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Title	A study of microbial communities on terracotta separator and on biocathode of air breathing microbial fuel cells
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Abstract

Recently, terracotta has attracted interest as low-cost and biocompatible material, to build separators in microbial fuel cells (MFCs). However, the influence of a non-conductive material like terracotta, on electroactive microbiological communities remains substantially unexplored. This study aims to study the microbial pools developed from two different seed inocula (bovine and swine sewage) in terracotta-based air-breathing MFC. A statistical approach on microbiological data confirmed different community enrichment in the MFCs, depending mainly on the inoculum. The results confirmed that terracotta separator impedes the growth of a biocathode. The biocathode-MFCs showed from 4 to 6-fold higher power densities that terracotta-MFCs. Both the thick biofilm formed on the surface (anolyte-side) of the terracotta separator and the biocathodes were analyzed by high-throughput Illumina sequencing applied to bacterial 16S rRNA gene. The results showed more abundant (3- to 5-fold) electroactive genera (mainly Geobacter, Pseudomonas, Desulfuromonas and Clostridia MBA03) in terracotta-free biocathodes than in terracotta biofilms. Nevertheless, terracotta separator induced only slight changes in anodic biofilms.

Keywords	Electroactive biofilms, biocathode, microbial fuel cell, terracotta, Illumina 16S rRNA gene sequencing
Manuscript category	Biofuel Cells and Signal Transduction

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Dear Scientific Committee of Bioelectrochemistry,

We gentle submit you the revision of the manuscript BIOELECHEM_2017_227 for the publication in the special issue of the journal Bioelectrochemistry on "*Electroactive microorganisms and microbial consortia*", as original research.

The English was improved with the help of a native speaker, according to the editors' suggestion.

Authors: Laura Rago, Sarah Zecchin, Stefania Marzorati, Andrea Goglio, Lucia Cavalca, Pierangela Cristiani, Andrea Schievano

The results included in the manuscript have not been published/submitted or are being submitted to another journal. This manuscript investigates the microbiological communities of four air cathode MFCs, which were enriched using two different inocula: bovine and swine sewages. Two of these MFCs, BM and SM, were built using terracotta between the anode and the cathode. The presence of terracotta avoided the formation of biocathode in BM and SM. In parallel, two MFCs (BC and SC) were built as control without the terracotta separator between the electrodes. The main difference was induced by the different inoculum enrichment. Moreover, the electroactive microorganisms are more present on conductive material, the cathode, than on non-conductive material, the terracotta. The OTUs of well-known or suspected electroactive microorganisms in BC and SC cathodic biofilms were respectively 3 and 5 times more than in BM and SM terracotta biofilms. Moreover, the changes in the cathodic part of the reactors induces only slight changes in the bioanodic communities. Statistical approach (through UPGMA and PCoA) confirmed different enrichment in microbiological biofilms according to the inoculum and to the different part of the reactor.

To the best of our knowledge, this is the first paper that deepen the microbiology of terracotta based MFCs.

The work done required relevant and different skills, for the tests and for the analyses, so a team of authors substantially contributed to reach the results, as detailed below, and all of them approved the version to be submitted:

- Laura Rago for acquisition and analyses of microbiological samples, coordinating the analyses and the results, and drafting the article
- Sarah Zecchin for bioinformatics and statistics of data, drafting relative parts of the article results

- Stefania Marzorati for acquisition and analyses of electrochemical data and drafting relative parts of the article;
- Andrea Goglio for building and maintain the reactors, for acquisition and analyses of electrochemical data;
- Lucia Cavalca for critical revision of the microbiological data and discussions
- Pierangela Cristiani for the revision of the article and for important intellectual content supervising bioelectrochemical results
- Andrea Schievano for the critical revision of the manuscript and the English.

I can confirm with the other authors that there are no conflict of interest, including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, our work. The authors believe in the high reputation of Bioelectrochemistry journal and approved the version submitted.

Hoping in a positive response, We look forward to the challenge of publishing with the journal.

Truly,

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Journal: Bioelectrochemistry

Dear Dr. Rago,

Thank you for submitting your manuscript to Bioelectrochemistry. We have completed the review of your manuscript. A summary is appended below. I emphasise the need for improved English. Also for the tables, please use black only and remove any shading. While revising the paper please consider the reviewers' comments carefully. We look forward to receiving your detailed response and your revised manuscript.

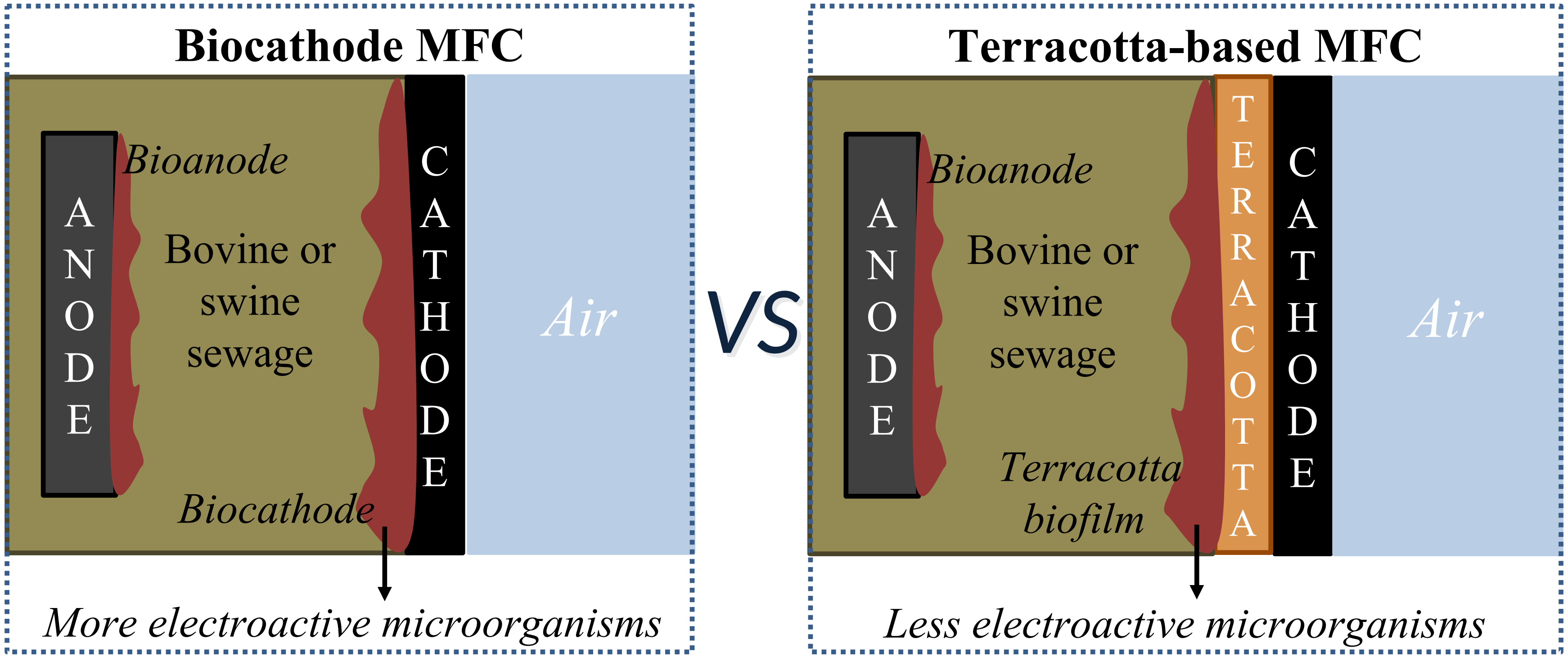
Comments from the editors and reviewers:
-Editor

- This version of the manuscript is a substantial improvement over previous versions and it is nearly ready for publication. There are however still English issues (grammar and syntax errors, plus awkward sentences) in the newly added text. I encourage the authors to review the text with the help of a native speaker.

Dear editors, thank you a lot for your help and suggestions. We improved the English of the manuscript and we corrected the tables.

Highlights

- Bovine and swine sewages were used as inoculum for microbial fuel cells (MFCs)
- Microbiology of terracotta-based MFCs was explored for the first time
- Illumina and statistics were used to characterize anodic and cathodic communities
- Terracotta-MFCs were compared with biocathode-MFCs
- Conductive material favored the presence of electroactive microorganisms


Biocathode MFC
Bioanode
ANODE
Bovine or swine sewage
CATHODE
Air
Biocathode
More electroactive microorganisms
VS
Terracotta-based MFC
Bioanode
ANODE
Bovine or swine sewage
TERRACOTTA
CATHODE
Air
Terracotta biofilm
Less electroactive microorganisms

A study of microbial communities on terracotta separator and on biocathode of air breathing microbial fuel cells

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Abstract

Recently, terracotta has attracted interest as low-cost and biocompatible material, to build separators in microbial fuel cells (MFCs). However, the influence of a non-conductive material like terracotta, on electroactive microbiological communities remains substantially unexplored. This study aims to study the microbial pools developed from two different seed inocula (bovine and swine sewage) in terracotta-based air-breathing MFC. A statistical approach on microbiological data confirmed different community enrichment in the MFCs, depending mainly on the inoculum. The results confirmed that terracotta separator impedes the growth of a biocathode. The biocathode-MFCs showed from 4 to 6-fold higher power densities that terracotta-MFCs. Both the thick biofilm formed on the surface (anolyte-side) of the terracotta separator and the biocathodes were analyzed by high-throughput Illumina sequencing applied to bacterial 16S rRNA gene. The results showed more abundant (3- to 5-fold) electroactive genera (mainly *Geobacter*, *Pseudomonas*, *Desulfuromonas* and *Clostridia MBA03*) in terracotta-free biocathodes than in terracotta biofilms. Nevertheless, terracotta separator induced only slight changes in anodic biofilms.

Keywords

Electroactive biofilms, biocathode, microbial fuel cell, terracotta, Illumina 16S rRNA gene sequencing

1. Introduction

In microbial fuel cells (MFC), electroactive microorganisms may colonize both electrodes and be responsible of most redox reactions. In recent years, anodic biofilms (bioanodes) and cathodic biofilm (biocathode) communities, colonized by electrogenic microorganisms, are increasingly studied in different MFC configurations [1]. In air-breathing MFC, the biocathode may consist of mixed consortia of anaerobic, microaerophilic and aerobic microorganisms in direct contact with the conductive surface [2–4]. In this case, the success of MFC relies not only on the exoelectrogenic activity of bioanodic community, but also on the development of a complex microbial community on cathodes [2,5,6]. The presence of a thick cathodic biofilm has a double function: a) impeding oxygen diffusion through the anodic chamber, thereby preventing the inhibition of the anodic anaerobic biofilm [7,8]; b) catalyzing oxygen reduction reaction (ORR), i.e. improving electron transfer chains from the conductor to intermediate electron acceptors and finally to O_2 [2]. The bio-catalytic mechanisms were previously associated to cyclic red-ox reactions with sulfur, iron and manganese compounds, which facilitate the dispatch of electrons to oxygen [2–4,8,9]. Specific microaerophilic, strong halophilic and alkalophilic conditions (high pH and salt concentration) at cathodic interface [10,11] are responsible for the selection of peculiar microbial populations [2,4]. These types of biocathodes can be considered as low-cost catalysts towards ORR in air-cathode MFC. Also, biocathodes may influence the selection of anodic biofilm communities [2].

One important constraint has been impeding the application of air-cathode MFCs to treat organic-rich wastewater at a large scale [12]: cathodes easily get clogged by both organic and inorganic deposits and, over relatively short periods (30-60 days), their catalytic activity gets deactivated and their internal resistance increased [13,14]. For this reason, structural parts of electrodes and separators should be fabricated using low-cost and easily recycled materials.

In recent studies, terracotta (earthenware) was introduced as low-cost structure material for MFC building [15–17]. Terracotta cylinders were used to build air-cathode MFC and to separate anode (external wastewater side) and cathode (internal air side) [18,19]. Due to the porosity of terracotta (60-500 nm [20]) that allows electrolytes mobility, terracotta cylinders were proposed for aims other than energy harvesting, such as nutrients [11] or precious [21] and heavy metals [22] recovery from wastewaters by electro-osmosis [23]. This configuration in MFCs gives the opportunity to exploit the electro-osmotic flow, created by the electric field, to extract cations from the anolyte, depositing as salts on the separator and the cathode [11,18,22,24]. Terracotta has the advantage to be environmentally compatible and at end-of-life can be re-used directly as agricultural soil conditioner. Unlike the materials normally used in air-cathode MFC (e.g. gas diffusion layers on carbon cloth), terracotta is a non-conductive material. This condition can deeply affect the electroactive biofilms that colonize the electrodes. However, the influence of terracotta separators on microbial communities remains substantially unexplored.

Due to the small size of terracotta pores (typically terracotta pores diameter is lower than 100 nm), terracotta separators tend to microbiologically separate the cathode (air-side) from the anolyte. Especially when the cathode is exposed to the air, the absence of cathodic inoculation and the typical alkaline (pH >12) conditions established by the accumulation of hydroxyl ions at the cathode, can impede the formation of a proper cathodic biofilm, according to what observed in previous studies [11]. Under these conditions, the ORR tends to be prevalently abiotic [18]. On the anolyte-side, the biofilm is in contact with the non-conductive terracotta surface. Thus, the function of this biofilm for the MFC is different as compared to biocathodes (biofilm growing in direct contact with the cathode). The microbial community might vary substantially, especially for what concerns electroactive microorganisms.

The present study aims at exploring how the presence of a terracotta separator can influence the microbial communities of air-cathode MFC. Lab-scale MFC reactors with terracotta separators were compared to identical terracotta-free reactors, using two different wastewaters as inocula. Particular attention was focused on microbial community diversity. The comparison also allows better identification of the electroactive microorganisms that colonize the MFC cathodes. In addition, anodic samples were analyzed and compared, to explore the influence of cathodic microbial community changes on anodic biofilm.

2. Materials and Methods

Two biocathode-MFCs (BC and SC) and two terracotta-MFCs (BM and SM) were operated for around three months. BC and BM were inoculated and fed using bovine sewage. Instead, swine sewage was used for SC and SM. Sewages were collected in a farm near Milan (Italy). DNA was extracted at the end of the experimental period, and it was processed by MiSeq 16S rRNA gene Illumina sequencing tools.

2.1. MFCs configuration

Four reactors were built using Simple Pyrex® bottles (125 mL volume). Two of them as traditional air-cathode single chamber MFCs were built as previously described [25] and called BC and SC. Electrodes were made of carbon cloth (SAATI C1, Appiano Gentile, Italy). Plain carbon cloth (3×10 cm) was rolled and placed at the bottom of the cell to serve as anode. Carbon cloth modified by a microporous layer made of activated carbon/PTFE mixture was used for cathodes [26]. Geometric surface area of the cathode exposed to the anolyte was 3.14 cm^2. Anode and cathode were electrically connected through an external copper circuit under a load of 100 Ω. Connections were insulated with non-conductive epoxy resin.

Other two MFCs (BM and SM) were built in similar way, but with a terracotta (non-conductive material) performing as membrane, between anode and cathode. The terracotta (25 cm^2 of area and 4 mm of thickness) was attached to the cathode using silicone. The other side of the terracotta was exposed to the anolyte (due to the reactor geometry, only 3.14 cm^2 of terracotta were directly exposed to the solution). Anode and cathode were positioned at a relative distance of around 4 cm.

FIGURE 1

2.2. Inoculation and experimental set-up

All MFCs anodic chambers were inoculated and fed in parallel with two different types of sewage as sole carbon source (except for the third cycle, named "Cycle 3 (acetate)" in Fig.2): bovine (BC and BM) and swine (SC and SM). Both sewages were filtered (0.5 mm mesh) and characterized before use (Table 1).

TABLE 1

MFCs were monitored during four batch cycles (90 days). The first acclimation cycle ("Cycle 1" in Fig. 2) lasted from day 1 to day 34. The second cycle ("Cycle 2" in Fig. 2) lasted from day 34 to day 56. After this period, 3 g L^{-1} of sodium acetate (2.4 gCOD L^{-1}) were added to all MFCs. This cycle ("Cycle 3 (acetate)" in Fig.2), from day 56 to 71, permitted to perform the electrochemical analysis reported in table 2. At day 72, all MFCs were refilled with sewage (20 mL), to replace the liquid phase evaporated during Cycle 3. This last cycle has been labelled as "Cycle 4" in Fig. 2.

2.3. Chemical analyses

Soluble fractions of Chemical Oxygen Demand (sCOD) were measured after filtration (0.2 µm nylon filters) using HACH COD vials and HACH DR220 Vis-spectrophotometer following the standard procedure. sCOD removal was calculated according to: $(1-sCOD_{final}/sCOD_{initial}) \bullet 100$. Total Chemical Oxygen Demand (tCOD) was obtain by the same procedure, but without filtration.
pH of the anodic compartment and the electrical conductivity were periodically monitored with an Amel Instruments pH-meter (combined glass electrode, daily calibrated with two buffers at pH=7 and pH=9) and an Amel Instruments conductivity meter (conductivity cell with a cell constant of 1 cm^{-1}), respectively.

Total Kjeldhal Nitrogen (TKN) was determined on fresh material, according to the analytical method for wastewater sludge, as previously reported [27].

2.4. *Electrochemical analysis*

For each MFC, the potential difference across a load of 100 Ω (R_{ext}) was recorded every 20 minutes using a multichannel Data Logger (Graphtech midi Logger GL820). The generated current density (j) was then calculated by the equation $j=IA^{-1}$, where A is the cathode's surface area and $I=V\,R_{ext}^{-1}$ is the current flowing through the external resistance; V is the potential.

Anodic open-circuit potentials (vs Ag/AgCl in KCl sat.) were periodically measured for each MFC system, after 30 min equilibration time. Power curves were recorded during Cycle 3 (Acetate) after 1 h equilibration time in open circuit condition to calculate the internal resistance of all MFCs.

2.5. *Brunauer–Emmett–Teller (BET) surface area and porosity*

The Brunauer–Emmett–Teller (BET) specific surface area was obtained from the N_2 adsorption/desorption isotherms at 77 K using a Micromeritics Tristar II apparatus. Specific surface area and porosity distribution were evaluated by BET and BJH theories using the instrumental software (Version 1.03). Before measurements, sample powders were heat-treated at 150 °C for 2 h under a N_2 flow to remove adsorbed and undesired species from the sample surface.

2.6. *DNA extraction*

DNA samples were obtained from each MFC at the end of the experiment. The anodic samples were obtained from all reactors. Small pieces of anodic carbon cloth were cut and combined for DNA extraction. The BC and SC cathodic samples were obtained scraping the cathodic biofilm from the carbon cloth with a sterile spatula. The same procedure was used to obtain terracotta biofilm samples for BM and SM reactors. Total DNA was extracted from approximately 0.25 g of samples using a PowerBiofilm DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Quantity and quality of the DNA were measured spectrophotometrically (BioPhotometer, Eppendorf,). DNA was visualized under UV light in a 1% gel electrophoresis with TBE 0.5× (Tris-Borate 50 mM; EDTA 0.1 mM; pH 7.5–8).

No samples were extracted from BM and SM cathodes, because the presence of terracotta separator with average pores size of 10 nm (maximum pores size of 100 nm) impeded the formation of a consistent cathodic biofilm in those reactors.

2.7. *Illumina MiSeq sequencing*

Genomic DNA was PCR amplified using a two-stage "targeted amplicon sequencing (TAS)" protocol [28,29]. The sequencing was performed as described previously [2]. The primers contained 5' common sequence tags (known as common sequence 1 and 2, CS1 and CS2) as described previously [30]. Two primer sets were used for this study, including CS1_341F/CS2_806R (*Bacteria*), CS1_ARC344F/CS2_ARC806R (*Archaea*) [2].

Library preparation and pooling was performed at the DNA Services (DNAS) facility, Research Resources Center (RRC), University of Illinois at Chicago (UIC). Sequencing was performed at the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign (UIUC).

Forward and reverse reads were merged using PEAR [31]. Ambiguous nucleotides and primer sequences were trimmed (quality threshold p = 0.01). After trimming, reads containing internal ambiguous nucleotides, lacking either primer and/or shorter than 300 bp were discarded. Chimeric sequences were identified with the USEARCH algorithm [32] and removed. Further analyses were performed with the QIIME tools [33]. Sequences with a similarity higher than 97% were grouped in Operational Taxonomic Units (OTUs) and representative sequences for each OTU were aligned to the SILVA SSU Ref dataset [34] using the PyNAST method [35]. The information concerning the taxonomic affiliations at phylum and genus level and the respective relative abundance, included in the OTU tables, is represented in Fig. 5 and 6. To compare the microbial diversity between samples, principal coordinate analysis (PCoA) and Unweighted Pair Group Method with Arithmetic mean (UPGMA) clustering were performed calculating weighted UniFrac analysis [36,37]. The significance between different clusters was tested with non-parametric multivariate analysis of variance (PERMANOVA) [38].

3. Results and discussion

3.1. Current generation of MFCs

Trends of the current density generated during Cycle 1, Cycle 2, Cycle 3 (acetate), and Cycle 4 are plotted in **Fig. 2**. For both BM and SM, current production was about 4-5 times lower than for biocathode-MFCs (respectively BC and SC), throughout the operation period of each system.

Focusing on BC module during Cycle 1, after 8 days of low current yield, the system started producing up to 200 µA cm^{-2}, until the soluble organics were consumed and the current production decreased. During Cycle 2, the current density reached slightly higher values and after 15 days started decreasing. During Cycle 3 (acetate), the current production reached again almost 200 µA cm^{-2} and the cycle lasted for less time than previous cycles. This is coherent with the lower amount of COD available (2.4 g_{COD} L^{-1} compared to the previous 13 g_{COD} L^{-1}). The last cycle (Cycle 4) yielded a prolonged current production, decreasing after 15 days.

The corresponding BM system (i.e. fed with the same bovine sewage but with the terracotta separator), produced almost a continuous but low current signal along 90 days of operation, around 50 µA cm^{-2}.

SC system (fed with swine sewage) started promptly producing current (at day 1 during Cycle 1), differently from the longer period needed by the corresponding system fed with bovine sewage. The swine sewage had a lower soluble COD, as compared to the bovine sewage (See Table 1) but the suspended organic matrix was likely less complex and more degradable. The presence of more readily available carbon might have helped during acclimation, hence yielding higher currents. Also, swine sewage is less viscous and diffusion processes are easier, which could facilitate mass transfer processes. An evident peak with a maximum of 350 µA cm^{-2} was recorded already at day 5. Then, a decreasing current trend lasted till the end of the cycle. Cycle 2 showed a similar behavior with a slightly lower current peak. Cycle 3 (acetate) showed a consistent drop in the peak maximum (200 µA cm^{-2}) compared to previous batch cycles. The last cycle (Cycle 4) yielded again a high and prolonged current production.

The corresponding SM system (fed with the same swine sewage, in presence of the terracotta separator) produced an oscillating and low current signal along 90 days of operation, around 50 µA cm^{-2} with some peaks of around 100 µA cm^{-2}.

FIGURE 2

3.2. High-throughput sequencing and microbial community analysis

The DNA was extracted from the biofilms sampled from all anodes, BC and SC biocathodes and BM and SM terracotta surfaces (anolyte-side). **Fig. 3** shows hierarchical clustering analysis via the unweighted pair group method with arithmetic mean (UPGMA). As expected, UPGMA demonstrated that the use of different sewages induced different enrichment in microbiological biofilms. The results are clustered mainly into two different clusters, highlighting that different communities were selected according to the different inoculum. However, the cathodic sample of BC diverged from BM. This aspect highlights strong differences in chemical and electrochemical conditions, between the two conditions. The same result was not confirmed for the MFCs inoculated with swine sewage, probably due to the lower and less constant current density obtained from SC reactor during the experiment (Fig. 2), that surely led to less extreme conditions.

As statistically confirmed by beta significance analyses, the anode samples of bovine sewage MFCs were grouped together and differed significantly from the swine sewage MFCs samples ($p \leq 0.01$).

FIGURE 3

Principal Coordinates Analysis (PCoA) confirmed the clustering identified between samples derived from the same inocula (Fig. 4). Furthermore, a separation from anodic samples to the cathodic and terracotta ones emerged.

FIGURE 4

The phylum distribution is presented in **Fig. 5**. *Bacteroidetes* phylum was the most abundant in all samples and it was present at higher percentages in both biocathode and terracotta samples (30-37%) than in all anodic samples (around 25-28%). The presence of *Bacteroidetes* was previously reported in fermentative bioelectrochemical biofilms [2,39] due to their ability to biodegrade polymeric proteins and carbohydrates.
Firmicutes phylum was often retrieved in bioelectrochemical systems, associated to electrogenic activity [40,41]. Also in this study, it was found in all samples: 18-20% for all anodes and BM terracotta biofilm; 14% for SC cathode and SM terracotta; and >37% for BC cathode.
Proteobacteria OTUs accounted for around 19-26% for almost all samples, but their presence was higher for SC cathode (31%) and very low for BC cathode (5%). Well-known electroactive genera of this phylum, (such as *Geobacter*, *Pseudomonas*, *Desulfuromonas* etc.) often play important roles in bioelectroactive biofilms [42,43].
Euryarchaeota (phylum of *Archaea* domain) presence was different in all samples. Anodic samples of the MFCs treating bovine sewage, BC and BM anodes, showed 11-12% of OTUs, due to the inoculum and the anaerobic condition of this part of the MFCs. *Archaea* were less presents in SC and BC cathodes, as well as in SM terracotta (1-2%), given the more aerobic conditions. A slightly higher number of OTUs was found in BM terracotta sample (5%), since this biofilm was thick enough to guarantee anaerobic conditions in inner layers.

FIGURE 5

The genus representation, according to the OTUs distribution is presented in **Fig. 6**. As commented before, the OTUs collected for genera of *Archaea* domain were more abundant in BC and BM anodes with respect to the other samples. The genera *Methanosaeta* (5% for SC anode, around 3% for BC and SC anodes and less than 2% for all other samples) and *Methanosarcina* (7% only in BC and BM anodes) are both acetoclastic methanogens. They are often found in the anodes of BES, when methanogenesis is not inhibited, competing with exoelectrogens for the same electron donor [44].
The abundance of uncultured *VC2.1 Bac22* ranged between 6 and 11% in all the samples of SC and SM MFCs. The uncultured *Bacteriodetes vadinHA17* was found in all samples (<2%).
vadinBC27 wastewater-sludge group is a genus belonging to the *Bacteirodetes* that was 8-9% for all the bovine sewage-MFCs samples. The distribution of this genus was higher for SC cathode (6%) than for other swine sewage MFCs samples (<3% for SC and SM anodes, <2% for SM terracotta). This genus was previously found in bioelectrochemical biofilms of similar MFCs [2].
Petrimonas, with higher presence in BC cathode than BM terracotta (respectively >6% and 3%), can be considered interesting since it is often found in bioelectroactive biofilms of MFCs [39,45]. Its abundance was less than 2% in all other samples.
Strictly anaerobic and nitrate-reducing species of *Vulcanibacillus* genus were previously reported in electroactive biofilm [46]. In this study, its presence was higher in SC cathodic biofilm (3.4%) than in SM terracotta biofilm (1.9%).
Desulfuromonas genus was one of the most important exoelectrogenic bacteria in all anodic samples. It was more abundant in bovine sewage-MFC anodes (>8% for BC and <6% for BM anodic samples) than in swine sewage-MFC anodes (<6% for SC and <4% for SM anodes). *Desulfuromonas* was also present in SC cathode sample with more than 3% of the total OTUs (less than 0.2% for SM terracotta sample).

Thiopseudomonas genus was found only in the anode of the terracotta-MFCs, BM (1.6%) and SM (4.2%). Facultative anaerobic *Thiopseudomonas* species were found oxidizing sulfide anaerobically with nitrate as electron acceptor in the sludge of an anaerobic, denitrifying, sulfide-removal bioreactor [47].

Several species of *Clostridia* were previously identified as electrogenic microorganisms [2,48]. *Clostridium sensu stricto 1* was found only in swine sewage-MFC anodes (6% for SC and 4% for SM). The same result was recorded in a recent study, in similar air-cathode MFC inoculated with swine sewage [2].

An important presence of *Clostridia MBA03* genera was reported in BC cathodic sample (2% MBA03 uncultured and 9% of other MBA03), but not in terracotta BM sample, which suggests an important electrogenic activity of these genera.

The well-known electroactive bacteria *Geobacter* and *Pseudomonas* were found with an abundance close to the 2% only in SC cathode (less than 0.3% for SM terracotta sample).

Halomonas and *Tissierella* genera were reported only in BC cathode (respectively 1.7% and 1.5%). Although, both genera have not been clearly reported as electroactive, they were previously found in electroactive biofilms of fermentative bioelectrochemical systems [2,49]. Similar conclusions can be drawn for *Erysipelotrichaceae UCG-004* (3.5% in BC cathode) since several genera of *Erysipelotrichaceae* family were previously reported in bioelectrochemical biofilms [2,49].

The results presented in this study were partially corresponding to the few previous reports that went into deep studying microbiological communities in MFCs fed with animal-manures. For example, in swine wastewater treating MFC was reported the presence of *Bacillus, Corynebacterium, Citrobacter, Staphylococcus, Micrococcus* and *Pseudomonas, Lactobacillus, Escherichia coli, Aspergillus*, and *Rhizopus* genera [50]. In another study [51], in MFC treating swine manure, the relative abundance of *Turicibacter*, *Alkaliphilus* sp. and *Bacteroidetes* were significantly higher in the two MFC compared to the raw swine manure samples, suggested an implication of these bacteria in electrogenesis.

Regarding MFC treating bovine wastewater, in Zhao et al. [52] the DGGE analysis showed the presence of genera *Trichococcus* sp., *Pseudomonas* sp., *Bacillus* sp., *Clostridium* sp. and *Alcaligenes* sp..

FIGURE 6

3.3 Comparison between the biofilms of biocathodes-MFCs versus terracotta-MFCs

In all reactors, both anodic and cathodic communities were more influenced by the inoculum than from the presence of terracotta. In particular, statistical analysis (UPGMA and PCoA in **Fig. 3** and **4**) confirmed that similar communities were obtained for anodes inoculated with the same sewage.

Better electrochemical performances (**Fig. 2**) were achieved by both biocathode-MFCs (BC and SC), as compared to the corresponding terracotta-MFCs (BM and SM). This can be due to the absence of a consistent biocathode, able to improve the sole abiotic catalytic activity for oxygen reduction. The consequent lower current generated in terracotta-MFCs, induced only slight changes in anodic microbial communities (**Fig. 6**). In fact, the full-blown or suspected electroactive genera of anodic communities were around 30% for both BC and BM MFCs and around 20% for both SC and SM MFC (**Fig. 6**).

On the contrary, the biofilm developed on BM and SM terracotta presented important differences towards the electroactive communities of BC and SC biocathodic samples. The total amounts of OTUs reported for well-known or suspected electroactive microorganisms in BC cathode was around 25% versus around 5% for BM terracotta. Over 15% of the total OTUs obtained for SC cathodic sample was attributable to either full-blown electroactive microorganisms (such as *Geobacter*, *Pseudomonas* and *Desulfuromonas*) or suspected electroactive genera (such as *Vulcanibacillus, vadinBC27 wastewater-sludge group* and *Petrimonas*). The amounts of all these genera in SM terracotta biofilm was <5%.

In bioanodes of all MFCs, *Desulfuromonas* mainly directed the exoelectrogenic activity, supported by an important presence of acetoclastic methanogens. In previous studies, Direct Interspecies Electron Transfer (DIET) between *Archaea* and exoelectrogenic bacteria was associated to the increasing of exoelectrogenic activity [53]. Here, in SC MFC, exoelectrogenic activity was performed also by *Clostridium sensu stricto I*, as recently reported for a similar MFC inoculated with swine sewage [2].
Thiopseudomonas genus was reported in the bioanode of both terracotta-MFCs (BM and SM). *Thiopseudomonas*, a genus of well-known electroactive *Pseudomodaceae* family, was often associated to the anaerobic oxidation of reduced sulfide compounds. Thus, its higher presence suggests a higher amount of reduced sulfur compounds in the terracotta-MFCs, also due to the presence of sulfur reducing bacteria (such as *Desulfuromonas*). In previous studies, it was hypothesized that the cathodic biofilm catalyzes ORR by promoting cyclic red-ox mechanisms, such as with sulfur compounds, facilitating the dispatch of electrons to oxygen [2,3,8,9]. The presence of terracotta separator with such small pores (10-100 nm), as detected by a BET and BJH analyses, impeded the formation of a consistent cathodic biofilm in BM and SM reactors. Thus, the absence of the cathodic biofilm was likely hindering the oxidation of sulfur compounds at cathode. This led to increase the amount of reduced sulfide and consequently enriching the microbial community in *Thiopseudomonas*. This result confirms that cathodic biofilms were not only consuming oxygen with traditional aerobic/microaerophilic metabolic pathways, but they also contributed to the electrochemical catalysis of cathodic ORR, according to more recent studies [2].
Deeply different were the microbial communities that colonized terracotta and cathodic biofilms. Several well-known electroactive bacteria as *Geobacter*, *Pseudomonas*, *Clostridia* and *Desulfuromonas* were found in biocathodic communities but not in terracotta biofilms. On the contrary, terracotta biofilms were colonized only by fermentative bacteria. That suggests that the possibility to interact with a conductive material, as cathodic carbon cloth, was ensuring better conditions for electroactive bacteria growth.
In the case of bovine sewage MFCs, the main difference between BC cathode and BM terracotta was represented by the high presence of genera of *Clostridia MBA03* only in BC cathode. In BC cathode, *Clostridia MBA03* represented >11% of the total OTUs, but it was not present at all in BM terracotta sample and it was <1% in the other samples. *Clostridia* are well known to be electroactive bacteria and their presence only in cathodic BC sample was definitely due to their capability to interact with conductive materials. In BC cathode, there were exclusively present other genera that were previously found in bioelectroactive biofilms and suspected to be electroactive (*e.g.* *Tissierella* and *Halomonas*) [2,49].

Conclusions

Different microbial communities were enriched in MFCs using bovine (for BC and BM) and swine (for SC and SM) sewages. The cathodic and terracotta biofilms in BC and SC MFCs were microbiologically different as confirmed by statistical analysis (unweight pair group method with arithmetic mean (UPGMA) and beta diversity calculated as principal coordinates analysis (PCoA)). In BM and SM MFCs, the presence of terracotta (pores size 10-100 nm) between the anode and the cathode impeded the biocathode development, undermining current production. The electroactive microorganisms were more enriched on conductive material, the cathode, than on non-conductive material, the terracotta. Indeed, the OTUs of well-known or suspected electroactive microorganisms in BC and SC cathodic samples (mainly *Geobacter*, *Pseudomonas*, *Desulfuromonas* and *Clostridia MBA03*) were respectively 3 and 5 times more than in BM and SM terracotta biofilms. The electroactive anodic communities were slightly influenced by the presence of terracotta, the increasing of internal resistance and the consequent lower current production than the cathodes.
In the next future, the use of terracotta as low cost and biocompatible material for MFC might be improved. We propose, for example, to investigate new composite porous materials with electro-conductive and electro-catalytic properties.

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Vitae

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Figure captures and figures

Fig. 1a. Schematic of the four reactors used in this study; and **1b.** Pictures of the biofilm growth on cathodes (BC-cat and SC-cat) or terracotta (BM-ter and SM-ter). In the pictures is possible to observe the white silicone circle used to attach the cathodes and the terracotta to the Simple Pyrex® bottles.

Fig. 2. Current density trends of MFCs during the operational period of 90 days.

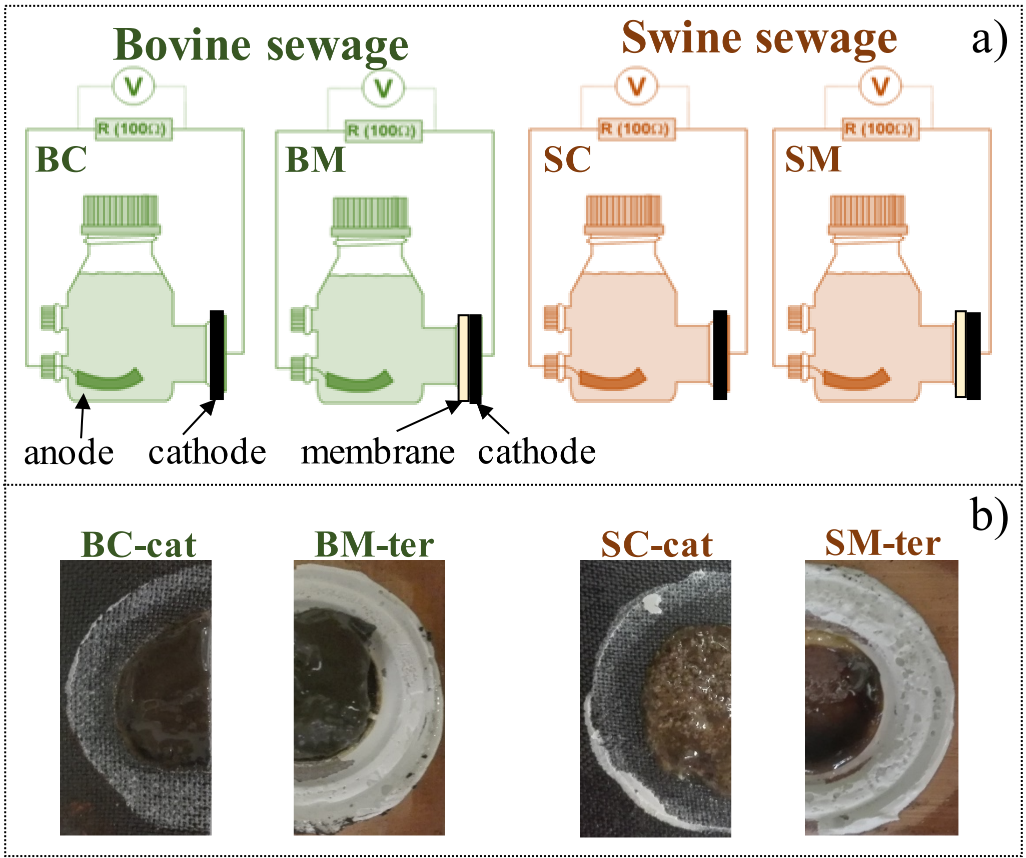
Fig. 3. UPGMA clustering analysis showing swine and bovine sewage samples belonging to significantly different groups ($p \leq 0.01$).

Fig. 4. Beta diversity calculated as principal coordinates analysis (PCoA) performed calculates weighted UniFrac distance between all samples. Grey dashed circles indicate groups of samples significantly correlated, according to Permutational Multivariate Analysis of Variance (PERMANOVA, $p \leq 0.01$).

Fig. 5. Relative abundance at phylum level obtained with high throughput Illumina amplicon sequencing.

Fig. 6. Genus representation of Illumina 16S rRNA gene amplicon sequencing results of anodic and cathodic biofilms. Red dashed squares indicate full-blown or suspected electroactive genera according to the literature.

FIGURE 1



Bovine sewage
Swine sewage
a)
V
R (100Ω)
BC
BM
SC
SM
anode
cathode
membrane
cathode
b)
BC-cat
BM-ter
SC-cat
SM-ter

FIGURE 2

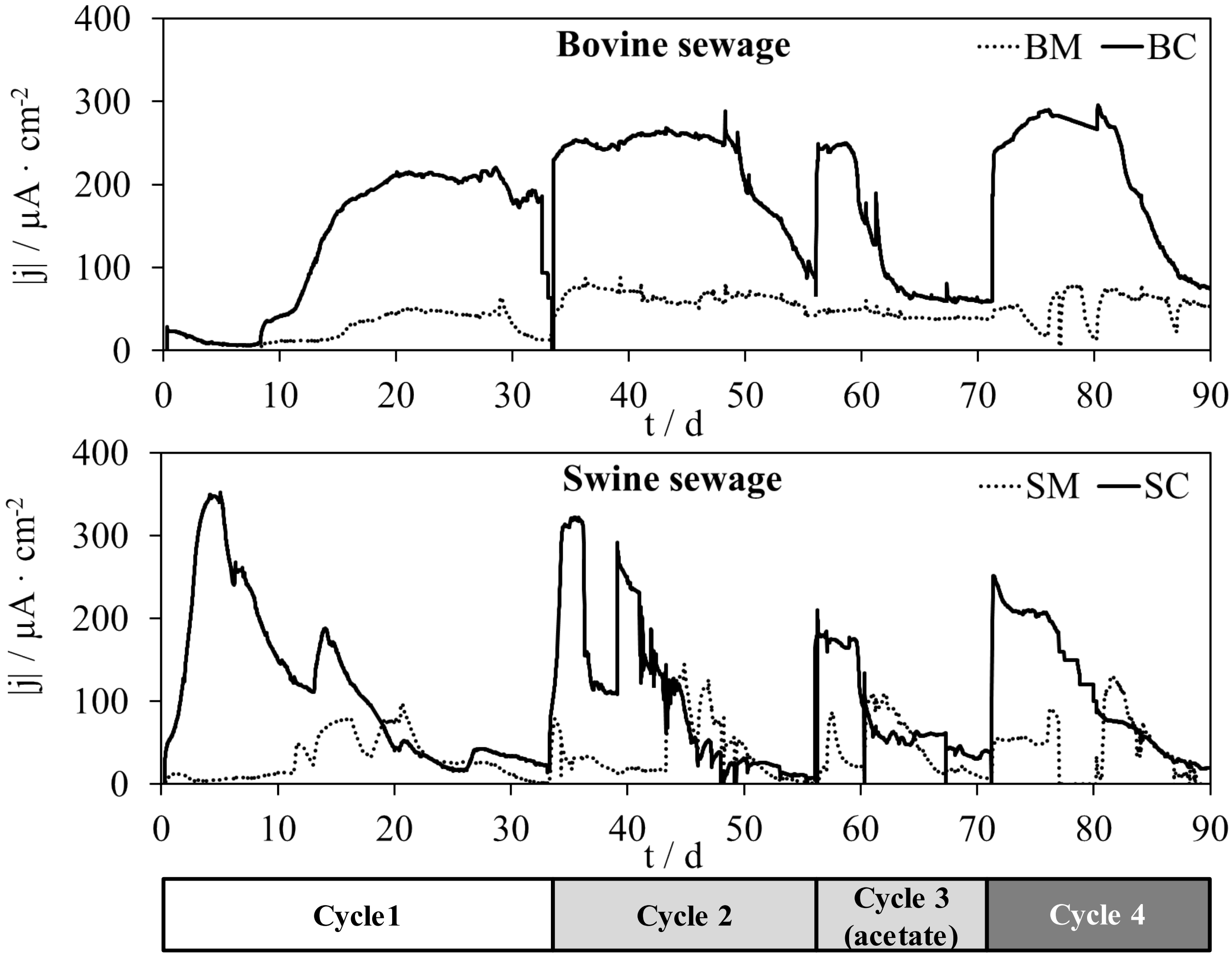

Bovine sewage
BM
BC
|j| / µA · cm-2
t / d
Swine sewage
SM
SC
|j| / µA · cm-2
t / d
Cycle1
Cycle 2
Cycle 3 (acetate)
Cycle 4

FIGURE 3

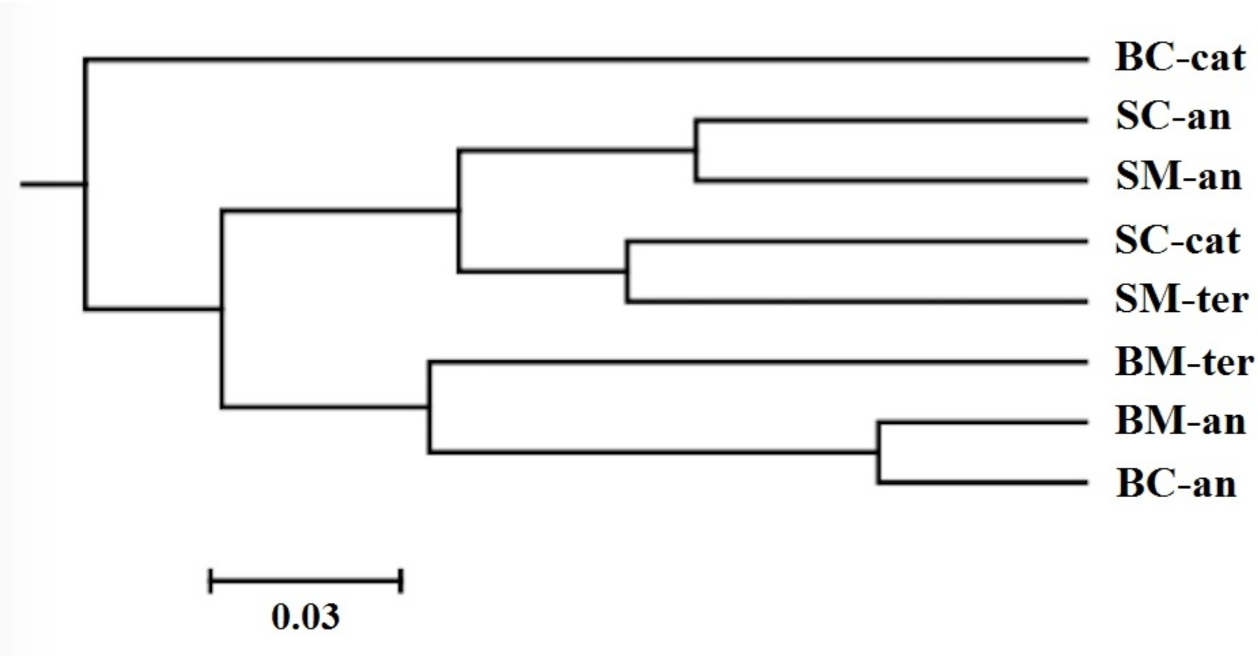
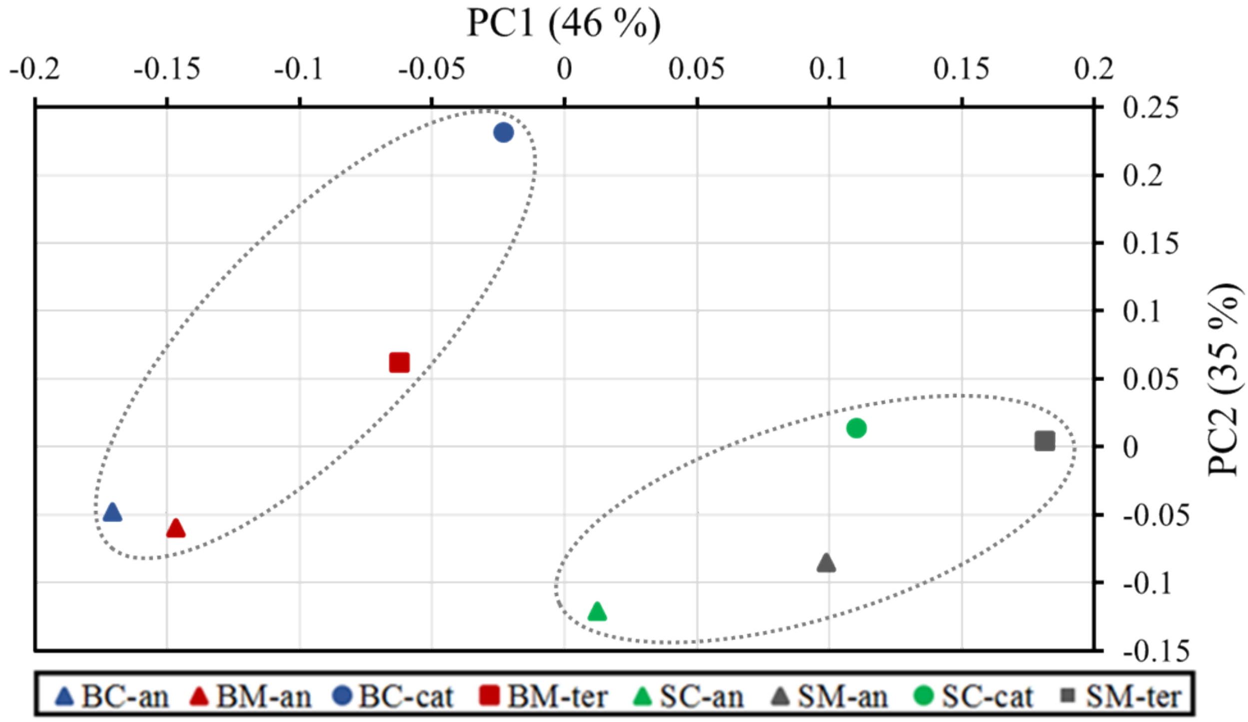
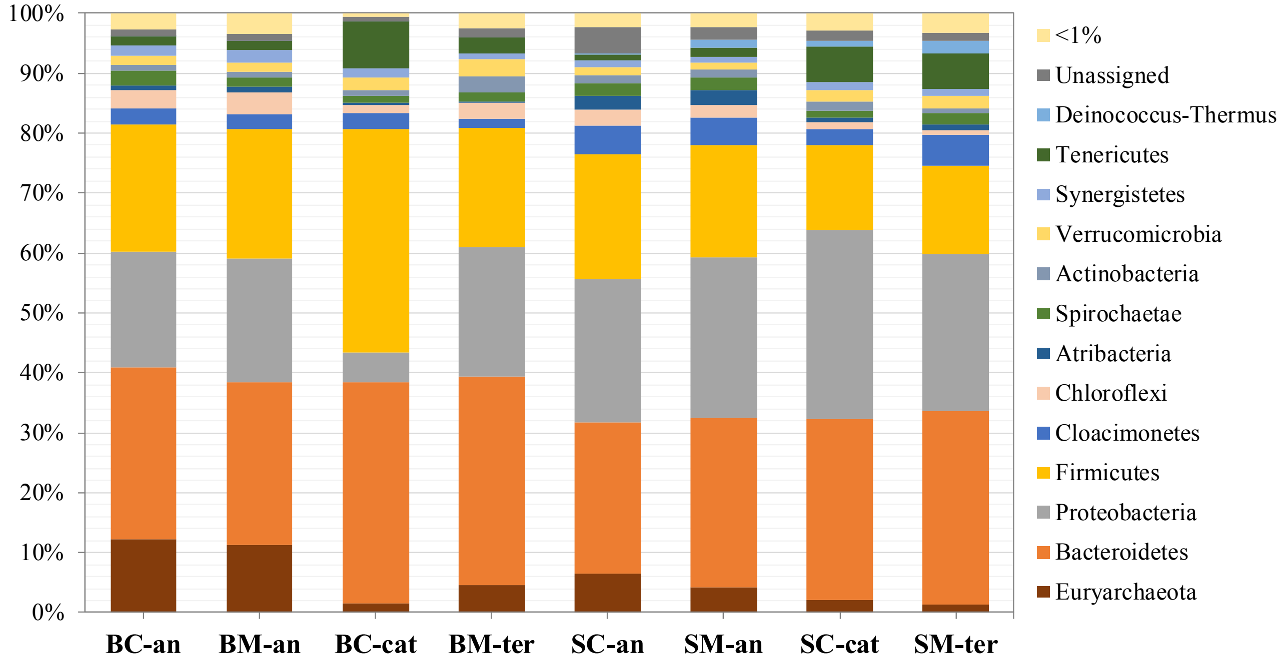


FIGURE 4



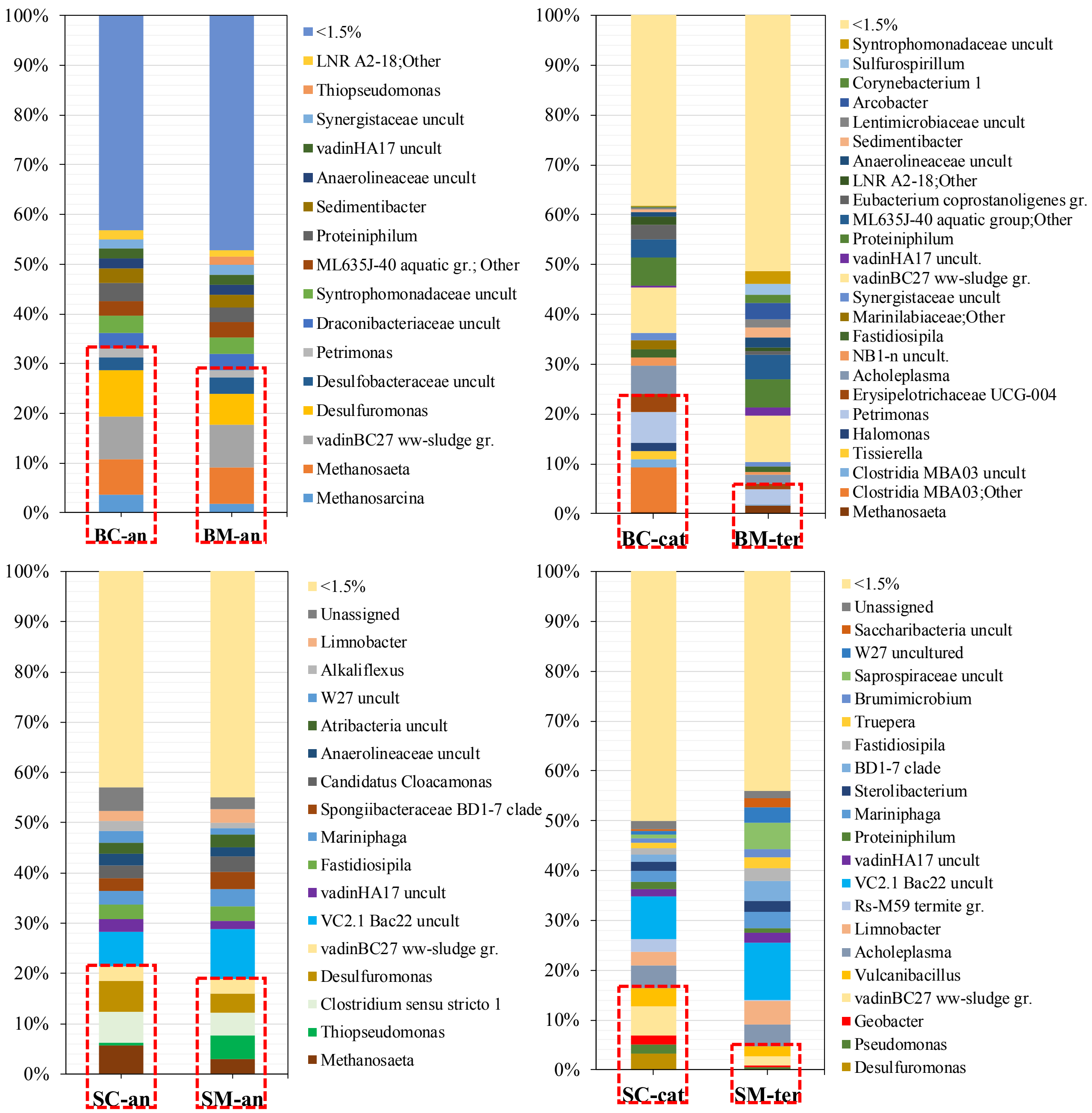
PC1 (46 %)
-0.2
-0.15
-0.1
-0.05
0
0.05
0.1
0.15
0.2
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
PC2 (35 %)
BC-an
BM-an
BC-cat
BM-ter
SC-an
SM-an
SC-cat
SM-ter

FIGURE 5



100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
BC-an
BM-an
BC-cat
BM-ter
SC-an
SM-an
SC-cat
SM-ter
<1%
Unassigned
Deinococcus-Thermus
Tenericutes
Synergistetes
Verrucomicrobia
Actinobacteria
Spirochaetae
Atribacteria
Chloroflexi
Cloacimonetes
Firmicutes
Proteobacteria
Bacteroidetes
Euryarchaeota

FIGURE 6


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
BC-an
BM-an
<1.5%
LNR A2-18;Other
Thiopseudomonas
Synergistaceae uncult
vadinHA17 uncult
Anaerolineaceae uncult
Sedimentibacter
Proteiniphilum
ML635J-40 aquatic gr.; Other
Syntrophomonadaceae uncult
Draconibacteriaceae uncult
Petrimonas
Desulfobacteraceae uncult
Desulfuromonas
vadinBC27 ww-sludge gr.
Methanosaeta
Methanosarcina
BC-cat
BM-ter
<1.5%
Syntrophomonadaceae uncult
Sulfurospirillum
Corynebacterium 1
Arcobacter
Lentimicrobiaceae uncult
Sedimentibacter
Anaerolineaceae uncult
LNR A2-18;Other
Eubacterium coprostanoligenes gr.
ML635J-40 aquatic group;Other
Proteiniphilum
vadinHA17 uncult.
vadinBC27 ww-sludge gr.
Synergistaceae uncult
Marinilabiaceae;Other
Fastidiosipila
NB1-n uncult.
Acholeplasma
Erysipelotrichaceae UCG-004
Petrimonas
Halomonas
Tissierella
Clostridia MBA03 uncult
Clostridia MBA03;Other
Methanosaeta
SC-an
SM-an
<1.5%
Unassigned
Limnobacter
Alkaliflexus
W27 uncult
Atribacteria uncult
Anaerolineaceae uncult
Candidatus Cloacamonas
Spongiibacteraceae BD1-7 clade
Mariniphaga
Fastidiosipila
vadinHA17 uncult
VC2.1 Bac22 uncult
vadinBC27 ww-sludge gr.
Desulfuromonas
Clostridium sensu stricto 1
Thiopseudomonas
Methanosaeta
SC-cat
SM-ter
<1.5%
Unassigned
Saccharibacteria uncult
W27 uncultured
Saprospiraceae uncult
Brumimicrobium
Truepera
Fastidiosipila
BD1-7 clade
Sterolibacterium
Mariniphaga
Proteiniphilum
vadinHA17 uncult
VC2.1 Bac22 uncult
Rs-M59 termite gr.
Limnobacter
Acholeplasma
Vulcanibacillus
vadinBC27 ww-sludge gr.
Geobacter
Pseudomonas
Desulfuromonas

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TABLES

Table 1. Characterization of raw wastewaters

	pH	*Total Kjieldahl Nitrogen / mg L^{-1}*	*Specific conductivity / mS cm^{-1}*	*Total COD / g L^{-1}*	*Soluble COD / g L^{-1}*
Bovine sewage	*7.41*	*2.35*	*9.70*	*29.15*	*12.66*
Swine sewage	*7.59*	*2.52*	*10.38*	*8.50*	*5.62*

Table 2. Performance of all MFCs during a representative batch cycle (Cycle 2).

	% COD removal	*Internal resistance/Ω*	*Max power density / mW m^{-2}*	*Anodic open circuit potential / mV vs Ag/AgCl*
BC	*55*	155	*283*	*-468*
BM	*35*	4876	*33*	*-492*
SC	*65*	261	*333*	*-503*
SM	*57*	2606	*67*	*-498*