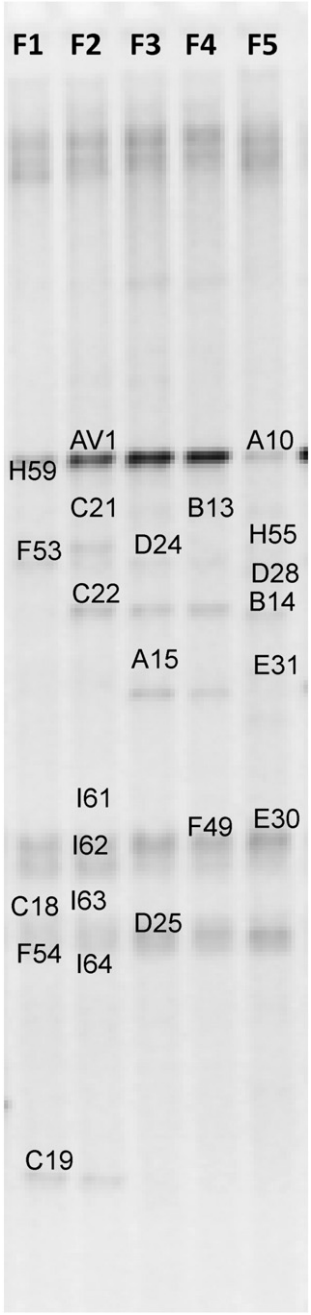

Fig. 1. DGGE profiles of 16S rRNA fragments amplified from DNA extracts from lake Furnas. Lane F1: 0–1 cm; Lane F2: 1–2 cm; Lane F3: 2.5–3.5 cm; Lane F4: 5–6 cm; Lane F5: 9–10 cm.

that the majority of the clones belonged to the *Proteobacteria* phylum, being the *Alfa*-subclass of *Proteobacteria* (5 clones) the most abundant one. *Delta*- (2 clones) and *Gamma*- (1 clone) subclasses of *Proteobacteria* sequences were also retrieved from the sediments. *Bacteroidetes/Chlorobi* group (4 clones) and *Chloroflexi* (3 clones) phyla were the other major groups identified. Literature studies on microbial diversity have found that bacteria from the *Bacteroidetes*, *Chloroflexi*, *Proteobacteria*, and *Gemmatimonadetes* phyla are quite common in lake and reservoir sediments [3,23,24].

Phylogenetic analysis indicated that clones retrieved from lake Furnas sediments belonging to *Delta*- and *Gamma*-subclasses of *Proteobacteria* were affiliated to iron-reducing *Geobacteraceae* (>90% similarity to *Geobacter bremensis*) and *Shewanellaceae* (>93% similarity to *Shewanella woodyi*) families, respectively, both well known as electroactive microorganisms [8]. Additionally, amplification of sediment DNA with primers directed toward conserved regions of the gene within the *Geobacteraceae* family (Geo564F, Geo840R) resulted in a discrete PCR product of the expected molecular weight (data not shown). This last result suggested that members of *Geobacteraceae* family populated sediments from lake Furnas.

The electroactivity of an enriched sediment bacterial suspension was assessed by cyclic voltammetry carried out at a scan rate of 50 mV/s (Fig. 2).

The voltammogram of the control experiment, depicted in Fig. 2, carried out with carbon paper (Toray) in anaerobic medium exhibited an oxidation peak at 750 mV vs. SCE with a moderate current density (125 mA/m^2) and a reduction peak at 320 mV vs. SCE. An important increase in current intensities between 480 mV and 740 mV vs. SCE was observed in the presence of sediment bacteria: oxidation and reduction peaks were observed at 675 mV (212.5 mA/m^2) and 250 mV vs. SCE, respectively (Fig. 2). The voltammogram of a pure culture of *Geobacter sulfurreducens* (data not shown) also exhibited an oxidation peak close to 675 mV vs. SCE with a current density slightly lower than the one obtained in the presence of the enriched sediment bacterial suspension (186 mA/m^2). Cyclic voltammetry suggested that the enriched sediment bacterial suspension presented electroactivity. The combination of electrochemical and molecular data suggested that bacteria from the *Geobacteraceae* and *Shewanellaceae* families might have contributed to the electroactivity presented by lake Furnas sediment.

To assess the electronic transfer processes between sediment bacteria and the anode and the reversibility of the electronic transfer, the oxidation peak potential (peak A in Fig. 2) was evaluated at different sweep scan rates. The curves representing the logarithm of the current intensity (i) as a function of the logarithm of the scan rate (v) and the dependence of the potential (E) on the logarithm of the scan rate (v) are depicted in Fig. 3.

The curve representing Log (i) as a function of the Log (v) (Fig. 3) gives information about the nature of the limiting step. The slope value of this straight line was close to 0.5, indicating that the mechanism of electron transfer between sediment bacteria and the anode was mainly diffusion controlled [21]. The dependence of E on the log (v) suggested that the electron transfer process was irreversible up to 250 mV/s and for scan rates in the range of 250 mV/s to 1000 mV/s was reversible. As suggested by literature studies, one possible explanation for this result is that primary metabolites (e.g. reduced fermentation products) are involved in irreversible mechanisms of electron transfer while reversible processes are mediated by extracellular compounds that are oxidized and reduced cyclically [25].

The electroactivity of lake Furnas sediments was also determined in a microcosm experiment simulating a benthic microbial fuel cell (BMFC) and voltage was recorded along time (Fig. 4).

The voltage was very low for the first 8 d increasing rapidly afterwards to a maximum value of 53 mV after 12 d, corresponding to a power density of 1 mW/m^2. This behaviour might be explained by the formation of an electrochemically active biofilm on the surface of

Table 1
Closest matches of excised, re-amplified, cloned and sequenced 16S rRNA-derived DGGE bands to sequences present in the NCBI database.

Clone	Closest relative (accession number)	Query coverage	Max identity	Taxonomy
AV1	Uncultured bacterium clone LWS-RSG-CH4-4075 16S ribosomal RNA gene, partial sequence (EU546553.1)	100%	98%	Unclassified
A10	Unidentified eubacterium clone vadinBA43 16S ribosomal RNA gene, partial sequence (U81652.2)	98%	92%	Unclassified
A15	Uncultured bacterium clone GASP-0 KB-587-C02 16S ribosomal RNA gene, partial sequence (EU043640.1)	98%	97%	*Bacteroidetes/Chlorobi group*
B13	Uncultured bacterium clone 101b1 16S ribosomal RNA gene, partial sequence (EF459907.1)	100%	99%	*Bacteroidetes/Chlorobi group*
B14	Uncultured bacterium clone PR_OTU-08 16S ribosomal RNA gene, partial sequence (EF165510.1)	100%	99%	*Chloroflexi*
C18	Uncultured bacterium clone 2G1-22 16S ribosomal RNA gene, partial sequence (EU160208.1)	96%	97%	*Gemmatimonadetes*
C19	Uncultured bacterium DGGE gel band ESR BR 17 16S ribosomal RNA gene, partial sequence (AF540052.1)	100%	99%	Unclassified
C21	Uncultured bacterium clone FL0428B PF55 16S ribosomal RNA gene, partial sequence (FJ716474.1)	100%	99%	*Bacteroidetes/Chlorobi group*
C22	Microcystis sp. AWT139 16S ribosomal RNA gene, partial sequence (U40331.2)	100%	100%	*Cyanobacteria*
D24	Uncultured alpha proteobacterium clone SA-B16 16S ribosomal RNAgene, partial sequence (DQ295442)	100%	99%	*Alpha-proteobacteria*
D25	Uncultured bacterium clone GD71 16S ribosomal RNA gene, partial sequence (EF613895.1)	98%	93%	*Bacteroidetes/Chlorobi group*
D28	Uncultured bacterium clone reef124 16S ribosomal RNA, partial sequence (EU121710.1)	98%	92%	Unclassified
E30	Uncultured bacterium clone Pro_CL-09054_OTU-5 16S ribosomal RNA gene, partial sequence (EU808746.1)	100%	100%	*Alpha-proteobacteria*
E31	Uncultured sphingomonas sp. partial 16S rRNA gene, clone 9.11 (AM293371)	100%	100%	*Alpha-proteobacteria*
F49	Uncultured bacterium clone Er-LLAYS-62 16S ribosomal RNA gene, partial sequence (EU542519.1)	98%	86%	Unclassified
F53	Uncultured bacterium HF0500_24B12 genomic sequence (EU795192.1)	100%	100%	*Alpha-proteobacteria*
F54	Uncultured bacterium clone FCPT525 16S ribosomal RNA gene, complete sequence (EF516073.1)	100%	97%	*Gamma-proteobacteria*
H55	Uncultured Sphingomonadales bacterium clone SHBZ696 16S ribosomal RNA gene, partial sequence (EU639137.1)	99%	100%	*Alpha-proteobacteria*
H59	Uncultured bacterium partial 16S rRNA gene, clone AV9-10 (AM181950.2)	100%	100%	Unclassified
I61	Uncultured bacterium clone SED1000_74 16S ribosomal RNA gene, partial sequence (EU557829.1)	100%	100%	*Chloroflexi*
I62	Uncultured bacterium partial 16S rRNA gene, clone c1LKS29 (AM086080.1)	100%	98%	*Delta-proteobacteria*
I63	Uncultured bacterium clone LaC15L90 16S ribosomal RNA, partial sequence (EF667608.1)	100%	100%	*Chloroflexi*
I64	Uncultured Syntrophaceae bacterium clone LCA1-1C 16S ribosomal RNA gene, partial sequence (EU522632.1)	98%	97%	*Delta-proteobacteria*

the anode. The open circuit voltage (OCV), representing the maximum voltage that is possible to obtain in optimum conditions, was 387 mV. The power density obtained in the present study was considerably lower than power densities reported in literature for BMFCs: typical values are in the range of 0.18 mW/m^2 to 49 mW/m^2 [5,26–28]. One possible explanation for this result is that fouling of the cathode occurred. After a few days of operation, it was observed the colonization of the cathode by a thick biofilm which probably consumed oxygen and reduced its availability to the cathodic reaction. Tender *et al.* [5] reported a decrease in power density in a BMFC due to the coverage of the cathode with sediments and macroalgal detritus. The highest power density reported in literature studies, 49 mW/m^2, was obtained in a BMFC operating with a rotating cathode that enhanced oxygen mass transfer to the cathode's surface.

The polarization curve depicted in Fig. 5 presents voltage as a function of current density obtained during the stable phase of power generation (12th day) for resistances varying from 60 kΩ to 275 Ω (current densities in the range of 1.6 mA/m^2 to 10.9 mA/m^2).

The initial steep decrease of voltage for low current densities depicted in Fig. 5 suggested that the BMFC had high activation losses [22]. For a current density higher than 3.73 mA/m^2, the slope of the curve decreased considerably which indicated the prevalence of ohmic losses [22]. The internal resistance estimated from the slope of the curve was high, 14.6 kΩ, meaning that optimization of the BMFC is required [22]. As depicted in the power curve (Fig. 5), the power

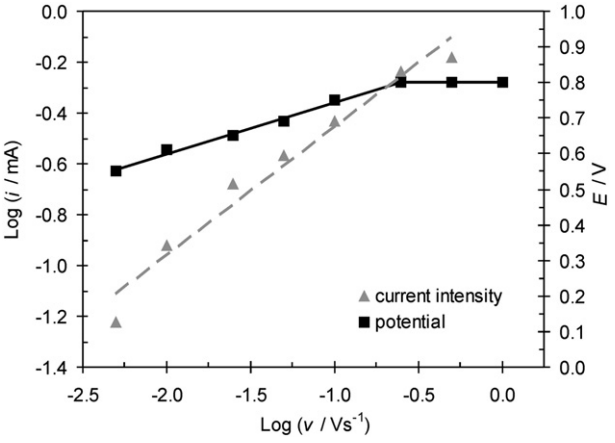

Fig. 3. Log *i* versus Log *v* (▲) and *E* versus log *v* (■) curves for the oxidation (peak A) of an enriched sediment bacterial suspension.

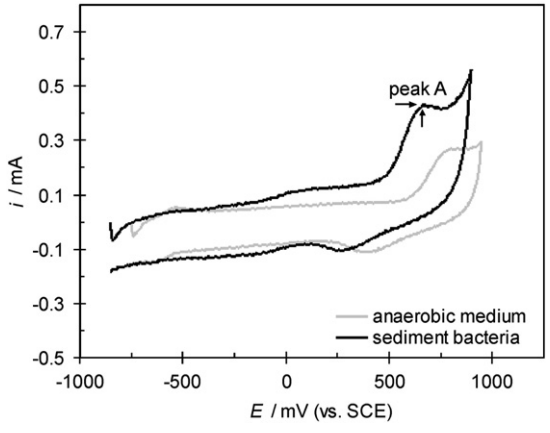

Fig. 2. Voltammograms of carbon paper in anaerobic medium and in an enriched sediment bacterial suspension.

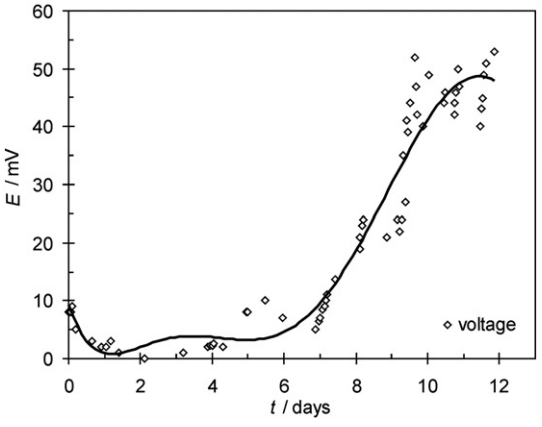

Fig. 4. Voltage along time obtained in the benthic microbial fuel cell.

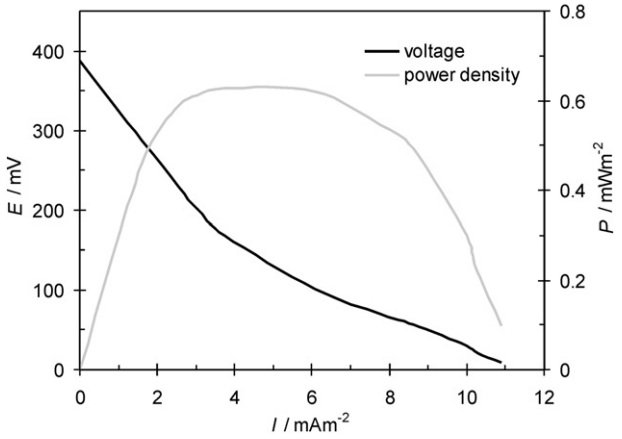

Fig. 5. Polarization and power curves obtained in a benthic microbial fuel cell during the stable phase of power generation (12th day).

density increased with current density to a maximum value of 0.62 mW/m^2, obtained at a current density of 3.73 mA/m^2 (15 kΩ), decreasing afterwards due to the increase of ohmic losses.

The microcosm experiment simulating a BMFC suggested that sediment bacteria from Lake Furnas are electroactive, transferring electrons resulting from the degradation of organic matter to a carbon anode surface. However, further work is required to optimise its power generation.

4. Conclusions

The present study provides insight into the composition of sediment bacteria from lake Furnas that were affiliated to the *Bacteroidetes*/*Chlorobi* group, *Chloroflexi, Alfa-*, *Delta-*, *Gamma*-subclasses *Proteobacteria, Cyanobacteria,* and *Gemmatimonadetes* phyla. The bacterial community present in the sediment was electroactive as demonstrated both by cyclic voltammetry and power generation in a simulated benthic microbial fuel cell. Members of the *Geobacteraceae* and *Shewanellaceae* families, from the *Delta-* and *Gamma*-subclasses of *Proteobacteria* respectively, might have contributed to the electroactivity presented by lake Furnas sediment. The BMFC operated with lake Furnas sediments presented a low power density (1 mW/m^2) indicating that further work is required to optimise its power generation.

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