Here we provide the reply to the reviewer comment.

Reviewer #1: I have read with interest this manuscript reporting on the 'Anodic and cathodic microbial communities in single chamber microbial fuel cells'. Though interesting, after going through the whole manuscript, I found several gaps which need to be addressed by the authors.

Just to give some examples, at several places random observations have been made without giving any proper reference for them. Similarly, there is problem with sentence formation and the quality of English is poor. The authors are advised to have their manuscript proof-read by a native English speaker. Also the discussion section is weak with authors mostly comparing the results among the two types of bacteria tested and little comparison has been made with previous MFC literature. Thus, I do believe that there is enough scope for improvement in the manuscript in its present form.

We thank the reviewer for the suggestions. The entire manuscript was proof-read by a native English speaker to correct the sentence formation and improve the overall quality of English. Furthermore we added an overall description of the previous findings regarding the microbial community composition associated to the electrodes in Bioelectrochemical Systems. This part was linked to the discussion requested at point 10 about the influence of the electrode potential in determining the microbial community structure.

Page 8, Line 162-172: "Interestingly only a small fraction of the sequences in the anodic community (<0.1%) belonged to the order *Desulfomonadales*, which usually dominates

the acetate oxidizing communities in BES [33]. The dominance of bacteria belonging to *Geobacter* genus was previously described to be unaffected by the anode potential [19]. However a recent study demonstrated that different *Geobacter* clades and different microbial associations were linked to specific potentials. In fact, *Geobacter metallireducens* clade appeared to be associated with more negative potentials, while *Geobacter* clades 1 and 2 were observed at more positive potentials [34]. The effect of the electrode potential on the cathodic communities is not well described. Several authors reported the presence of different microorganisms in the cathodic biofilm and changes in community composition according to the cathode potential [8].”

Besides this points, I also have some other remarks which I mention here-

1. Abstract, Page 2, Line 19 - Replace the word 'new' with 'rapidly growing'. I don't think that after 15 years of intensive research, the MFC is still a new technology. It is in fact now on the verge of commercialization.

We replaced the word as the reviewer requested.

2. Abstract, Page 2, Line 19 - Please replace the word 'biomasses' with 'wastewater and biomasses'. Please don't omit the fact that the first application of MFC is wastewater treatment and other applications are secondary.

We replaced the word as the reviewer requested.

3. Abstract, Page 2, Line 20 - It should be 'a MFC'.

We changed the text as requested.

4. Abstract, Page 2, Line 24 - Please replace 'ETT' with 'EET'.

We replaced the word as requested.

5. Introduction, Page 3, Line 35-36 - Please provide an appropriate citation for this sentence. Authors may refer to and cite here - "2013. Valorization of cereal based biorefinery by-products: Reality and expectations. Environmental Science & Technology. 47(16): 9014-9027."

We thank the reviewer for the suggestion and we added the reference as requested.

6. Page 5, Line 99 - It should be '-20 °C'. Mind the space between the number and unit. Please correct at all places in the manuscript.

Thanks. We corrected this mistake in the whole manuscript.

7. Page 7, Line 130-143 - All these results are with acetate as the substrate. How did this performance compared with real wastewater. Please refer to these recent papers and cite here - "1/ 2013. Integrated conversion of food waste diluted with sewage into volatile fatty acids through fermentation and electricity through a fuel cell. Environmental Technology. 34(13-14): 1935-1945". 2/ 2014. Bioelectro-catalytic valorization of dark fermentation effluents by acetate oxidizing bacteria in bioelectrochemical system (BES). Journal of Power Sources. 262: 183-191.

We thank the reviewer for the suggestion. We discussed the electrochemical performance of the reactors referring to the two recent papers as suggested.

Page 7, Line 146-154: "All the studies described above used acetate as carbon source, but in studies where wastewater was used to feed the reactors, the electrochemical performance further decreased. One study showed the performance of a SCMFC fed

with the effluent from a wastewater fermentation reactor where a current density of only 65 mA/m^2 was reached [31]. A similar substrate was used by the same authors in a further study in which a two chamber MFC was inoculated with cattle manure at different loading rates. This reactor performed better than the previous. The maximum power output was 165 mW/m^2 at a loading rate of 190 g COD/m^3, but decreased to 39 mW/m^2 when the loading rate was increased to 570 g COD/m^3 [32].”

8. Page 7, Line 146 - What is RDP?

The Ribosomal Database Project (RDP) Bayesian Classifier is a database commonly used for the taxonomic assignments of the 16S rRNA gene sequences. In order to be more clear we reported the name in extenso in the Materials and Methods section and we added the reference: Wang, Q, G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Appl Environ Microbiol. 73(16):5261-7.

9. Page 8, Lines 176 - Sulfate-reducing bacteria has been reported in bioelectrochemical systems especially for bioelectrosynthesis; Please refer to this paper and cite here - "2013. Bioelectrocatalyzed reduction of acetic and butyric acids via direct electron transfer by a mixed culture of sulfate-reducers drives electrosynthesis of alcohols and acetone. Chemical Communications. 49(58): 6495-6497".

We cited the paper as suggested by the reviewer.

Page 9, Line 196-199: “PNS were hypothesized to take part in oxygen reduction by a cycling oxidation of sulfide to sulfate through the cathode in a synergistic mechanism together with sulfate-reducing bacteria, described to have a role both in the anodic [34] and in the cathodic EET [41], and spirochetes [11].”

10. Authors reported about the different microbial communities at the anode and cathode of a MFC. Is there a way to influence this microbial population such as by setting a particular pH or posing these electrodes at a set potential so that the desired species preferably grow there? Also are all the species reported here electroactive in nature and do they all contribute to the current generation by the MFC? Please discuss these points as well to make your discussion part stronger.

We thank the reviewer for the suggestion to extend the discussion section. We reported here on the effect of pH and electrode potential.

Page 8, Line 165-172: "The dominance of bacteria belonging to *Geobacter* genus was previously described to be unaffected by the anode potential [19]. However a recent study demonstrated that different *Geobacter* clades and different microbial associations were linked to specific potentials. In fact, *Geobacter metallireducens* clade appeared to be associated with more negative potentials, while *Geobacter* clades 1 and 2 were observed at more positive potentials [34]. The effect of the electrode potential on the cathodic communities is not well described. Several authors reported the presence of different microorganisms in the cathodic biofilm and changes in community composition according to the cathode potential [8]."

Page 10, Line 207-212: "The pH value played a crucial role in selecting the electroactive biofilm composition. Patil and coworkers demonstrated that varying the pH in the anodic chamber lead to a change in the performance of the reactor, producing higher current densities at pH 7. The highest bioelectrocatalytically active biofilms were dominated by *Geobacter sulfurreducens*, while the microbial communities with the lower performance showed greater diversity [44]."

Furthermore for each of the most abundant genera an outline of the principal electron acceptors used in nature was provided.

***Highlights (for review)**

- Microbial communities developed in an SCMFC inoculated with biogas digestate
- Illumina sequencing of the 16S rRNA gene allowed the description of the communities
- The anodic community was dominated by Fe(III) reducers belonging to *Geovibrio* genus
- On the cathode *Nitrincola* genus dominated the microbial community
- Results suggest that an oxygen gradient influenced the composition of the biofilm

ANODIC AND CATHODIC MICROBIAL COMMUNITIES IN SINGLE CHAMBER MICROBIAL FUEL CELLS

Authors: Matteo Daghio[a], Isabella Gandolfi[a], Giuseppina Bestetti[a], Andrea Franzetti[a], Edoardo Guerrini[b], Pierangela Cristiani[c]

Affiliations:

[a]Dept. of Earth and Environmental Sciences – University of Milano-Bicocca, Piazza della Scienza 1, 20126 Milan, Italy

[b]Dept. of Chemistry, University of Milano, Via Golgi 19, 20133, Milan, Italy

[c]RSE — Ricerca sul Sistema Elettrico S.p.A., Environment and Sustainable Development Department, Via Rubattino 54, 20134 Milan, Italy

Corresponding author: Andrea Franzetti; phone number: +39 0264482927; e-mail address: andrea.franzetti@unimib.it

Abstract

Microbial fuel cells (MFCs) are a rapidly growing technology for energy production from wastewater and biomasses. In a MFC, a microbial biofilm oxidizes organic matter and transfers electrons from reduced compounds to an anode as the electron acceptor by extracellular electron transfer (EET). The aim of this work was to characterize the microbial communities operating in a Single Chamber Microbial Fuel Cell (SCMFC) fed with acetate and inoculated with a biogas digestate in order to gain more insight into anodic and cathodic EET. Taxonomic characterization of the communities was carried out by Illumina sequencing of a fragment of the 16S rRNA gene. Microorganisms belonging to *Geovibrio* genus and purple non-sulfur (PNS) bacteria were found to be dominant in the anodic biofilm. The alkaliphilic genus *Nitrincola* and anaerobic microorganisms belonging to *Porphyromonadaceae* family were the most abundant bacteria in the cathodic biofilm.

Keywords: microbial characterization, microbial fuel cells, next generation sequencing, *Geovibrio*, *Nitrincola*

Introduction

Microbial fuel cells (MFCs) are innovative systems for energy production from renewable biomass sources and from biomass derived wastes [1]. In an MFC bacteria can oxidize organic matter in anaerobic conditions and transfer the electrons to an anode that serves as solid electron acceptor. The electrons then pass through a circuit and combine with protons and a terminal electron acceptor at the cathode [2] where the process can be mediated by microorganisms [3]. The processes involved in the transfer of electrons to/from the electrodes are known as External Electron Transfers (EET). The anodic communities can transfer the electrons by direct contact using membrane cytocromes or conductive pili, or by using shuttles that can be reduced on the cellular surface then diffuse to the anode where they are oxidized thus transferring the electrons to the electrode [4–7]. Although the EET involving the cathodic communities may be similar to those used to transfer the electrons to the anode [8], more insights are still needed to globally describe these mechanisms and the microorganisms involved.

The most typical tools used to characterize the microbial communities in MFCs use a molecular approach. The 16S rRNA gene is generally used as a molecular marker in performing the fingerprinting of the communities. In a previous study a Denaturing Gradient Gel Electrophoresis (DGGE) technique was used to describe both anodic and cathodic communities in Single Chamber MFCs (SCMFCs) fed with acetate dissolved in an inoculum of raw municipal wastewater. The results suggested that the sulfur cycle could have a crucial role in cathodic EET [9–11]. Other studies used DGGE for molecular fingerprinting to assess the effect of the sediment matrix, the inoculum [12], the operational time [13], the electron donors [14] and to understand how the taxonomic composition can affect the power density [15]. Other molecular techniques adopted to describe microorganisms colonizing the

electrodes are Fluorescence In Situ Hybridization (FISH), which uses specific probes that allow quantification of specific populations within the whole bacterial community [16], or Terminal Restriction Fragment Length Polymorphism (T-RFLP) [12]. Moving beyond these techniques, the recent development of Next Generation Sequencing (NGS) technologies has greatly improved the capability to describe microbial communities. In recent years, sequencing costs have rapidly declined and consequently the amount of available data has increased exponentially. Owing to their high throughput and the decreasing cost per sequence, NGS techniques have great potential to describe the diversity and composition of microbial communities [17]. For example, Illumina and 454 pyrosequencing technologies can generate up to millions of amplicon sequences in a single run, thus providing high coverage both to amplicon-based and whole metagenomic studies of microbial communities. Thus, this technology could be used to help fill the gaps in the current knowledge of microbial community structure involved in EET mechanisms [18]. This approach has been applied in recent studies reporting that the anode potential [19] and the sampling point position on the electrode surface [20] did not affect the microbial composition of the anodic communities, whereas different chemical treatments of the anode surface can lead to the development of biofilms with different taxonomic compositions [21]. However, there is still a considerable lack of knowledge in this field particularly regarding the microbial communities operating at the cathodes.

In this work we described the anodic and cathodic bacterial communities in a SCMFC operated with digestate from a biogas plant, using Illumina sequencing of the 16S rRNA gene in order to gain insight into the processes that select bacterial populations on MFC electrodes. The interest in biogas digestate as a matrix for MFC operation is particularly boosted by the

fact that its treatment is one of the most promising applications for MFC technology to remove nitrogen and phosphate pollutants [22].

Materials and Methods

SCMFC operation

The experiment was carried out using an SCMFC (solution volume: 125 mL) operated with an external resistor (R_{ext}) of 100 Ω, at a temperature of 30±2 °C. A Pt-free cathode (10 cm^2 projected area) was made with carbon cloth (30 wt.% PTFE, FuelCellEarth) and a Micro-Porous Layer (MPL) of 30–50 μm thickness was applied since it was found to enhance oxygen exchange and facilitate the biofilm growth [23]. The MPL was made from carbon black particles (VulcanXC-72R), PTFE (60% emulsion, Sigma Aldrich), distilled water and a non-ionic surfactant (Triton X100, Sigma Aldrich) as previously described [24,25]. Untreated carbon cloth (SEAL, Legnano, Italy) of 10 cm^2 was used as anode and acetate was added periodically as the carbon source at a concentration of 3g/L. The cell was inoculated with biogas digestate (pH 8.2, conductivity 15 mS/cm, soluble COD 2380 mg/L, soluble BOD_5 200 mg/L) and current was monitored over time. Anodic and cathodic biofilm samples were collected after 41 days in order to describe in detail the communities by NGS of the 16S rRNA gene.

Amplification of the 16S rRNA gene, sequencing and sequence analyses

Samples of anodic and cathodic biofilm were aseptically removed from the electrodes and stored at -20 °C until further processing. Total bacterial DNA was extracted from the samples using the FastDNA Spin for Soil kit (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions.

104 The V5-V6 hypervariable regions of the 16S rRNA gene were amplified in 3 x 80 µL volume
105 reactions with GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA) and 1
106 µM of each primer. 783F and 1027R primers were used [26,27] and the cycling conditions
107 were: initial denaturation at 94 °C for 5 min; 29 cycles of 94 °C for 50 s, 47 °C for 30 s, and
108 72 °C for 30 s and final extension at 72 °C for 5 min. At the 5' end of 783F primer, a 6-bp
109 barcode was also included to allow sample pooling and subsequent sequence sorting. The
110 amplified products were purified with the Wizard® SV Gel and PCR Clean-up System
111 (Promega Corporation, Madison, WI, USA) and DNA quantity and purity were
112 spectrophotometrically evaluated by NanoDrop™ (Thermo Scientific, USA).
113 Purified amplicons with different barcodes were pooled in 100 µL samples with a DNA
114 concentration of 40 ng/µL. Multiplexed sequencing of all the pooled samples were performed
115 on a single Illumina Hiseq 1000 lane, using a paired-end 2x100 base-pair protocol and the 4.0
116 sequencing chemistry. The cluster extraction and base-calling processing analyses were
117 performed using the Illumina CASAVA Analysis software, version 1.8. Illumina Hiseq 1000
118 sequencing was carried out at BMR Genomics, Padua, Italy.
119 Each sequence was assigned to its original sample according to its barcode. A quality cut-off
120 was then applied in order to remove sequences i) that did not contain the barcode, and ii) with
121 an average base quality value (Q) lower than 30. The barcode was removed from sequences
122 before further processing. The reverse read of each paired-end sequence was reverse-
123 complemented and merged with the corresponding forward read, inserting 10 Ns in between
124 [18]. The taxonomic attribution of filtered sequences was carried out using the stand-alone
125 version of the Ribosomal Database Project (RDP) Bayesian Classifier [28], using 50%
126 confidence, as suggested for sequences shorter than 200 bp [18].
127

Results and Discussion

Electric output from SCMFC

After 2 days the current density profile rose from negligible values up to 2810 mA/m^2 then dropped down to zero at day 6 (Figure1). After feeding the SCMFC with 3 g/L of acetate the current rose again and reached a maximum of 3800 mA/m^2 (corresponding to a maximum power density of 1444 mW/m^2) after 16 days of incubation during the third batch cycle. After this peak the current decreased to a stable value (1840±220 mA/m^2) and the following additions of acetate (days 23, 29 and 39) produced only small peaks of current. This current density profile and the maximum current observed are consistent with previous observations from reactors with the same architecture, but different wastewater inoculum [16]. The performance was considerably higher compared to other studies carried out using SCMFCs inoculated with anaerobic sludge and using different cathode materials. In these cases, maximum current densities (and maximum power densities) of 350 mA/m^2 (109.5 mW/m^2), 210 mA/m^2 (32.7 mW/m^2), 18 mA/m^2 (3.1 mW/m^2) were reached using graphite felt, carbon paper and stainless steel mesh respectively as cathode material [29]. In another study using wastewater as the inoculum, maximum current densities between 3440 mA/m^2 and 2040 mA/m^2 (corresponding to power densities of 802 mW/m^2 and 584 mW/m^2) were observed with a cathode made by rolling activated carbon and PTFE at different ratios [30]. All the studies described above used acetate as carbon source, but in studies where wastewater was used to feed the reactors, the electrochemical performance further decreased. One study showed the performance of a SCMFC fed with the effluent from a wastewater fermentation reactor where a current density of only 65 mA/m^2 was reached [31]. A similar substrate was used by the same authors in a further study in which a two chamber MFC was inoculated with cattle manure at different loading rates. This reactor

performed better than the previous. The maximum power output was 165 mW/m^2 at a loading rate of 190 g COD/m^3, but decreased to 39 mW/m^2 when the loading rate was increased to 570 g COD/m^3 [32].

Microbial community characterization

The classification of the sequences was performed with a RDP Bayesian classifier (50% confidence) and a comparison between the anodic and cathodic communities was performed at the fourth taxonomic level (Figure2). The most abundant orders in the anodic community were *Deferribacterales* (51.6% of the sequences) and *Rhodospirillales* (9.0% of the sequences). In the cathodic biofilm the main taxonomic groups were *Oceanospirillales* (37.8% of the sequences) and *Bacteroidales* (20.4% of the sequences). Interestingly only a small fraction of the sequences in the anodic community (<0.1%) belonged to the order *Desulfomonadales*, which usually dominates the acetate oxidizing communities in BES [33]. The dominance of bacteria belonging to *Geobacter* genus was previously described to be unaffected by the anode potential [19]. However a recent study demonstrated that different *Geobacter* clades and different microbial associations were linked to specific potentials. In fact, *Geobacter* metallireducens clade appeared to be associated with more negative potentials, while *Geobacter* clades 1 and 2 were observed at more positive potentials [34]. The effect of the electrode potential on the cathodic communities is not well described. Several authors reported the presence of different microorganisms in the cathodic biofilm and changes in community composition according to the cathode potential [8].

A more in depth characterization of the most abundant orders was performed at a family and genus level. Almost all the detected sequences classified as *Deferribacterales* belong to the family *Deferribacteraceae* and to the genus *Geovibrio* (98.1% of the *Deferribacterales*)

(Figure3). This genus is characterized by gram-negative, strictly anaerobe bacteria able to couple the oxidation of acetate to Fe(III), S^0, Co(III) and Se(VI) reduction [25,35]. *Geovibrio* genus is not related to the other metal reducing bacteria in *Proteobacteria* phylum and forms a separate line [36]. *Geovibrio ferrireducens* was previously detected by DGGE in dual chamber microbial fuel cells fed with slaughterhouse wastewater [37]. The importance of *Deferribacteraceae* as bioelectrogenic active microorganisms was also reported for the anodic biofilm of a five face parallelepiped SCMFC inoculated with an Fe(III)-reducer enrichment. In that case, clones close to *Geovibrio ferrireducens*, *Geovibrio thiophilus* and *Denitrovibrio acetiphilus* (all members of the *Deferribacteraceae*) were found to be the most abundant in the microbial community colonizing the electrode surface [38]. Among the *Rhodospirillales* two main families were detected: *Acetobacteraceae* (4.1% of the sequences) and the purple non-sulfur (PNS) bacteria *Rhodospirillaceae* (94.4% of the *Rhodospirillales*) (Figure4). Within the latter family different genera were detected and the most abundant were *Caenispirillum*, *Roseospira*, *Skermanella*, and *Rhodospira* (respectively 35.9%, 26.7%, 9.5% and 4.1% of the *Rhodospirillales*). PNS are a non-taxonomic group with a versatile metabolism [39], they can grow as photoheterotrophs, but can also use reduced forms of sulfur such as S, H_2S and $S_2O_2^{3-}$ or Fe(II) [40] as an electron donor, switching from one mode to another depending on available conditions such as oxygen concentration, carbon source and light source [39]. The oxidation of H_2S leads to the formation of S_0 which is then converted to SO_4^{2-} [40]. The role of PNS bacteria in the electron transfer mechanisms in SCMFCs was previously reported. PNS were hypothesized to take part in oxygen reduction by a cycling oxidation of sulfide to sulfate through the cathode in a synergistic mechanism together with sulfate-reducing bacteria, described to have a role both in the anodic [34] and in the cathodic EET [41], and spirochetes [11]. *Rhodopseudomonas palustris*, a PNS bacterium, was also

found to be dominant together with *Geobacter sulfurreducens* in the anodic biofilm of an SCMFC in which power production increased when exposed to high light intensities [42]. Among the *Oceanospirillales*, the most abundant taxon in the cathodic community, the biodiversity was very low since 98.4% of the sequences belonged to the genus *Nitrincola* (family *Oceanospirillaceae*) (Figure5). Microorganisms belonging to this genus were previously isolated from an alkaline, saline lake. *Nitrincola lacisaponensis,* for instance, shows its highest growth at a pH of 9.0, and it is able to use a wide range of carbon sources using both O_2 or NO_2^- as electron acceptors [43]. The pH value played a crucial role in selecting the electroactive biofilm composition. Patil and coworkers demonstrated that varying the pH in the anodic chamber lead to a change in the performance of the reactor, producing higher current densities at pH 7. The highest bioelectrocatalytically active biofilms were dominated by *Geobacter sulfurreducens*, while the microbial communities with the lower performance showed greater diversity [44]. In SCMFC the pH increase can affect both the anodic and the cathodic reactions [16], while the best performance is achieved between a pH of 8 and 10 [45,46]. Due to the oxygen reduction at the cathode pH can increase to alkaline values in the cathodic chamber [47], thus influencing the microbial community composition. This is consistent with the fact that the most abundant PNS bacteria identified in our SCMFC belonged to the genera *Caenispirillum* and *Roseospira*, whose members are often described as halophilic and/or moderately alkaliphilic [48–50].

The microbial diversity in the cathodic populations was higher within the order *Bacteroidales*, with 79.3% of the sequences belonging to *Porphyromonadaceae* family (59.4% of the sequences belonged to *Paludibacter* genus) (Figure6) and 18.9% to the *Marinilabiaceae*. The high presence of microorganisms belonging to the *Bacteroidales* could be correlated with the biogas digestate used as inoculum. In fact, members of this order were previously proven to be

dominant in a biogas plant, since their abundance increased during the fermentation process [51]. Particularly, the family *Porphyromonadaceae* was used as an indicator for fecal contamination because of its common presence in fecal samples from many host animals [52]. *Porphyromonadaceae* were also described to play an important role in the anodic community of microbial fuel cells [53] but to our knowledge this is the first time that this taxonomic group was described in the cathodic community. Microorganisms belonging to *Paludibacter* genus are fermentative obligate anaerobes [54,55]. Their presence on the cathodic biofilm of an SCMFC with an air cathode could indicate that complex interactions occurred between different populations. Considering also the high abundance of *Nitrincola* it is possible to hypothesize that the microbial community colonized the cathode on the basis of an oxygen gradient, with aerobic microorganisms located close to the external surface and a small number of anaerobic bacteria facing the inner side of the biofilm.

Conclusions

After 41 days of operation, the microbial anodic and cathodic communities in an SCMFC inoculated with biogas digestate were characterized in depth by Illumina sequencing of the V5-V6 hypervariable regions of the 16S rRNA gene. The anodic community was dominated by Fe(III) reducers belonging to *Geovibrio* genus, confirming the results obtained in previous studies [37,38]. The presence of alkaliphilic microorganisms in both the communities suggested that pH had a strong influence in determining the microbial composition, but the large presence of microorganisms belonging to *Nitrincola* genus in the cathodic biofilm could be due to more alkaline conditions near the cathode. The air cathode community was also characterized by both aerobic microorganisms and anaerobic microorganisms, suggesting that

an oxygen gradient influenced the composition of the biofilm. Further studies will help to completely understand the influence of oxygen on the cathodic community and on EET.

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FIGURE CAPTIONS

Figure 1 – Current density profile of the tested SCMFC. The black arrows indicate different additions of acetate (3 g/L)

Figure 2 – Taxonomic classification of the sequences using an RDP Bayesian classifier with 50% of confidence. The classification is at the fourth taxonomic level

Figure 3 – Taxonomic classification of the sequences belonging to *Deferribacterales* order in the anodic community

Figure 4 – Taxonomic classification of the sequences belonging to *Rhodospirillales* order in the anodic comunity.

Figure 5 – Taxonomic classification of the sequences belonging to *Oceanospirillale*s order in the cathodic community

Figure 6 – Taxonomic classification of the sequences belonging to *Bacteroidales* order in the cathodic community.

Figure1

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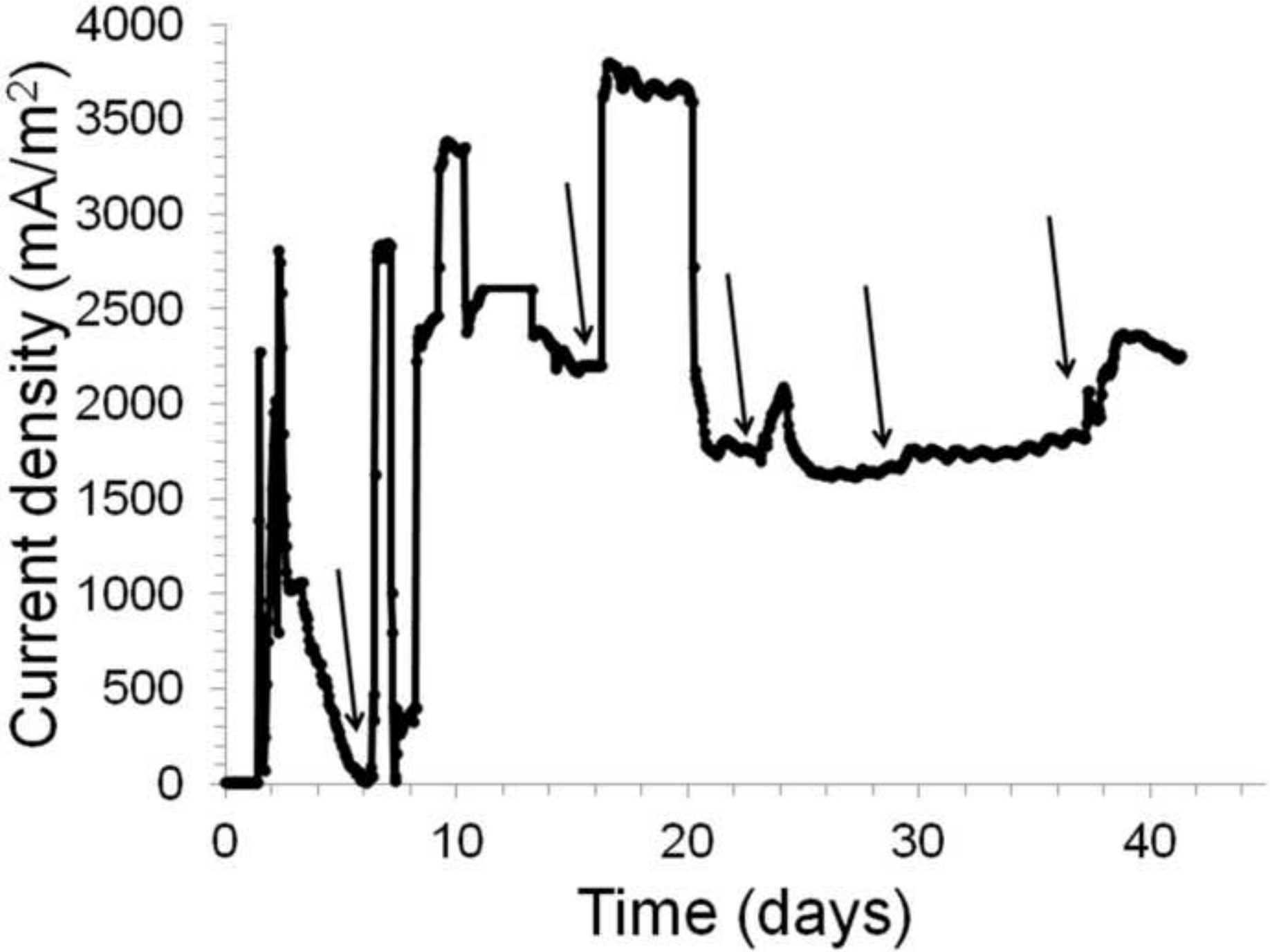

Figure2

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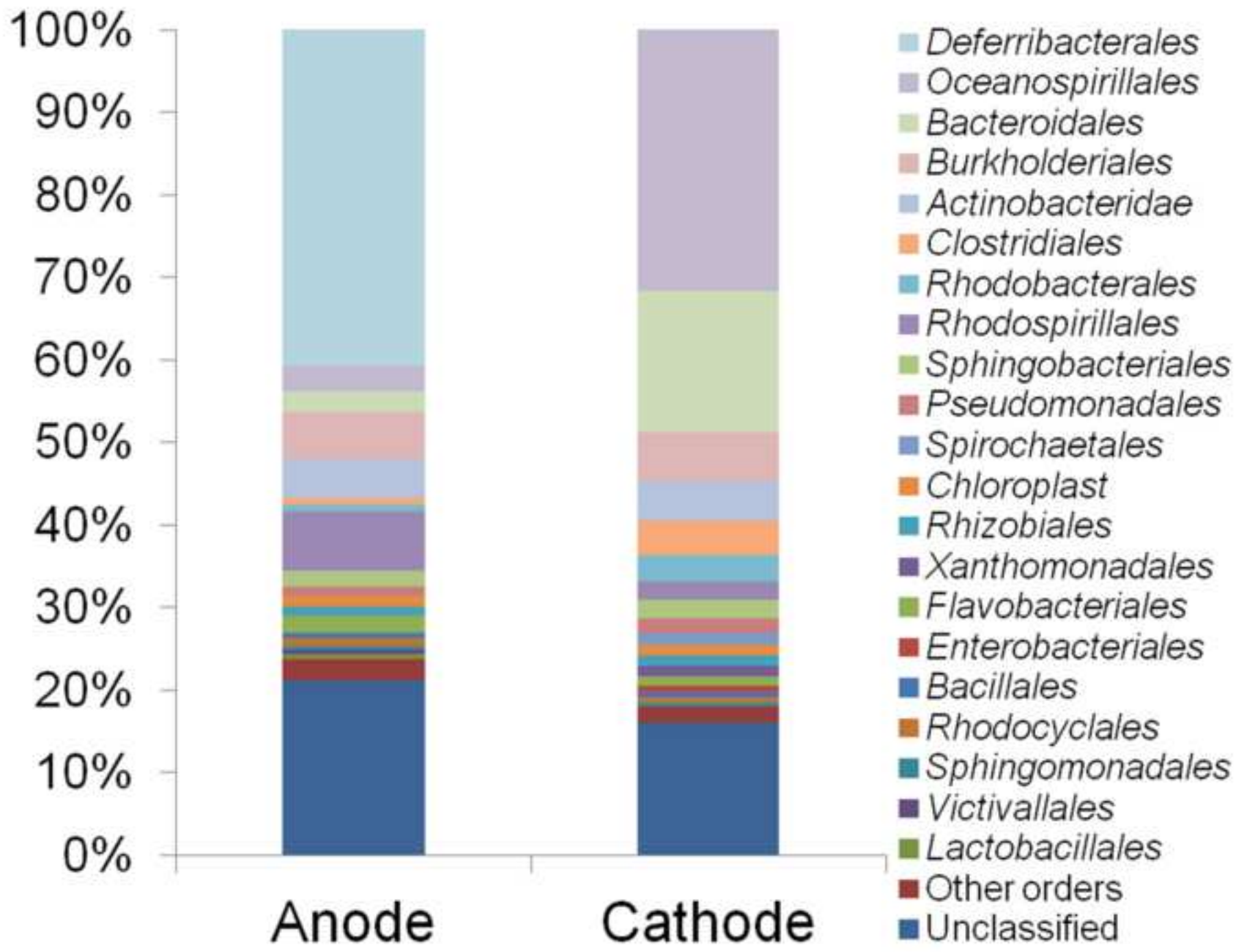

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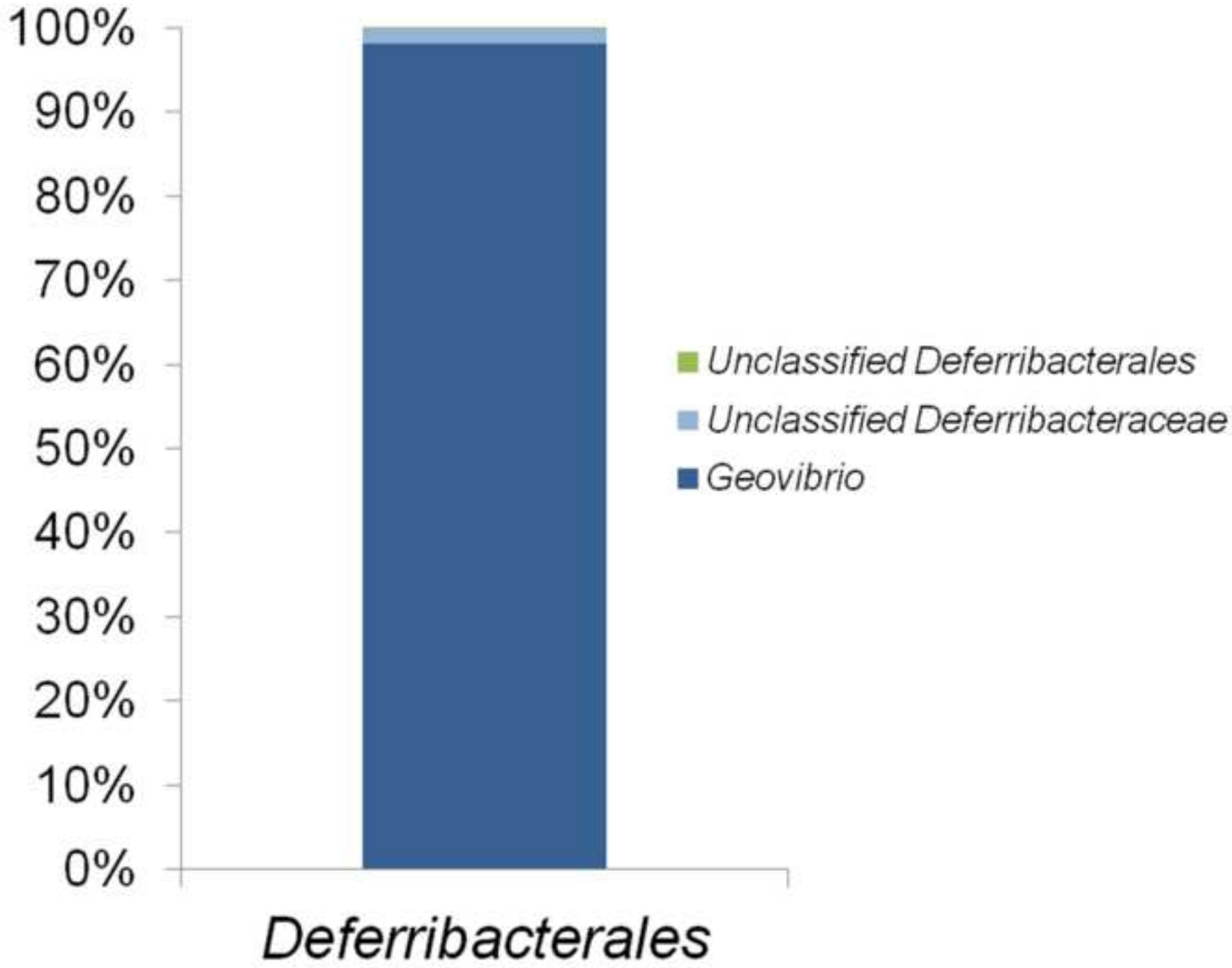

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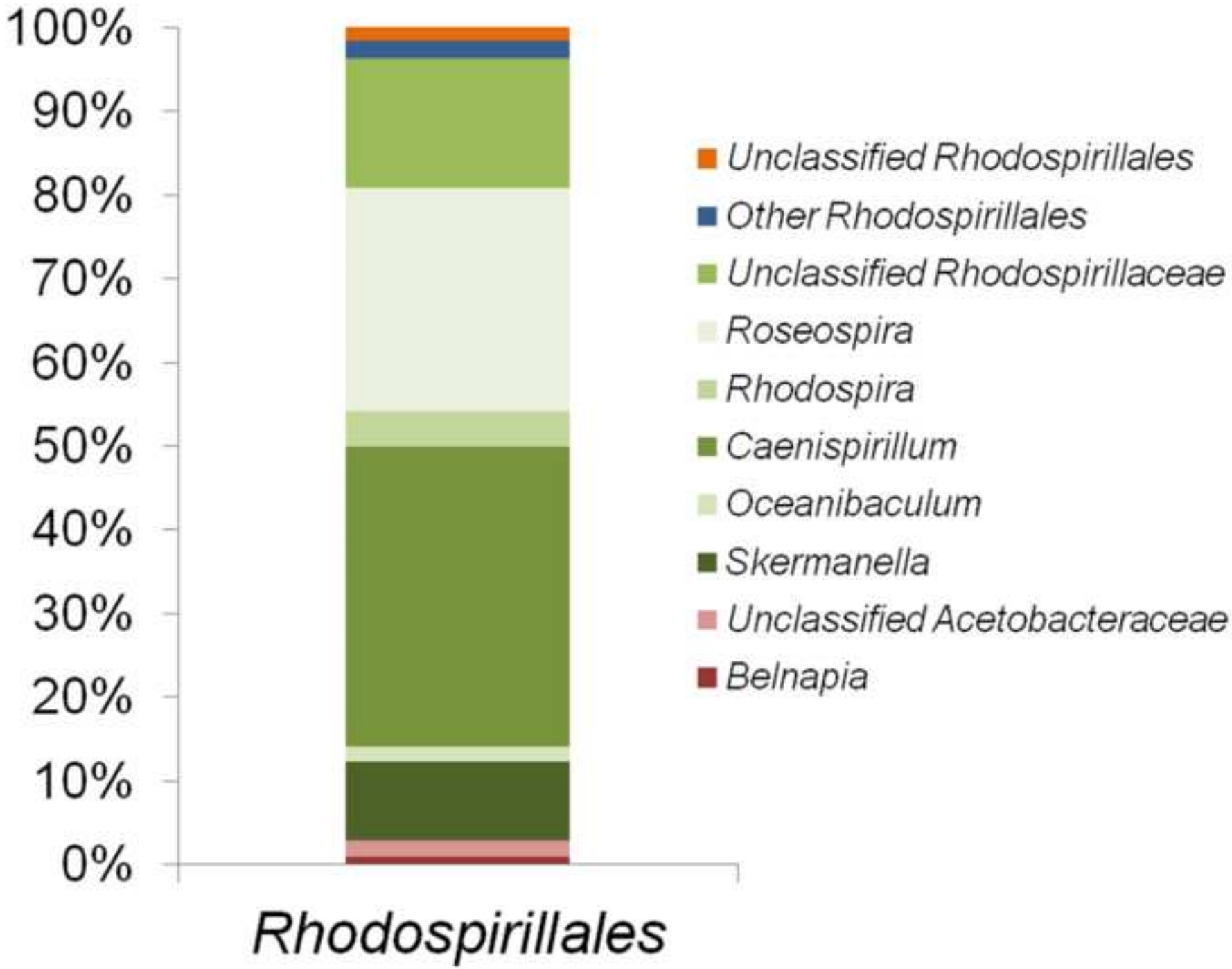

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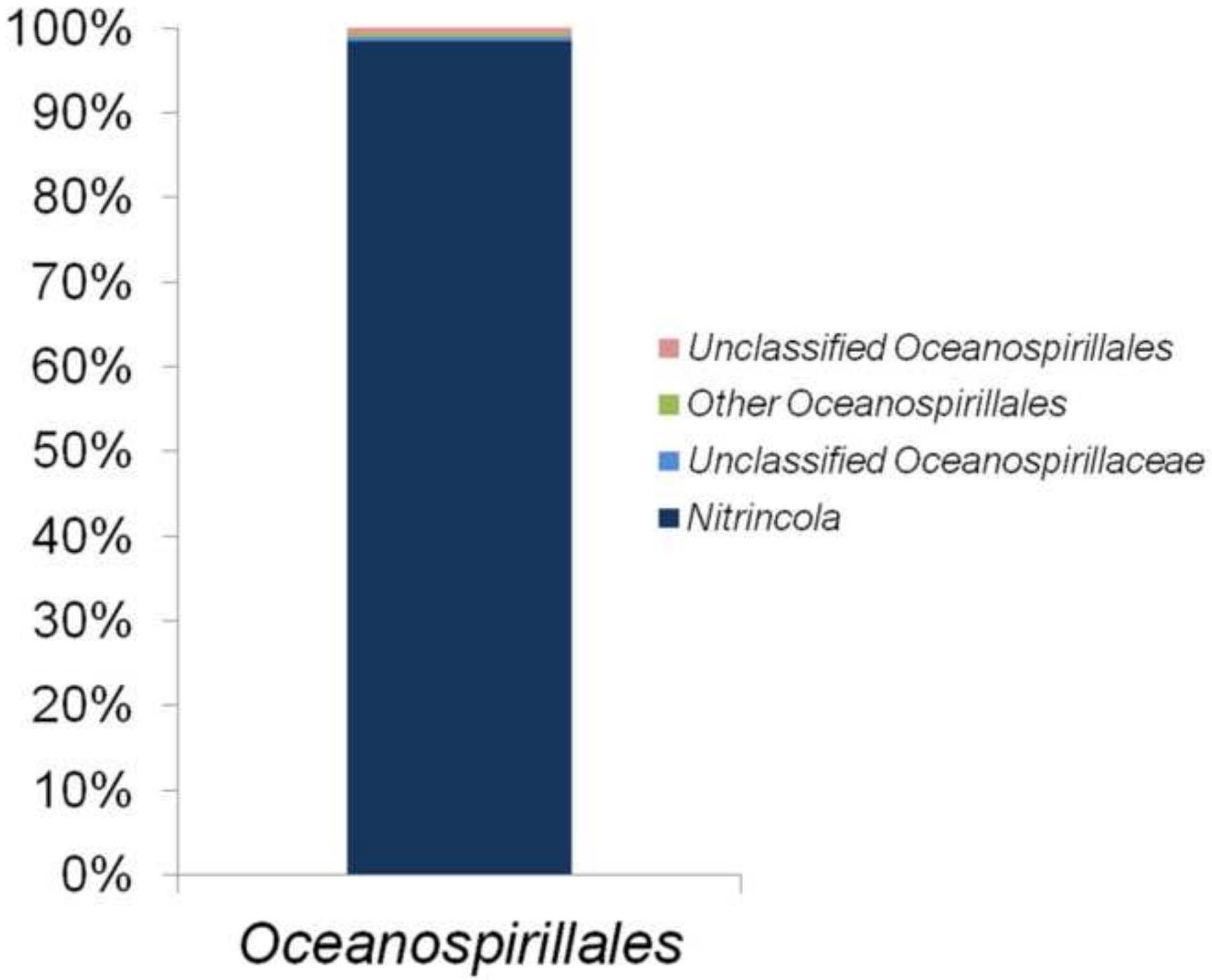

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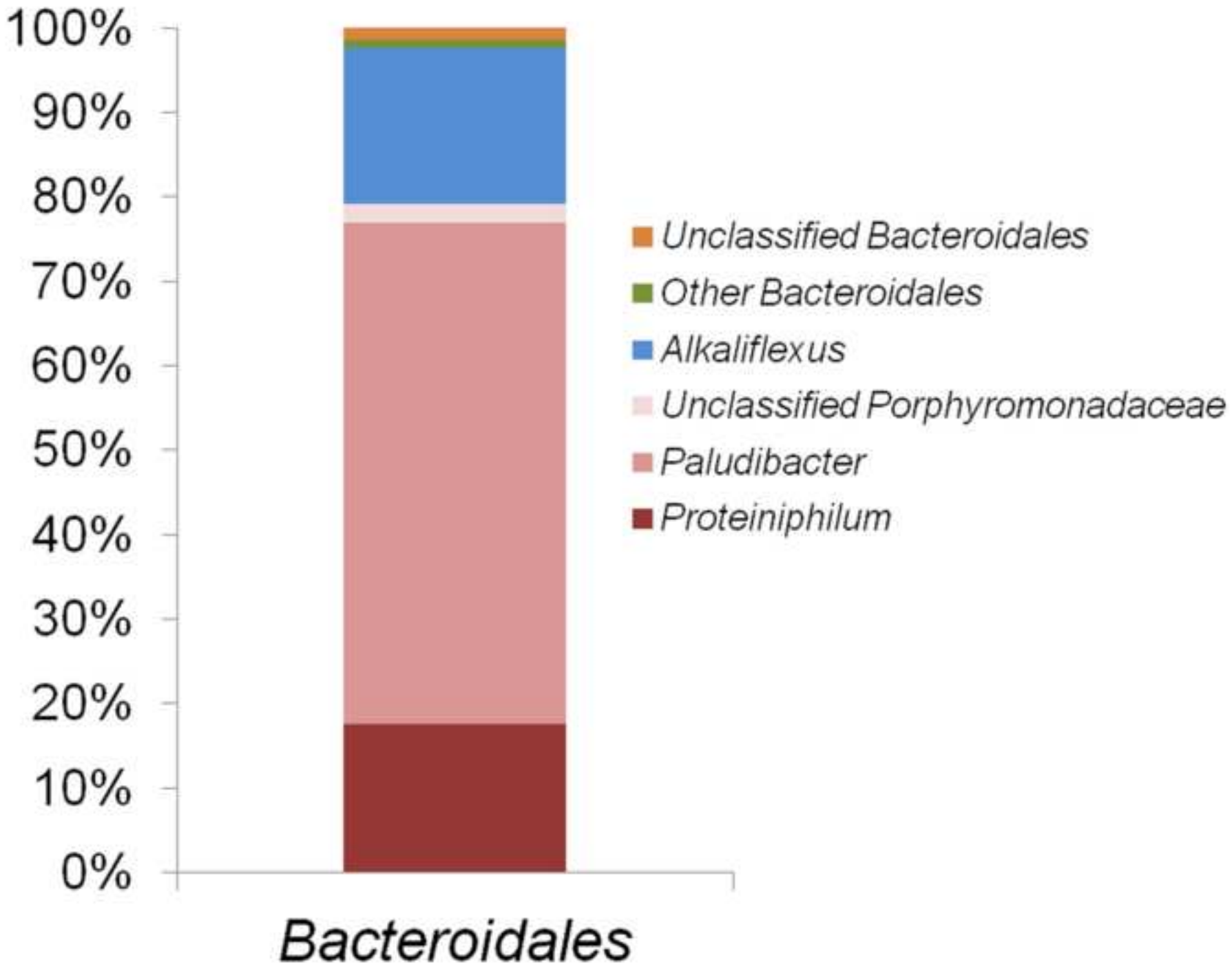

Graphical Abstract