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Microorganisms playing key roles in bioelectrogenesis

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DEDICATORIA

A mi familia y a esos seres queridos que ahora forman parte de otro universo.

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RESUMEN

Hoy en día la búsqueda de microorganismos productores de electricidad o exoelectrogénicos se ha intensificado debido al gran potencial que poseen para el desarrollo de dispositivos conocidos como Celdas de combustible Microbianas (MFC por sus siglas en inglés). Estos microorganismos exoelectrogénicos se pueden encontrar en nichos que van desde el fondo marino hasta cráteres de volcanes activos. El análisis metagenómico de las comunidades microbianas se ha convertido en una de las herramientas más poderosas y útiles para identificar consorcios microbianos productores de electricidad. En el presente estudio se caracterizó las comunidades microbianas de MFCs ensambladas con sedimentos de lagunas salinas de la isla de San Cristóbal y otras con consorcios microbianos cultivados en medios mínimos, mediante el análisis bioinformático de la región V4-V5 del gen ARNr 16S, en los *software QIIME2* y *Phyloseq*. Los resultados denotan la presencia de microbiota halófila, quimiorganótrofa y fotoautótrofa con capacidad exoelectrogénica. Familias bacterianas como Cyanobacteriaceae, Desulfobulbaceae, Desulfobacteraceae, a las cuáles pertenecen los géneros *Halothece, Electrothrix* y *Desulfobacter* respectivamente, han sido previamente descritas en bioceldas altamente electrogénicas, lo cual sugiere que podrían ser candidatos para el desarrollo de plantas de biorremediación autosustentables en el archipiélago de Galápagos.

Palabras clave: exoelectrogénico, extremófilos, análisis metagenómico, comunidades microbianas, bioceldas microbianas, región V4-V5 del gen ARNr 16S.

ABSTRACT

Nowadays, exoelectrogenic microorganisms from diverse habitats have been subjected to extensive research, due to their potential to generate electrical current on devices known as Microbial Fuel Cells (MFCs). These exoelectrogenic microorganisms are found in niches ranging from the ocean floor to the craters of active volcanoes. Metagenomic analyses of microbial communities have become one the most powerful and useful tools in detecting electrogenic populations in a determined niche. Therefore, this study aimed to explore the microbial communities from MFCs assembled with microorganisms from athalassic lagoons of the San Cristóbal Island, Galapagos. The MFCs contained either sediment from the lagoons or a microbial consortium cultivated from the sediments on minimal growth media. We conducted a metagenomic analysis of the region V4-V5 from 16S rRNA from the MFCs' bacterial communities using *QIIME2* and *Phyloseq* software. We found that most microorganism were halophiles with a photoautotrophic and chemoautotrophic metabolism; bacterial families such as Cyanobacteriaceae, Desulfobulbaceae, Desulfobacteraceae, with genera *Halothece, Electrothrix* and *Desulfobacter,* respectively, have been previously described on microbial fuel cells with high energy input, representing excellent candidates for the development of auto sustainable biorremediation plants on the Galapagos archipelago.

Keywords: exoelectrogenic, extremophiles, metagenomic analysis, microbial communities, microbial fuel cells, rRNA 16S V4-V5.

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INTRODUCTION

Microbial fuel cells (MFCs) are devices that can generate electricity based on the metabolism of exoelectrogenic microbes (Santoro et al., 2017). These devices are a promising tool for fighting against climate change, not only for clean energy production but also for CO_2 consumption, wastewater bioremediation and other applications. The increasing interest in MFCs' microbes has raised due to environmental pollution by fossil fuel consumption (Azevedo-Santos et al., 2016).

The consequences of climate change such as food insecurity, extreme weather events and sea level rising (Hoegh-Guldberg *et al.,* 2018) could also harm the development and stability of entire ecosystems, such as the Galápagos Archipelago. Since the current rate of greenhouse emissions, mainly caused by fossil fuels and other human activities, is expected to increase the average temperature by 0.2°C per decade (IPCC, 2018), it is imperative to research more about improving new sources of sustainable energy, such as bio-electrochemical energy in the case of MFCs.

Nowadays, the seeking for clean energy has encouraged scientists to look down intosoils of extreme environments for microorganisms, who can supply electrical current feeding on organic substrates (Logan et al., 2019). Some studies have found exoelectrogenic microbes in niches ranging from volcanic lagoons to anaerobic sludge (Saratale et al., 2017).

The Galapagos Archipelago is of great interest because it has been considered a paradise to study the evolution of species since the Darwin era. On these days, this paradise is seriously harmed by human activity, mainly the pollution caused by oil spillovers into deep

waters (Alava, Palomera, Bendell & Rose, 2014). These oil leaking could be prevented if the Islands' population would rely its energy supply on sustainable sources like bio-electrochemical systems.

The MFCs implementation at large scale is still a challenge, basically for the weak MFCs' current production compared to electro-chemical systems, and lack of knowledge of the interactions between exo-electrogenic microbes. Most studies are based on the culture of electroactive microbes such as *Geobacter sulfurreducens* (Wei, Liang, Cao, & Huang, 2010), and *Shewanella oneidensis* (Li et al., 2017) into the anode of MFCs. Few or almost none researches have focused on the microbial interactions as a whole ecosystem, so this could be the bottleneck of MFCs' development. Besides, most of the MFCs researches followed-up the development of MFCs in a short scale of time, meanwhile this research tries to study the microbial communities playing key roles after a year of cultivation of sediments from athalassic lagoons into Microbial Fuel Cells (MFCs).

The main purpose of this study is to understand the biological system carrying out biochemical reactions that generate electrical current into Microbial Fuel Cells assembled with sediments from athalassic lagoons from San Cristobal Island,Galápagos Archipelago.

LITERATURE REVIEW

Microbial fuel cells (MFCs)

Microbial fuel cells (MFCs) are defined as systems that can transform chemical energy into electricity by microbial metabolism (Santoro *et al.,* 2017). The microbial communities of MFCs may be forming biofilms to retain or expel electrons from the soil populations (Saratale *et al.,* 2017). Extracellular Electron Transfer (EET) biofilms have been studied by some authors (Gimkiewicz & Harnisch, 2013; Jain *et al.,* 2011; Khan *et al.,* 2013). Among MFCs biofilm populations, some EET microbes have been identified and isolated, such as *Aeromonas hydrophila; Citrobacter* sp., *Clostridium butyricum; Enterococcus gallinarum; Geobacter* spp., *Pseudomonas aeruginosa; Rhodobacter sphaeroides; Rhodoferax ferrireducens; Shewanella* spp. Some studies have found that populations with deletion in biofilm promoting genes, such as *PilA*, had decreased energy production inside the MFC's (Richter *et al.,* 2009). Hence the importance of the EET biofilm formation in the functioning of microbial fuel cells. On the other hand, there are many types of microbial fuel cells, according to their design and assembly, on this research we assembled two types of MFC, single-chamber (scMFCs) and double-chamber (dcMFCs) (Javed *et al*., 2018).

Single chambered MFCs

The single-chamber MFC was designed to have an aerobic cathode (carbon cloth) in the upper place of the MFC compartment. Down the cathode, a layer of sediment is placed, then the anode (carbon cloth) and another layer of sediment -are put at the bottom of the

MFC. This design has the purpose of decrease diffusion of oxygen to the anode (anaerobic) and has a low cost and high energy input compared to other designs (Javed et al., 2018).

Double chambered MFCs

These MFCs are made of two chambers linked to each other by a tube containing an ion exchange membrane made of nafion. Glass bottles were designed to have a tube on one of their sides, so linking to each other could be easily made with the help of a clip or harness. One chamber contains the anolyte (carbon cloth) with inoculated sediment and its growth media, and the other chamber contains the catholyte (carbon cloth) with saturated salt solution (Javed *et al*., 2018).

Parameters affecting electricity production on MFCs

The complex system that is conferring electrogenic abilities to the MFCs' microorganisms is under constant research. Thanks to these studies, parameters affecting electricity production into the MFCs have been documented (Saratale *et al.,* 2017). Among these parameters are included: Electrogenic communities' diversity, biofilm production on anode, system design, operating and environmental conditions. Since we are focus on the biological system of the MFCs, we are going to briefly describe some of the parameters that affect the microbiological development into the MFC chambers the most.

Electroactive biofilms

One of the main parameters affecting MFCs' energy input is biofilm formation and availability, diversity and abundance of electroactive populations (Logan *et al.,* 2019). The capacity to form biofilms is given by the presence of certain genes, such as those that codify *Quorum sensing* signals, for example, *LasI, RhlI* genes and *PqsABCDH* operon from

*Pseudomonas aeruginos*a (Wolska *et al.,* 2016); another important parameter is the microbial ability to synthesize redox mediators or the now-known as nanowires or electron shuttles (figure 1.a). Those mediators might be acting as electron bridges or wires to transfer electrons from the intracellular to the extracellular matrix (Liu *et al*., 2018).

EET mechanisms

Inside the MFCs' biofilms, it is known that extracellular electron transfer (EET) is one the main process performed by electrogenic microbes (Reguera, 2018). EET mechanisms (Figure 1) have been mainly described from bacteria of the genus *Geobacter* (Poddar & Khurana, 2011), although in recent years other microorganisms have been studied because of their potential role in electroactive systems, such as *Desulfovibrio desulfuricans* (Kang *et al*., 2014), *Desulfovibrio alaskensis* (Keller *et al.,* 2014), *Thermincola ferriacetica* (Parameswaran *et al.,* 2013).

One mechanism is directly mediated by membrane-bounded cytochromes, also known as short-range electron transfer (Kumar *et al.,* 2015*)*. This mechanism helps microorganisms to perform Extracellular Electron Transfer (EET) using redox-active proteins like *Geobacter*' c-type cytochromes containing heme groups in their motifs (Logan *et al*. 2019); another direct mechanism involves the presence of conductive pili, known as long-range electron transfer, although these conductive pili have been only found on *Geobacteraceae* and *Shewanellaceae* bacterial families (Kumar *et al.,* 2015). Finally, the other is an indirect mechanism or mediated by secondary metabolites, such as magnetite from Fe (III) reduction carried out by *Geobacter metallireducens* (Reguera, 2018), pyocianin from *Pseudomonas aeruginosa* (Sheng *et al.,*

2014), riboflavin secreted by *Geothrix fermentans* which promotes the reduction of Fe (III) oxides (Mehta-Kolte & Bond, 2012), among others.

Short Range Electron Transfer

The direct electron transfer via cytochromes has been profoundly studied in *Geobacter sulfurreducens* (Liu *et al.,* 2015). The cytochromes act in an orchestrated way with other proteins of the electron transport chain (ETC) like b-type cytochromes, quinones and iron-sulfur proteins (Aklujkar *et al.,* 2013). Among the major proteins involved in EET are the Outer Membrane c-type Cytrocromes (Omc), especially OmcZ, OmcB and OmcE, which were found to be the most abundant in current harvesting cells (Inoue *et al*., 2011). The importance of OmcZ inside the MFCs could be due to its intervention on the electron transfer through the biofilm, meanwhile, OmcB helps transfer electrons across the biofilm to the electrode interface or resistance (Richter et al., 2009). Little is known about the function of OmcE but it is suggested to play a secondary role in the electron transfer through the biofilm (Voordeckers *et al.,* 2010).

Long Range Electron Transfer

Long range electron transfer is one of the main topics of interest inside the EET mechanism researches. This fact could be explained because of the high current production associated with this EET mechanism (Liu & Bond, 2012). This form of EET is mediated by a vast network of conductive pili, produced mainly by bacterial families *Geobacteraceae* and *Shewanellaceae* (Kumar *et al.,* 2015). Nowadays, it is known that conductive pili, especially *G. sulfurreducens'* pili, are Type IV pili composed by monomers of PilA proteins (Richter *et al.,* 2009). The conductive features of the pili are given by a conserved sequence of aromatic amino acids (Trp, Phe, Tyr, His and Met) located at the C terminus region of PilA proteins

(Vargas *et al.,* 2013). Although long range electron transfer is associated to conductive pili, another mechanism has been shown, the pilus-associated c-type cytochrome OmcS seemed to work together with pili to transfer electrons in an interspecies manner, thus it is called DIET (Direct Interspecies Electron Transfer) (Shrestha *et al.,* 2013).

The so-called electron shuttles could facilitate the electron transfer independently from pili or outer membrane cytochromes. These soluble electron shuttles are released by some microorganisms, they help by promoting redox reactions and electron transfer to the anode (Brutinel & Gralnick, 2012). Some examples of these shuttles are pyocianin and phenazine-l-carboxamide produced by *Pseudomonas aeruginosa* (Wang, Kern & Newman, 2010), Riboflavin produced by *Geothrix fermentans* (Mehta-Kolte & Bond, 2012), 2-amino-3-dicarboxy-1,4-naphthoquinone from *Lactococcus lactis* (Freguia, Masuda, Tsujimura & Kano, 2019), and 2,6-di-tert-butyl-p-benzoquinon from *Klebsiella pneumonia* L17 (Deng *et al.,* 2010).

Thanks to these mechanisms, microbes are capable of transferring electrons to the extracellular matrix, forming some kind of network between other microorganisms, resulting in “electrogenic symbiotic relationships” among communities in a determined habitat, such as soil (Wolińska *et al*., 2014).

Electroactive Gram positive-like microorganisms

Even though EET mechanisms have been well documented, most of those studies have been carried out on Gram negative mesophilic bacteria, such as *Geobacter* and *Shewanella* spp. Recent studies have tried to explore electroactive features of thermophilic microorganisms with Gram positive-like cell wall because of their potential use into MFCs coupled with bioremediation systems (Lusk, 2019). Due to the intrinsic characteristics of the

cell wall, it is expected that EET mechanisms vary between Gram positive and Gram negative microorganisms.

In this scenario, we placed some of the features that made Gram positive-like thermophiles EET mechanisms differ from those of the Gram negative mesophilic bacteria. Thermophiles have smaller genomes than the organisms that thrive below 45°C; hence Gram positive thermophiles have reduced protein length and family size, a lower rate of non-synonymous substitutions in protein-coding regions (Wang, Cen & Zhao, 2015) thus increasing stabilizing selection, which implies that thermophilic protein structure and function are under a strong selective pressure (Berezovsky & Shakhnovich, 2005). This low non-synonymous mutation rates result in a functional stability better than mesophilic Gram negative bacteria, so thermophiles are the best candidates to build bioreactors for long-term bioremediation with high reproducibility (Lusk, 2019).

Advantages of MFCs' usage

Electric current production is just one of the main benefits of using Microbial fuel cells as an alternative fuel to fight against the use of fossil fuels and greenhouse gases pollution. One of the most interesting applications of MFCs is their potential in the bioremediation of wastewaters (Li, Yu, & He, 2014), application as a biosensor (Yang *et al.,* 2015), plant-based power generation (Deng, Chen & Zhao, 2012), water desalination plants (Luo *et al.,* 2012), electrolysis for H_2 recovery (Wang *et al.,* 2011), among the major ones.

Thanks to these noticeable features, MFCs have been drawing attention from scientific community in the past few years. The race for clean energy development to fight climate change has speeded up exponentially. In this scenario, scientist have tried to find improved

electricity production of bioelectrochemical systems but approaches to biological systems involved in electricity production are less known (Javed *et al.,* 2018).

MATERIAL AND METHODS

Sample collection

The samples used for this study were collected from two athalassic lagoons at San Cristobal Island in the Galapagos archipelago; one of brackish features, Cerro Brujo, and a man-made hypersaline pond, Punta Pitt (Figure 2). Approximately 10 pounds of each sediment were collected using sterile instruments. Two sites from each lagoon were sampled, hence we call the samples from Cerro Brujo: CB 1 and CB 2; and the samples from Punta Pitt: PP 1 and PP 2. Once the samples were taken, they were kept at 4°C until they arrived at the laboratory. *In situ* parameters (GPS coordinates, salinity, pH and temperature) were taken using a portable YSY model water quality sonde. Furthermore, conductivity, pH and Dissolved Oxygen (DO) were measured *in vitro* using the Thermo Scientific™ Orion™ Versa Star Pro™ pH/ Conductivity/Dissolved Oxygen Multiparameter Benchtop Meter (Table 1).

Microbial fuel cells (MFC) assembly

Approximately 40 grams from each sediment were placed into the single chamber MFC (scMFC), 20 grams under the anode and 20 grams below the cathode (Figure 3); from each sediment, two MFCs were assembled, and thus we called the MFCs: CB 1.1, CB 1.2, CB 2.1, CB 2.2, PP 1.1, PP 1.2, PP 2.1 and PP 2.2. Energy production was measured once a week using a standard voltmeter. Sterile water was added to the MFC at least once per month or when low energy production and dry appearance of the sediment were detected.

Furthermore, based on the previous results of the microbial diversity from the sediments (data not shown) we tried to isolate certain microbial communities because of their relative abundance into the sediments. For this purpose, we inoculate approximately 10

grams of each sediment into 90 ml of the following growth media: m9 (minimal), BG11 (cyanobacteria), Chlorobium (Green sulfur bacteria), and Chromatium (Purple sulfur bacteria). After one month of culture, we proceed to place 10 ml of the primary culture into fresh growth media to renew the cultures and propagate them. After 6 months from the first isolation, we selected 8 samples for dual-chamber MFC (dcMFC) assembling (Figure 4); those samples and their growth medium were added into the anolyte or anode as follow: PP 1 - Chlorobium, PP 2 – Chlorobium, PP 2 – BG11, PP 2 – Chromatium, PP 2 – M9, CB 1 – M9, CB 1 – Chromatium, and CB 1 – Chlorobium; on the other chamber or catholyte, we poured 200 ml of a saturated salts solution (M9 salts 5x). Once the MFCs were assembled, we followed-up the current production during the first month, later we measured energy production once per week and then once per two weeks until six months were completed.

DNA extraction

One year after single-chamber MFC assembly, we selected the MFCs that produced the highest and constant amount of energy, which was CB1.1 and PP2.2 (Figure 5). Four samples of the cathode were collected and mixed into a pool. DNA extraction was carried out using the QIAGEN™ DNeasy PowerSoil kit® following manufacturer's instructions. Once the DNA was extracted, quality parameters were measured using fluorometry. DNA was stored at -20°C for later use. On the other hand, six months after dual-chamber MFC assembly, we chose PP2.BG11 dcMFC for DNA extraction due to its higher input compared to the other dcMFCs (Figure 6)

Sequencing data processing

Library preparation and sequencing runs were performed at Macrogen in South Korea. Paired-end runs were sequenced by Illumina technology.

Sequencing data analyses

Sequencing results came as fasta.gz files, one per forward and reverse read of each sample. Raw data was imported to QIIME2 (Quantitative Insights into Microbial Ecology) (Bolyen *et al.,* 2018) program using the function qiime tools import. The sequenced raw data contained quality information that could be depicted by the function qiime demux summarize, figures 8 and 9 shows forward and reverse reads quality information at base 240, just before quality score started to fall. Primers were removed with qiime cutadapt trim-paired, to prevent the formation of chimeras.

Quality control and denoising, which are removing sequences with low quality, chimeras, and redundant, were performed using the dada2 pipeline. Function qiime dada2 denoise-paired was set to admit 2 errors from each read and truncate the sequence at base 240. Three archives were obtained from this analysis, a feature table with the number of reads from each sample, the representative sequences and the denoising stats, all these data can be found on supplemental information. Representative sequences were aligned using MAFFT (Katoh & Standley, 2013), with the following command line: qiime alignment mafft.

Once the sequences were aligned, we proceed to assign taxonomy to each sequence. rRNA 16S Green Genes database (Balvočiūtė & Hudson*.,* 2017) was used as the reference database. The sequence classifier was generated using the Naive-Bayes method; function qiime feature-classifier fit-classifier-naive-bayes was set to train the classifier on the V4-V5 region of the rRNA 16S gene, which was the targeted gene region of the primers we used on this research (Hughert *et al.,* 2015). Representative sequences classification was done thanks to the program Scikit-learn (Pedregosa et al., 2011), this was called by the function qiime feature-classifier classify-sklearn.

Microbiome Diversity Analysis

The classified sequences and feature table were used to generate a relative abundance taxa barplot. Figure 7 depicts the relative abundance of taxa in each sample. QIIME2 command line for bar plotting was qiime taxa barplot.

Alpha diversity indexes were calculated to see the species richness and evenness inside of each sample. Shannon index was calculated using qiime diversity alpha-rarefaction.

Beta diversity was calculated as *weighted UniFrac* index, line command: qiime diversity beta-rarefaction. *Weighted UniFrac* includes phylogenic relationships as a parameter, so we created a phylogenetic tree using the program MAFFT-FAST TREE; line command: qiime phylogeny align-to-tree-mafft-fasttree.

Moreover, to obtain high quality plots we exported QIIME2 files to R, to analyze the data in Phyloseq pipeline (McMurdie & Holmes, 2013). In Phyloseq, we created a heatmap of the 20th most abundant families and re-run the diversity index.

DATA ANALYSIS

Sample parameters

Physic-chemical parameters were measured at the time of sampling (*In situ*) and after one month of arriving at the lab (*In vitro*) (Table 1). The main differences between *In situ* and *In vitro* measures could be due to several factors, such as the variation of the measuring instruments because *in situ* measures were taken by a portable sonde meanwhile *In vitro* measures were taken by a specific benchtop. Changes in temperature and altitude also could be explained by the fact that MFCs were assembled in Quito, which is 2890 meters above sea level (m.a.s.l.) on the highlands of Ecuador, while the samples of sediment were taken on Galapagos islands whose altitude is around 350 m.a.s.l. Finally, reduced dissolved oxygen (DO) availability on *In vitro* measures might be due to anoxic conditions of the MFCs (Saratale *et al.,* 2017) as a consequence of microbial metabolism**.**

MFC's energy production

Bioelectrochemical activity of the MFCs was measured as voltage generation; measures were taken using a standard voltmeter. After one year of assembly, we chose the scMFC with the higher and constant energy input for DNA sequencing, these MFCs were CB1.1and PP2.2, whose average input was 441 and 314 mV, respectively (Figure 5). Same as single-chamber MFCs, we chose the dual-chamber PP2.BG11 that produces the highest and most constant energy input, (Figure 6). One interesting fact is that the energy production of the MFCs was reestablished after the addition of sterile water (Supplement info) or when the MFCs were opened for sampling purposes; this could indicate oxygenic metabolism and a burst of Extracellular Electron Transfer (EET).

Microbiome Diversity Analysis

Relative frequency or abundance of taxa showed us the microbial composition of each microbial fuel cell (Figure 7). The single-chamber MFCs are more diverse (CB1.1, PP2.2) than dual-chamber MFC (PP2.BG11). This was corroborated by the number of sequences reads on each sample, being CB1.1 the sample with the higher count of reads: 88k, followed by PP2.2: 85k reads and PP2.BG11; 76k reads. Besides, these data were confirmed by alpha and beta diversity analysis. Alpha diversity is a measure of diversity from each sample. Figure 8. A depicts alpha diversity index, Shannon index calculated the distribution of microbial communities into each sample (Kim *et al.,* 2017). Single chamber MFCs had higher Shannon index as sequencing depth was increasing, CB1.1 had the highest Shannon score: 6, while PP2.2 and PP2.BG11 had Shannon scores of 5.5 and 3.5, respectively, meaning that CB1.1was the most diverse of all MFCs and also had more evenly distributed communities.

Beta diversity index displayed dissimilarity between samples, which meant that samples did not share representative amounts of microbial abundance or phylogenetic relationships between them (Figure 8.B). Weighted UniFrac based its index on phylogenetic distances and relative abundance (Schroeder & Jenkins, 2018). The differences between samples were mainly explained by the type of MFC (60.9%) because of PP2.BG11 dcMFC was assembled with enriched sediment and scMFCs (CB1.1 and PP2.2) were assembled with raw sediment; the other 39.1% of dissimilarity between samples was explained by the origin of the sediments, Cerro Brujo for CB and Punta Pitt for PP.

Microbial communities evolving into the MFCs

Since we had the information of microbial communities abundance and taxonomy assignment from the sediments or starting point (data not shown), we proceeded to compare the communities that were at the starting point and a year after they were inoculated into the MFC's. These results suggest the microbial consortia could be playing a major role in this bioelectrochemical system. The relative abundance of the communities that were found at the two points of analysis can be seen on supplemental info.

Differences in the relative abundance at different points of analysis might help us to infer microbial activities happening inside the MFCs. Some microbial families had decreased in relative abundance after one year of scMFCs culturing, such as the following ones: Chromatiaceae, Flavobacteriaceae, Geobacteraceae and Rhodospirillaceae in both scMFCs, CB1.1 and PP2.2; while almost the rest of the families had increased their abundance over the year (Supplemental info), especially the bacterial families Anaerolineaceae, Caldilineaceae, Cyanobacteriaceae, Chlorobiaceae, Planctomycetae, Spirochaetaceae and arqueal Halobacteriaceae on CB1.1 scMFC and bacterial families Cyanobacteriaceae, Chlorobiaceae, Desulfobacteraceae, Desulfohalobiaceae, Ectothiorhodospiraceae and arqueal Halobacteriaceae on PP2.2 scMFC.

The most abundant microbial families on the MFCs were Alteromonadaceae, Balneollaceae, Cyanobacteriaceae, Hyphomicrobiaceae, Marinicellaceae, Phycisphaeraceae, Pirellulaceae, Pseudanabaenaceae, Rhodobacteraceae and arqueal Halobacteriaceae that were present in all MFCs (CB1.1, PP2.2, and PP2.BG11), bacterial families Flammeovirgaceae and Xanthomonadaceae were only present in Punta Pitt MFCs (PP2.2 and PP2.BG11), Coriobacteriaceae, Erysipelothricaceae, Lachnospiraceae were only found on PP2.2; on the

other hand, Spirochaetaceae and Priscirickettsiaceae were shared by PP2.2 and CB1.1. Finally, Chromatiaceae, Desulfobulbaceae and Nitrospiraceae were only present in Cerro Brujo MFC (CB1.1). (Figure 9).

An interesting finding was the presence of Desulfobulbaceae, and Nitrospiraceae only in CB1.1, which was the most electrogenic MFC (441mV). Delsufobulbaceae members such as Candidatus *Electrothrix* and *Electronema* had been proposed as new genera of EET bacteria (Trojan *et al.,* 2016). On the other hand, members of Nitrospiraceae family, like genera *Leptospirilum* and *Thermodesulfovibrio*, are known to be potentially useful for wastewater treatment, acid mine drainage and extracellular polymeric substance production (Daims, 2014) this could lead us to test MFC as a sustainable way of bioremediation, biomass and energy production.

In contrast, family Lachnospiraceae was found only in PP2.2, this family had been characterized in soils with high energy production on MFCs (Jiang, Zhong, Han & Deng, 2016). Cultivation on BG11 medium might inhibit the growth of this and other families, resulting in the decreased energy input of PP2.BG11 MFC.

Also, we notice that some microbial families were found most abundantly on PP2.BG11 dcMFC (Figure 9), we compared PP2.BG11 relative abundance with PP2.2 because both samples were inoculated with the same sediment. An interesting finding was the increased abundance of the family Alteromonadaceae, which includes the genus Marinobacter hydrocarbonoclasticus, an old extremophile bacteria which can degrade hydrocarbons (Vance et al., 2019).

DISCUSSION

In this study, we were trying to understand the microbial ecosystem inside the new devices called microbial fuel cells (MFC). For this purpose, we compare the most prevalent microbial community's relative abundance from sediments of athalassic lagoons (data not shown) versus the same communities found a year after cultivation on single-chamber MFC and six months on dual-chamber MFC. Besides characterizing those communities, we manually search for the kind of metabolism and carbon source to have an idea of what it's happening inside the MFC's in terms of biochemistry (supplemental info).

Microbial families found in this study might be replicating the natural cycles of chemical elements. In this case, oxygenic photosynthesis carried out by Cyanobacteria such as *Haloteche, Nostoc, Cyanothece* might be participating in the cycling of hydrogen and carbon, this could be exploited by other microorganisms inside the MFC systems, (Pisciotta, Zou & Baskakov, 2010). The sulfur cycle could be carried out by members of the family Desulfuromonadales, they might be reducing elemental sulfur from the sediments to H_2S; Thiotrichaceae family could oxidize H_2S to sulfate (SO_4), Desulfobacteraceae and Desulfobulbaceae, might be reducing sulfate to H_2S (Kuever, 2014), and then, that H_2S molecule is recycled, preventing its lethal action on the MFCs microhabitat. Finally, Nitrogen cycle might be executed in the first place by nitrogen fixers like Clostridiaceae, Cyanobacteriaceae, Rhodospirillaceae, they fix nitrogen from the atmosphere and make it available to Nitrogen reducers such as Pirellulaceae. Nitrogen reducers form NH_3 that might be nitrificated by Chromatiaceae and Nitrospiraceae families. Nitrate formed by nitrogen reducers could be de-nitrificated by Hyphomicrobiaceae and Rhodobacteracea families, preventing NO_3 accumulation and hence eutrophication of the microhabitat.

Besides replicating natural cycles of chemical elements, families that had increased their relative abundance over the time were most likely to be halophiles with photolithoautotropic metabolism, which means they can use light as their main source of energy, carbon dioxide as carbon source and also use an inorganic electron donor (Stambler & Dubinsky, 2007). These types of metabolism are likely to be found on MFCs coupled with bioremediation systems. For instance, family Chlorobiaceae are predominant on benzene and ammonium-contaminated groundwater MFCs (Wei *et al.,* 2015). Likewise, some genus of the family Anaerolineaceae such as *Anaerolinea thermophila* has been found on activated sludge and oil spillover treatment plants (Sekiguchi *et al.,*2003), Another interesting discovery was made on PP2.BG11 dcMFC, a bacterial genus increased its abundance once was cultivated on BG11 medium, *Marinobacter hydrocarbonoclasticus*, an old bacteria that can degrade hydrocarbons (Vance *et al.,* 2019). This could lead us to test BG11 growth medium on bioreactors to propagate *M. hydrocarbonoclasticus* and analyze the bioremediation potential of this bacteria. All these findings can suggest MFCs as a possible source of auto-sustainable bioremediation plants.

The fact that CB1.1 produced higher electrical current input than the other MFCs could not be elucidated with this analysis, but we could have an initial approach looking at the communities that have survived all this time after MFC's culturing. In this scenario, Desulfobulbaceae and Nitrospiraceae might be playing a key role in bioelectrogenesis, this is because they have increased on relative abundance compared to the Starting Point and were only found on CB1.1 (Supplemental info). Moreover, Alpha diversity showed us that single-chamber MFCs are more diverse than dual-chamber MFCS, which could be explained since PP2.BG11 dcMFC was under selective pressure of MFC conditions and the presence of a

specific growth media (BG11), meanwhile, scMFCs allowed the growth of more taxa due to the absence of a specific growth media. On the other side, Beta diversity depicted the difference between all of the MFCs, meaning that every MFC has a low proportion of shared taxa and phylogenetic relationships. A fact that could explain the difference in diversity between sites is that Cerro Brujo is a pristine habitat where humans are not allowed to enter without special permission, and Punta Pitt is an old saltern where humans used to extract salt for consumption.

The change in relative abundance and biochemical characteristics of the most prevalent microbes could give us an idea of the type of reactions that are happening on the MFCs, but we need to seek deeper into this microhabitat to clarify its functioning. Lower abundant taxa that could not be detected in this study might be fine-tuning bioelectrochemical reactions. Transcriptomic and metabolomic tools could show us what reactions are predominating between microorganisms, not only the predominant taxa, leading to discoveries about how to improve MFC electrical current in a short period (Logan *et al.,* 2019).

CONCLUSIONS

In this study, we found that microbial communities might have evolved into the MFCs in a syntrophic way. Most microbial communities play key roles in biogeochemical cycles, hence maintaining the functioning of microbial fuel cells over time. The most prevalent class of metabolism among microbial families are anaerobic photolithotrophy. This type of metabolism allows microbial communities to obtain energy from the sun while using an inorganic electron donor and could be the MFC's main source of energy. Alongside photolithotrophs, heterotrophs might be reusing microbial debris and consuming oxygen from the environment, thus preventing its lethal action over the anaerobic populations; chemolithoautotrophs might be oxidizing and reducing chemical compounds present in the sediments and making them available to the other communities and Cyanobacteria could be providing protons to the habitat while fixing CO_2 from the atmosphere. Also, almost all the families found in the MFCs were halotolerant or halophile, with high bioremediation potential, which is not surprising due to the conditions of the athalassic lagoons we sampled; these findings might drive us to test MFC as a bioremediation process of wastewaters, reducing CO_2 environmental levels, draining acid mines, and production of non-oil derivate polymers. All these phenomena make us think about what it's happening inside the microbial fuel cells, and that not only Geobacteraceae and Shewanellaceae are the rare microorganisms that can produce significant amounts of energy. Although we could only infer these phenomena until we establish a "core microbiome" of the MFCs and their transcriptome and metabolome involved in energy production.

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TABLES AND FIGURES

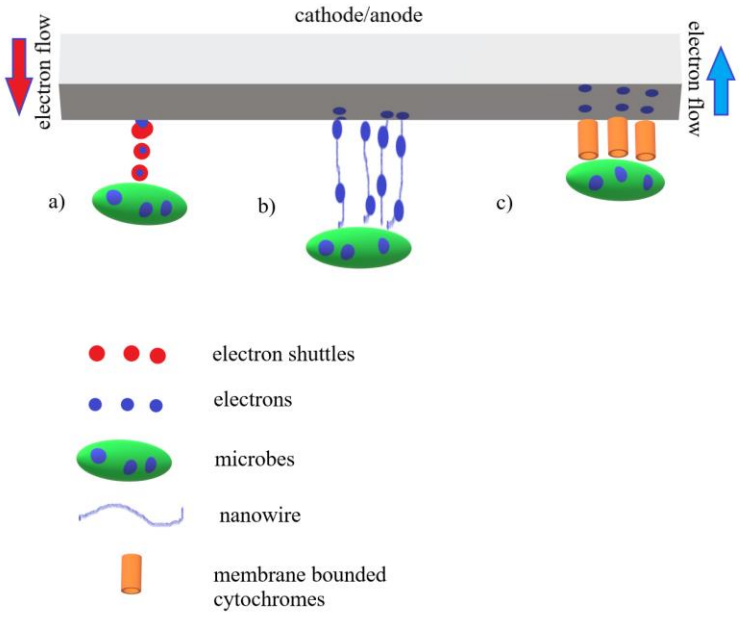

Figure 1. - Different pathways and mediators for EET. a) Electron shuttles; b) pili-like nanowires; c) outer-membrane redox proteins. (Logan et al, 2019)

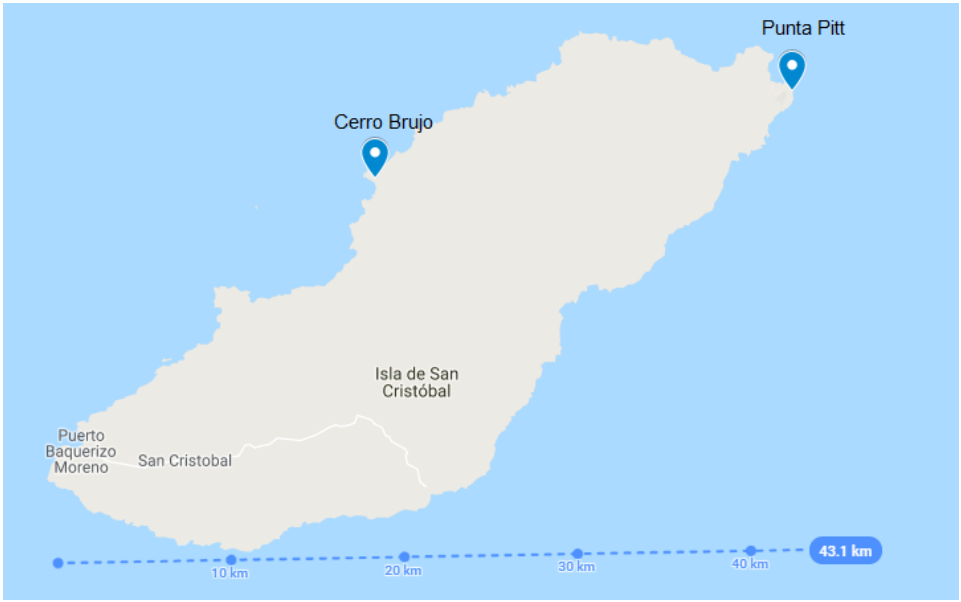

Figure 2. – Sampling sites on San Cristobal Island. Two athalassic lagoons, Cerro Brujo and Punta Pitt (Shown with blue mark).

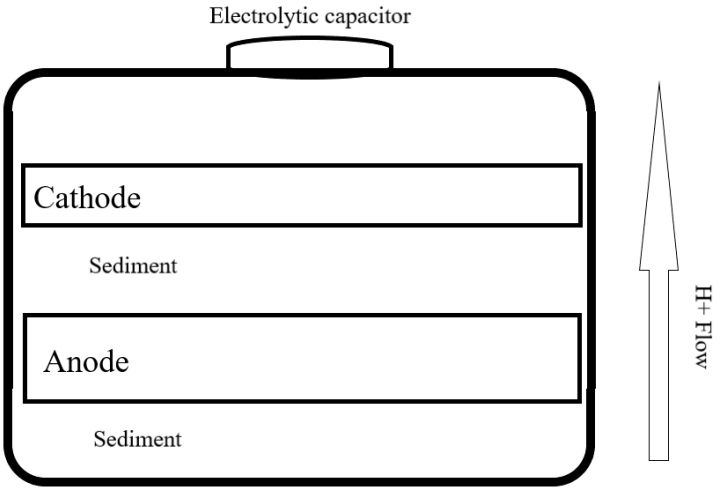

Figure 3. – Single chamber Microbial Fuel Cells working scheme**.**

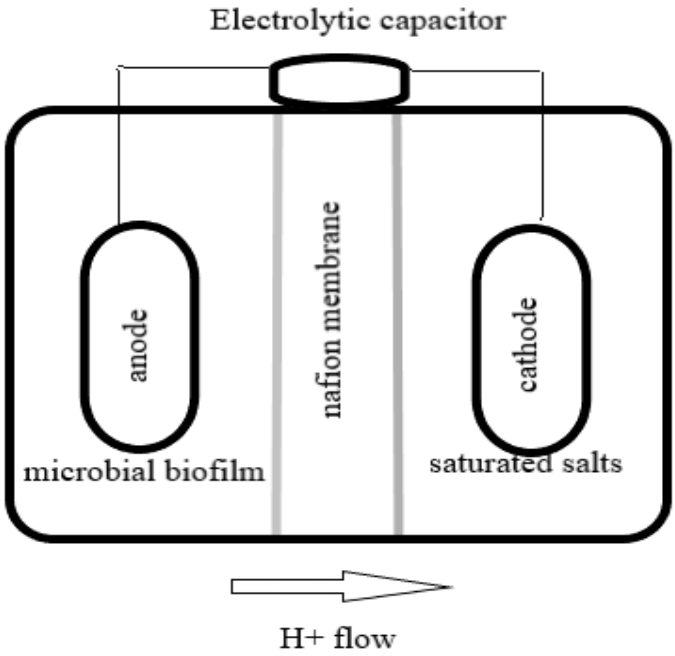

Figure 4. –Dual-chamber MFC's scheme.

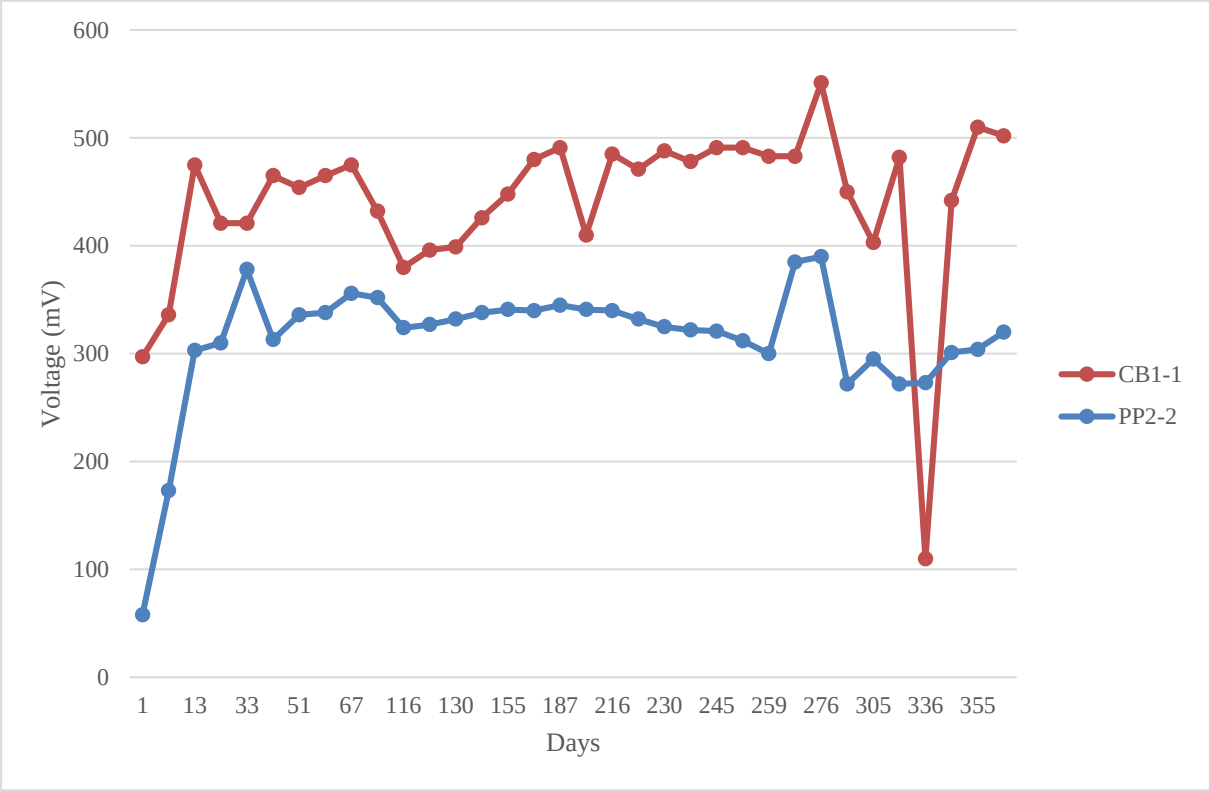

Figure 5. - Voltage data of sequenced Single Chamber MFCs. Data from the period 07/2018 - 07/2019. Data from all scMFCs can be seen on supplemental info**.**

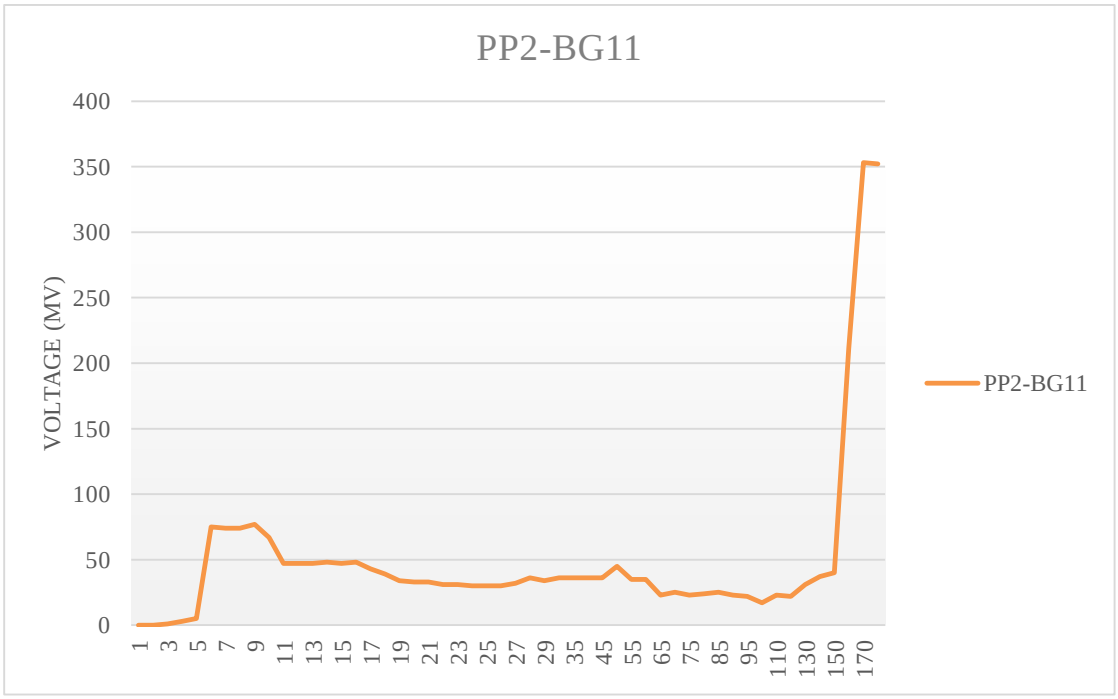

Figure 6. – Voltage from sequenced Dual Chamber MFC. Data of the period 02/2019 - 08/2019. Information from all dcMFCs can be seen on supplemental info.

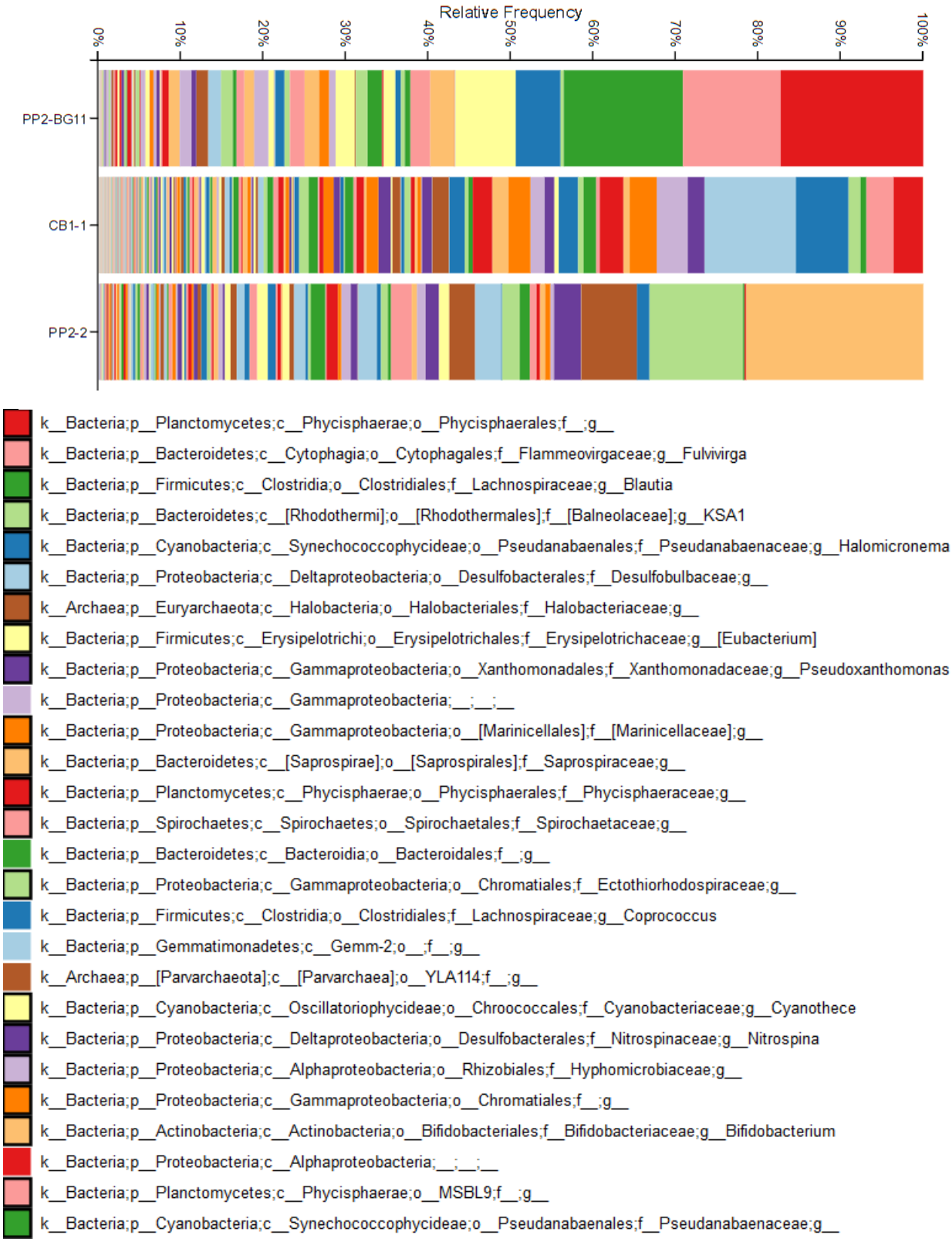

Figure 7. - Relative frequency or abundance of taxa on each MFC sample. Not all taxa are shown.

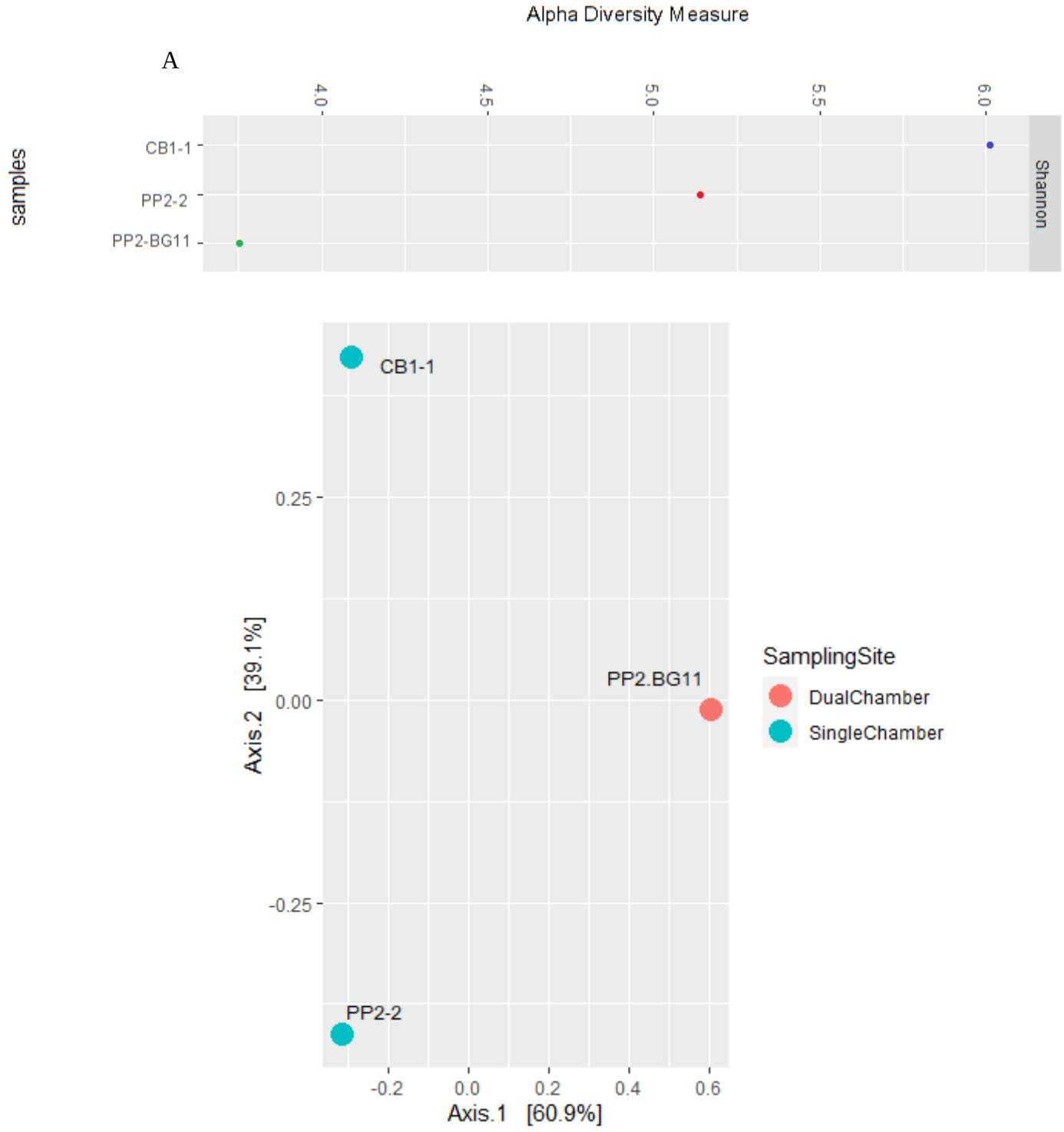

Figure 8. - **Diversity Indexes**. A-Alpha Diversity: Shannon. B-Beta Diversity: Weighted Unifrac. These indexes were measured to see how microbial communities differ between samples. Rarefaction value: 60k sequence reads.

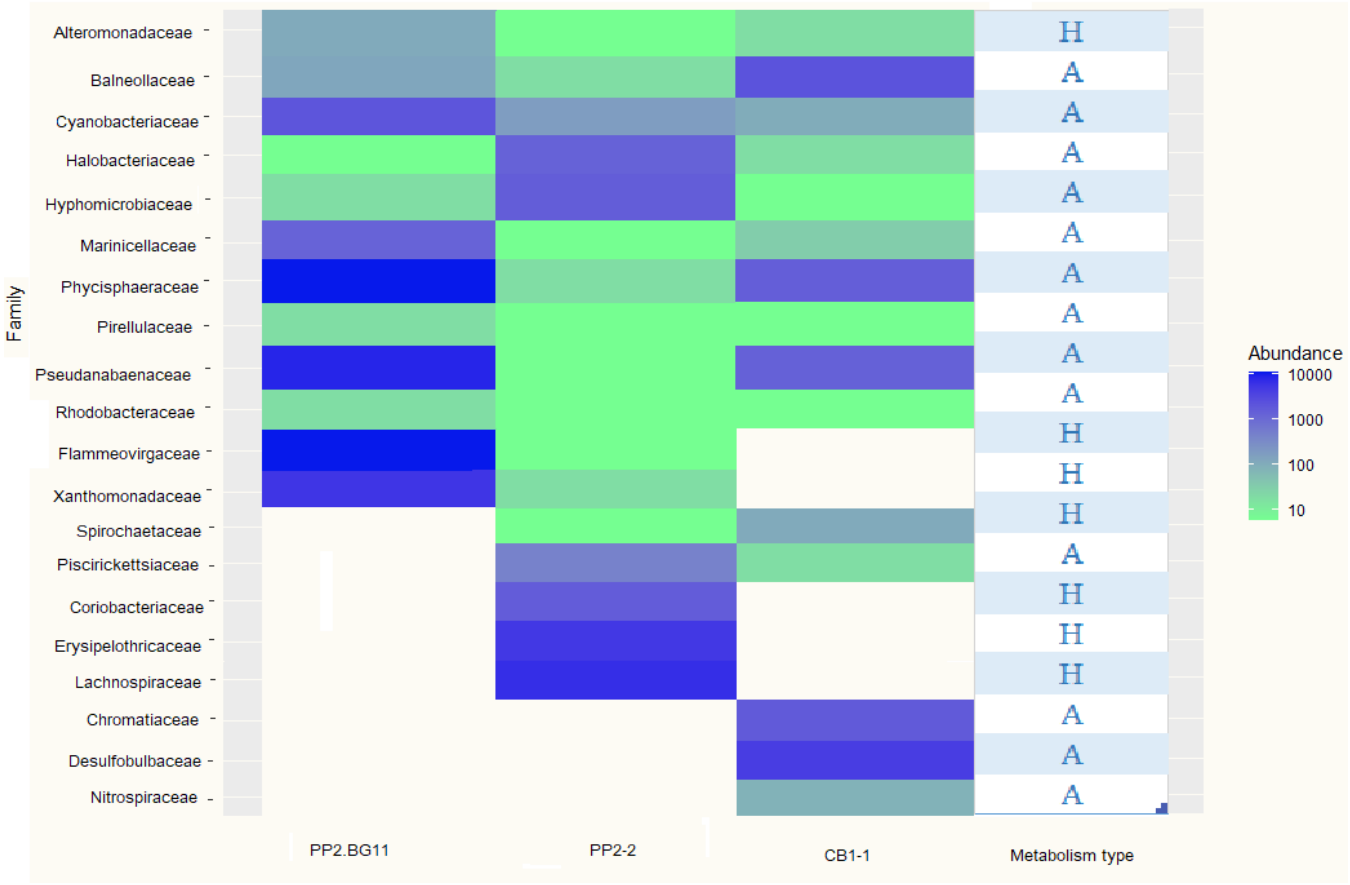

Figure 9. - Differential abundance heatmap. Blue marks show more counts while green shows fewer. Blank gaps show no counts for the sample. H: Heterotrophy; A: Autotrophy.

Table 1. - Physic-chemical parameters of the sediments. Measures were taken "In situ" on the sampling area, and "In vitro" in the laboratory

***In situ* measures**

Sample	Salinity (ppt)	DO (mg/ml)	pH	Temperature (°C)
Cerro Brujo 1	19,70 ± 7,29	7,97± 0,38	7,78 ± 0,85	31,10 ± 2,07
Cerro Brujo 2	19,70 ± 7,30	7,97± 0,39	7,78 ± 0,86	31,10 ± 2,08
Punta Pitt 1	72,09 ± 11,74	8,09 ± 0,56	5,92 ± 0,76	33,10 ± 2,07
Punta Pitt 2	72,09 ± 11,75	8,09 ± 0,57	5,92 ± 0,77	33,10 ± 2,08

***In vitro* measures**

Sample	Conductivity (ms/cm)	DO (mg/ml)	pH	Temperature (°C)
Cerro Brujo 1	40,105	0,27	7.25	22.2
Cerro Brujo 2	34,82	0,29	7.72	22.2°C
Punta Pitt 1	77,9575	1,35	7.28	22.2°C
Punta Pitt 2	71,3775	0,17	7.57	23°C