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Research Article

Influence of Fe^{2+} and Fe^{3+} on the Performance and Microbial Community Composition of a MFC Inoculated with Sulfate-Reducing Sludge and Acetate as Electron Donor

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A sulfidogenic sludge supplemented with acetate was evaluated in the anodic chamber of microbial fuel cells (MFCs) in the presence of sulfate (SO_4^{-2})/Fe^{3+} and sulfate (SO_4^{-2})/Fe^{2+} to investigate the MFC performance and the effect of the iron ions on the composition of the microbial community since sulfate and iron ions are frequently present in wastewater derived from several anthropogenic activities. The current densities were up to 0.025 mA/cm^2 and 0.017 mA/cm^2 for MFCs with Fe^{2+} and Fe^{3+}, respectively. Accordingly, the redox activity was slightly higher in the presence of Fe^{2+} than Fe^{3+}. In general, the metabolic activity of the MFC supplemented with Fe^{2+} was higher than the system with Fe^{3+} reaching a percentage of sulfate reduction (% SR), sulfide concentration (mg/L HS^-), and removal of chemical oxygen demand (% COD removal) of 35.2 ± 0.75 , 450.3 ± 3.6, and 50.05 ± 0.24 for % SR, HS^-, and % COD, respectively, whereas in the MFC with Fe^{3+}, the percentages were of 30.1 ± 1.076, 220.6 ± 2.0, and 11.78 ± 10.81 for % SR, HS^-, and % COD, respectively. The microbial population determined in each system was also correlated to the metabolic activity. *Rhodospirillales, Caulobacterales*, and *Burkholderiales* were the most abundant orders of bacteria in the MFC with Fe^{3+}, whereas with Fe^{2+}, *Rhodobacterales, Sphingomonadales*, and *Rhizobiales*. *Desulfohalobiaceae* and *Desulfovibrionaceae* were identified in the presence of Fe^{2+}. Unexpected interactions and combinations of microorganisms were observed in a relatively short culturing time, demonstrating the importance of characterizing the anode biofilm prior to shifts in iron ion concentrations on a long-term basis.

1. Introduction

The performance of a microbial fuel cell (MFC) depends on several factors; some important factors to consider in regard to the design of an MFC are the configuration and arrangement of the cell (i.e., single, dual, or multichamber), the electrode structure and material, and also the microbial community that is utilized to inoculate the MFC and that eventually develops a biofilm on the anode or cathode (in the case of a biocathode) of the cell [1–4]. According to research on microbial communities in MFCs, the sources of electroactive microorganisms utilized in these systems are mostly found in natural environments, for example, in marine and river sediments and soils [5–8], whereas niches of potentially electroactive microorganisms are also developed in activated sludge, either aerobic or anaerobic in

bioreactors utilized for wastewater treatment [9–14]. These electroactive microorganisms can also be cultivated from wastewater effluents of bioreactors when these effluents are cultured in the anodic or cathodic chambers of MFC utilizing a variety of electron donors, for example, volatile fatty acids, sugars, and alcohols [9, 15–17]. Some known electroactive iron reducing bacteria that have been isolated from sediments, soils, and sludge and have been utilized in MFCs belong to the following genera: *Geobacter, Shewanella*, and *Citrobacter* [2], whereas some of the sulfate reducing bacteria (SRB) that have been studied and reported as electroactive bacteria are found in natural environments and bioreactors and belong to the genera *Desulfuromonas* and *Desulfovibrio* [2]. Although the electroactive nature of the aforementioned bacteria has been proved individually, at large scale, it may be economically and technically difficult to sustain a pure strain culture on a long-term basis. Furthermore, the interaction that occurs in consortia of bacteria in a biofilm may favor the complete consumption of organic matter and contribute to the electron transfer mechanisms through the release or production of redox mediators, such as for example, ferrous iron (Fe^{2+}) or sulfide (S^{-2}) that are used as potential electron donors in MFCs when SRB (i.e., *Desulfuromonas* sp.) and sulfide oxidizing bacteria (SOB, i.e., *Desulfobulbus* sp.) are present in the anodic chamber [18]. This release or utilization of potential redox mediators can occur in addition to direct electron transfer mechanisms to the electrode via pili (Geobacter sp.) or outer membrane cytochromes that are in contact with the anode surface (*Shewanella* sp.) [2].

A combination of heavy metals—iron ions included—with sulfur species and organic compounds can be found in several wastewater streams such as for example, in effluents from paper, pharmaceutical, alimentary, metals processing, and waste derived from acid mine drainage (AMD); therefore, it is important to investigate the interactions that occur between iron reducing (IRB) and sulfate reducing (SRB) bacteria in biotechnological processes that may offer a recovery of energy, such as MFCs. In view that MFC technology is useful in wastewater treatment and that the wastewater composition may vary from one process to another, these variations need to be taken into consideration for the MFC performance. The study of this technology has included to some extent the influence of ferric iron, ferrous iron, and sulfate on the performance of MFCs although they have been investigated in separate experiments. For example, it has been reported that the addition of ferric iron favors the degradation of organic matter and generation of electricity in MFCs and also promotes the enrichment of the microbial communities in electroactive bacteria such as *Geobacter* sp. [13, 15, 19]. On the other hand, it has been suggested that ferrous iron enhances the performance of MFCs when it is present in the cathode (biocathode) [17]. The biosynthesis of iron sulfide nanoparticles that seem to promote the extracellular electron transfer in MFCs has been confirmed by including ferric iron and thiosulfate in the experiments [20], but the studies have not been linked to sulfate or COD removal. In regard to sulfate removal and electricity generation, research has been conducted on sulfate removal and the role of sulfide on the anode [21, 22] and also on the analysis of the shifts in the microbial community with iron and sulfur species, although the effect of iron and sulfur species has been evaluated in separated experiments in the anodic chamber of MFCs [7]. However, the combination of iron species with sulfate in MFCs linked to COD removal, which is a combination that may have implications on metal removal in wastewater treatment, has not been studied. Therefore, the aim of this work was to evaluate the effect of iron ions (Fe^{2+} and Fe^{3+}) in combination with sulfate (SO_4^{-2}) and acetate as the sole source of carbon and energy on the performance and microbial community composition developed on the anode of MFCs inoculated with sulfidogenic sludge from a UASB (upflow anaerobic sludge blanket) reactor.

2. Materials and Methods

2.1. Mineral Medium. The composition of the culture medium utilized in the entire experimental work was as follows (g/L): $CaCl_2{\cdot}2H_2O$, 0.02; $MgCl{\cdot}6H_2O$, 0.166; NaCl, 2; NH_4Cl, 0.56; K_2HPO_4, 1.2; $NaH_2PO_4{\cdot}2H_2O$, 1.6; and yeast extract, 0.04. Vitamins solution is 10 mL/L, and trace metal solution is 2 mL/L. The composition of the trace metal solution was as follows (g/L): H_3BO_3, 0.05; $FeSO_4{\cdot}7H_2O$, 2.8; $ZnSO_4{\cdot}7H_2O$, 0.106; $MnSO_4{\cdot}7H_2O$, 0.70; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.05; $AlK(SO_4){\cdot}12H_2O$, 0.175; $Na_3Co(NO_2)_6$, 3.4; $NiSO_4{\cdot}6H_2O$, 0.026; $CuSO_4{\cdot}5H_2O$, 0.175; EDTA, 1; and resazurin, 0.2. The composition of the vitamins solution was as follows (g/L): biotin, 0.02; folic acid dehydrate, 0.02; pantothenic acid, 0.05; nicotinamide, 0.05; p-aminobenzoate, 0.05; thiamine, 0.05; lipoic acid, 0.05; and piridoxine, 0.1.

2.2. MFC Setup. The microbial fuel cell system (type "H") consisted of two 130 mL chambers (125 mL working volume), an anodic chamber (anaerobic), and a cathodic chamber (aerobic) as shown in Figure 1. The anodic chamber was sealed, and the cathodic chamber was open to the atmosphere. The area of the graphite electrodes (graphite cloth) was 16 cm^2 (geometric area), and it was activated by immersion in HCl 1 M for 24 h, followed by washing it with distilled water and a final immersion in a NaOH 1 M for another 24 h; finally, the pH was adjusted to 7. The distance between both electrodes was of 4 cm. The two chambers were connected through a cationic membrane Ultrex (CMI-7000S Membrane International Inc.) that was arranged to a diameter of 4 cm. The membrane was sterilized (121°C for 15 min) and activated in a 2% NaCl solution at 37°C per 2 h.

2.3. Batch Experiments in the MFCs with Fe^{2+} and Fe^{3+} and Sulfidogenic Sludge. The sulfate reducing inoculum (sulfidogenic sludge) and the electrodes with biofilm (graphite cloths) had been previously adapted to concentrations of sulfate (SO_4^{-2}) of 6000 mg/L and to acetate as electron donor (at a chemical oxygen demand concentration of 4000 mg COD/L) according to González-Paz et al. [23]. The mineral medium in which the inoculum was maintained contained

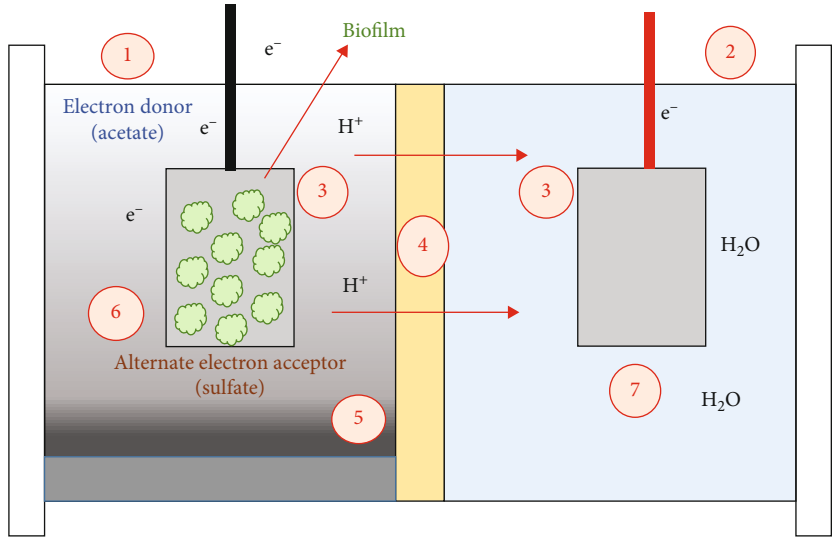

Figure 1: MFC type "H": anodic chamber (anaerobic) (1); cathodic chamber (aerobic) (2); graphite cloth (electrode) (3); cation exchange membrane (4); sulfate reducing inoculum (5); mineral medium (6); distilled water (7).

trace amounts of Fe^{2+}. The batch experiments (15 days each) were conducted to evaluate the effect of Fe^{2+} and Fe^{3+} and carried out in two MFCs. The two MFCs were inoculated with the sulfidogenic sludge. One of the MFC was supplemented with sulfate, acetate, and Fe^{+2} and the other one with sulfate, acetate, and Fe^{3+}. The MFC in which Fe^{2+} was added utilized the same graphite cloth (anode electrode) that was already covered with biofilm, a biofilm that was developed previously [23] and was tested apart to observe if the mature biofilm presented different behavior. The MFCs in which Fe^{2+} and Fe^{3+} were supplemented were inoculated with sulfidogenic sludge utilized in previous work [23], and the anode electrode did not present biofilm at the beginning of these batch experiments.

The anodic chambers of the MFCs were prepared as follows: one of them was inoculated with sulfidogenic sludge, the anode electrode with biofilm, sulfate, acetate as electron donor and iron, which was added as Fe^{2+} from a stock solution of $FeSO_4{\cdot}7H_2O$, to a final working volume of 125 mL and 5 mL of headspace. The other MFC was supplemented with sludge, sulfate, acetate as electron donor, and iron as Fe^{3+} from a stock solution of $FeCl_3{\cdot}6H_2O$ to a final working volume of 125 mL and 5 mL of headspace. The cathodic chamber only contained distilled water and was exposed to air. The pH was not adjusted with buffer solutions in any of the MFCs. The analytical determinations were conducted in all the MFCs for initial and final concentrations of sulfate, sulfide (as HS^-), pH, COD, and voltage. Table 1 shows the conditions for each MFC; besides the biotic MFCs, Table 1 also shows the conditions for the abiotic MFCs that were also prepared; for these MFCs, the determinations made were only the initial and final voltage. All the experiments were set in duplicates and conducted at room temperature (18–22°C).

2.4. Cyclic Voltammetry. In order to evaluate the redox reactions carried out in the MFCs with iron and the role of the biofilm in the reactions, cyclic voltammetry was conducted in the MFCs. The complete analysis of cyclic voltammetry required the preparation of additional MFC systems that are shown in Table 2. The cyclic voltammetry was conducted for all the MFCs, that is, those ones prepared according to Table 1 and the ones prepared according to Table 2. In addition to the biotic MFCs, a series of controls (Table 2) were also prepared in order to clarify the role of the microorganisms in the MFCs. The measurements were made with a potentiostat–galvanostat Metrohm, Autolab ®, US (73925). The working electrode was the graphite cloth in the anodic chamber, the reference electrode was Ag/AgCl, and the counter electrode was the cathode electrode (graphite cloth) in the cathodic chamber. The measurements were carried out in a range of -1.5 to 1.5 V at a scan rate of 0.1 V/s. The same conditions were used for the measurements in all the MFCs.

2.5. Analytical Methods. The sulfate (SO_4^{-2}) concentration and pH were analyzed according to standard methods [24]. The soluble sulfide (HS^-) was analyzed by the colorimetric method [25]. The Fe^{2+} concentration was determined according to the ferrozine method adapted from Stookey (in a concentration range of 0.009–1.4 mg/L). Acetate as the only electron donor was analyzed as the COD content (HACH HR^+ range 0–15000 mg/L, US and HACH, DR 2700 Germany). The voltage was measured with a commercial multimeter (Steren US) MUL605.

2.6. DNA Extraction and Sequencing of the Samples for Identification of Bacteria. At the end of the batches, samples of the biofilms developed on each of the MFCs that were supplemented with Fe^{2+} and Fe^{3+} were taken to analyze the microbial community. The DNA was extracted from the biofilm samples using a CTAB protocol [26]. The purified DNA was eluted with 40 mL of Milli-Q water and kept at -20°C before using it as template DNA for

Table 1: Experimental conditions for the batch tests for each MFC.

Parameter	MFC with sludge and Fe^{2+}	MFC with sludge and Fe^{3+}	MFC abiotic with Fe^{2+}	MFC abiotic with Fe^{3+}
Mineral medium	Yes	Yes	No	No
Volume	125 mL	125 mL	125 mL	125 mL
Sulfate (SO_4^{-2})	6000 mg/L	6000 mg/L	6000 mg/L	6000 mg/L
Acetate	4000 mg/L	4000 mg/L	4000 mg/L	4000 mg/L
*Iron concentration	Fe^{2+}10 mM	Fe^{3+}10 mM	Fe^{2+}10 mM	Fe^{3+}10 mM
Temperature (°C)	25	25	25	25
Time	15 days	15 days	15 days	15 days
Inoculum 15% *v*/*v*	0.0097 g VSS/g inoculum	0.0088 g VSS/g inoculum	No	No
Graphite cloth area: 16 cm^2	With biofilm	With biofilm	No biofilm	No biofilm

*10 mM (0.56 g/L).

Table 2: Operation conditions for the MFC that served as controls in order to evaluate the cyclic voltammetry (CV) determinations of each MFC.

Parameter	MFC acetate	MFC sulfate	MFC medium	MFC Fe^{2+}	MFC Fe^{3+}
Mineral medium	*No	No	Yes	No	No
Working volume	130 mL	130 mL	130 mL	130 mL	130 mL
Sulfate (SO_4^{-2})	No	6000 mg/L	No	No	No
Acetate	4000 mg/L	No	No	No	No
Iron concentration	No	No	No	10 mM	10 mM
pH	4.5	4.5	7	4.5	7
Temperature (°C)	25	25	25	25	25
Time	—	—	—	—	—
Inoculum (sludge)	No	No	No	No	No
Graphite cloth area: 16 cm^2	No biofilm	No biofilm	No biofilm	No biofilm	No biofilm

*In the case of "no," the mineral medium was replaced with distilled water; 10 mM (0.56 g/L).

sequencing analysis. DNA samples were analyzed at RTL Genomics (RTL, TX, US) for 16 rRNA gene sequencing using the pair of primers 28F-519R for bacteria. Bioinformatic data analysis was performed according to the RTL's protocol. An additional data processing was performed by using the metagenomic analysis server (MG-RAST, http://metagenomic.anl.gov) [27]. Data were submitted (Project ID Mgp 99135) for online annotation using the quality control (QC) pipeline.

3. Results and Discussion

3.1. Batch Experiments in the MFCs with Sulfate, Fe^{2+}, Fe^{3+}, and Acetate as Electron Donor and Sulfidogenic Sludge. The results obtained from the batch experiments of the MFCs supplemented with sulfate, Fe^{2+}, Fe^{3+}, and acetate are presented in Table 3. After 15 days, it was found that sulfate reduction, COD removal, and voltage were higher in the case of MFCs supplemented with Fe^{2+} than with Fe^{3+}. In regard to Fe^{2+} and Fe^{3+}, none of them were detected after 15 days in any of the MFCs. In the case of the MFC supplemented with Fe^{3+}, it was assumed that Fe^{3+} was precipitated by the sulfide produced, and if it was reduced, ferrous sulfide was formed, which made difficult to detect it. Furthermore, it has been reported that precipitates of Fe^{3+} and several mineral forms of it may hinder the capability of SRB to be active [28]; also, the formation of FeS and FeS_2 is feasible [20]. It may also be possible that either sulfide or Fe^{2+} or both had been oxidized on the anode, although sulfide to a minor extent in view of the voltage that was reached in that MFC. Oxidation of Fe^{2+} could lead to a difficult detection of Fe^{2+}, whereas sulfate concentration did not decrease further in the anolyte, which may have been due to sulfide oxidation (to sulfate) even at a low extent.

In the case in which Fe^{2+} was added to the MFC, at the end of the batch, no significant amount of Fe^{2+} was detected either; therefore, it was assumed that part of the Fe^{2+} was oxidized to Fe^{3+} in this MFC, and part of it formed some iron sulfides with the sulfide that was in the sludge at the beginning of the experiment (150 mg/L). This sulfide was in the sulfidogenic sludge and was taken as the background concentration of sulfide for that sample of sludge. In the abiotic MFCs, the voltage measured was never higher than ~0.16 V and did not change over time (Table 3). The voltage observed in the abiotic MFCs demonstrated that the inoculum (sludge) in the cells actually promoted the exchange of electrons in the system. Despite the fact that the inoculum (sludge) utilized to inoculate, the MFCs were previously acclimated to acetate to sustain sulfate reduction at a percentage of 70% [23] and derived up to 0.788 V in a MFC;

Table 3: Results obtained from the batch tests for each MFC with Fe^{2+} and Fe^{3+} inoculated with sludge and from the abiotic controls.

	Days	Sulfate (mg/L)	Sulfate reduction (%)	HS^-(mg/L)	*Iron (Fe^{2+}/Fe^{3+}) (10 mM)	COD (mg/L)	COD removal (%)	pH	Voltage (V)
MFC with (inoculum) sludge and Fe^{3+}	0	6234.5 ± 89.3		0		4384.2 ± 13.9		4.5	0.184
	15	4358.2 ± 8.1	30.1 ± 1.076	220.6 ± 2.0	No Fe^{2+} detected as a result of Fe^{3+} reduction	3895.6 ± 15.04	11.78 ± 10.81	7.4	0.360
MFC with (inoculum) sludge and Fe^{2+}	0	6408.4 ± 75		150.2 ± 1.1		4522.5 ± 23.4		4.2	0.452
	15	4154.8 ± 1.4	35.2 ± 0.75	450.3 ± 3.6	The Fe^{2+} was not detected in the anolyte of the MFC	2248.16 ± 2.61	50.05 ± 0.24	7.4	0.647
MFC abiotic with Fe^{3+}	15	—	0	0	10 mM	—	0	4.5	0.158
MFC abiotic with Fe^{2+}	15	—	0	0	10 mM	—	0	4.5	0.120

The results presented correspond to the average ± SD of duplicate samples. *10 mM (0.56 g/L).

in the present work, it was notorious that sulfate reduction was modified by the addition of Fe^{3+} (as $FeCl_3.6H_2O$). It has also been proved that an iron reducing sludge generated from the same seed sludge that was used in the present work can function in MFCs in which Fe^{3+} reduction was coupled to acetate oxidation (16, 27, and 55 mM concentrations of Fe^{3+}) yielding up to 90% of Fe^{3+} reduction (iron supplemented as ferric citrate) along with 80% of acetate consumption [16]. Therefore, the results obtained in the present work indicate that the combination of the two potential electron acceptors, sulfate and Fe^{3+}, may lead to iron sulfides production, which causes a diminished sulfate reduction. Iron reduction cannot be completely ruled out either, since the electrons may have been directed towards both, sulfate and Fe^{3+}, as demonstrated by the lower voltage obtained which is still higher than in the abiotic control. Furthermore, biosynthesis of sulfide nanoparticles with iron has been obtained over longer periods of incubation in MFCs that were supplemented with iron (as $FeCl_3$) and sulfide (as sodium thiosulfate $Na_2S_2O_3$) in order to enhance their performance; in those cases, the mature anode biofilms did not exhibit sulfate reducing activity prior to the addition of the iron and sulfide [20], and it was obtained a maximum voltage of 675 mV, a value that is comparable to 647 mV that was obtained in the present work in the cell amended with Fe^{2+}. Thus, it is possible that nanoparticle biosynthesis occurs in any SRB–IRB consortium under the appropriate conditions (i.e., time of incubation and steady supplement of iron, for example).

Under the evaluated conditions of this work, there was no concern on toxicity of Fe^{3+} to the SRB or IRB that could be present in the consortium utilized. The results in regard to percentages of sulfate reduced and acetate consumed are in agreement with studies in which the interval of Fe^{3+} concentrations has been between 18 mM (as $FeCl_3.6H_2O$ [29]) and 50 mM (as ferrihydrite [28]) during evaluations of sulfate reduction using acetate as electron donor, which is an interval that is close to the concentrations used in the present work. In the case of the MFC in which Fe^{2+} (as $FeSO_4.7H_2O$) was added, it was noticed that sulfide was in a higher concentration in the cell (Table 3) and that the electron transfer was slightly higher than in the cell amended with Fe^{3+}; therefore, sulfide concentration in the range obtained (~200–450 mg/L) was not a cause of concern in regard to toxicity to the microorganisms, which is in agreement with previous studies with this sludge and similar concentrations of sulfate [23]. The work presented by Kikuti-Mancílio et al. [7] indicated that the MFC amended with sulfate at an initial concentration of 6000 mg/L presented a 0.43 V (open circuit voltage) which is similar to the obtained in the present work, although the concentration of sulfide and the percentage of sulfate reduction were not reported in their work to compare them with the present work; on the other hand, the toxicity to the inoculum due to sulfide was not mentioned either.

3.2. Electrochemical Behavior of Each MFC without Sludge (Inoculum) for with Each Component of the Anolyte Was Evaluated Individually. It is important to evaluate each component that was utilized in the anolyte of the MFCs in order to emphasize the role of the consortium in the reduction of sulfate and iron and also in the acetate or ferrous iron oxidation. Thus, cyclic voltammetry was used to evaluate each MFC configuration presented in Table 2. That is, acetate, sulfate, Fe^{2+}, Fe^{3+}, and mineral medium were evaluated individually in different MFCs in absence of sludge (inoculum). The results of this evaluation are shown in Figure 2 (current vs. potential (V)). It can be observed that the trend in current is very similar in all cases. This can be due to the adsorption of the soluble components to the graphite electrode, as suggested by Uria et al. [6] or to an absence of a redox pair that promotes oxidation or reduction. The electrochemical response for each one of these control MFCs was smooth; it was only appreciated the double layer capacitance, since there were no peaks that indicate a redox reaction in the MFCs. In all cases, after -1 V, the hydrogen evolution reaction can take place. In Figure 2(a), the control MFC–acetate displays a maximum oxidation current of 0.21 mA, whereas for sulfate (control MFC–sulfate,

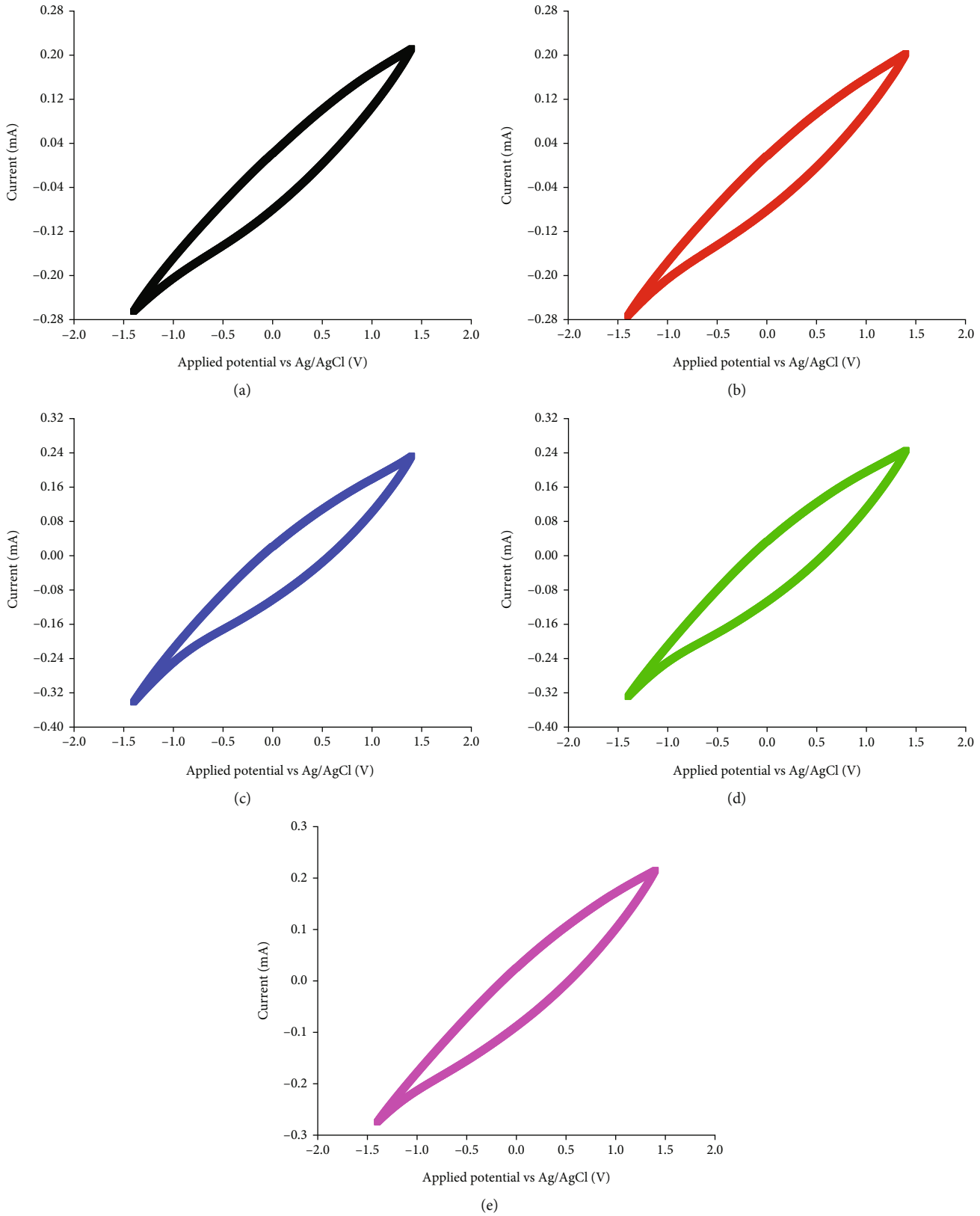

Figure 2: Cyclic voltammetry (CV) for each MFC without sludge (inoculum): (a) CV of MFC with acetate; (b) CV of MFC with sulfate; (c) CV of MFC with mineral medium only; (d) CV of MFC with Fe^{2+} only; (e) CV of MFC with Fe^{3+} only.

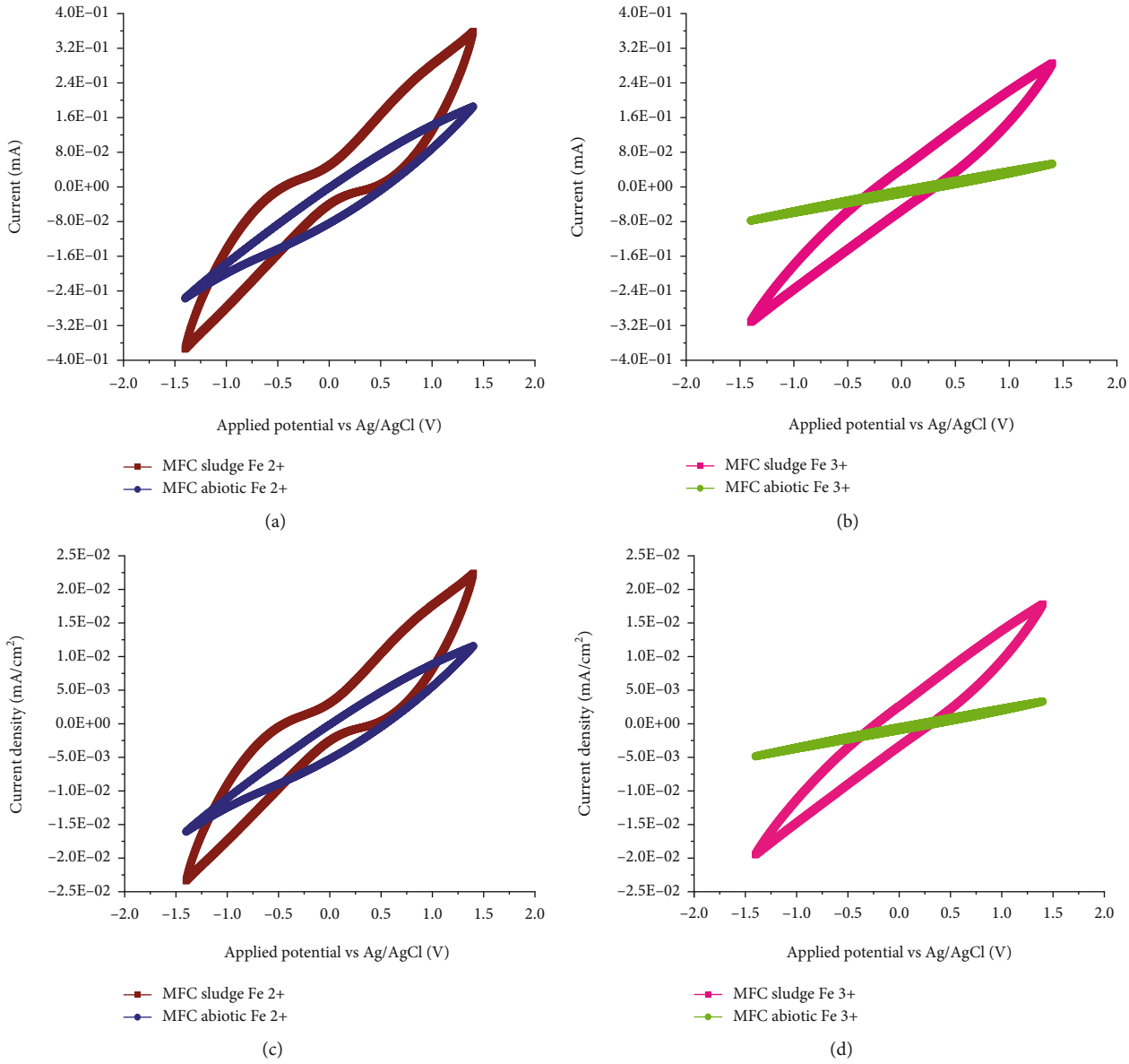

Figure 3: Cyclic voltammetries for the MFCs: (a) comparison of the voltammograms (current) of abiotic MFC and MFC with sludge and Fe^{2+}; (b) comparison of the voltammograms (current) of abiotic MFC and MFC with sludge and Fe^{3+}; (c) comparison of the voltammograms (current density) of the abiotic MFC with the MFC with sludge and Fe^{2+}; (d) comparison of the voltammograms (current density) of the abiotic MFC with the MFC with sludge and Fe^{3+}.

Figure 2(b)), the maximum reduction current was of 0.27 mA. The mineral medium composed of salts and trace metals (control MFC–mineral medium, Figure 2(c)) presented a maximum current of reduction of 0.34 mA and a maximum current of oxidation of 0.23 mA. In this control MFC, the reduction process was more pronounced that in the control MFC–acetate and control MFC–sulfate. Figure 2(d) shows the behavior of the MFC system with Fe^{2+} where no coupled redox reaction occurred and the double layer capacitance was observed, and a maximum oxidation current of 0.24 mA was observed in comparison with the 0.21 mA observed for the MFC system with Fe^{3+} (Figure 2(e)). In regard to the oxidation of acetate and Fe^{2+}, the Fe^{2+} presented a higher oxidation current (-0.21 vs. 0.21 mA), which indicates that Fe^{2+} can be a better electron donor (particularly to the anode). In the case of the reduction (sulfate and Fe^{3+}), both presented a similar current (-0.27 vs. 0.27 mA); thus, they may compete to be taken as electron acceptors (besides the electrode) in the MFC.

3.3. Electrochemical Behavior of the Abiotic and Biotic (Inoculated with Sludge) MFCs Using Acetate as Electron Donor and Sulfate Supplemented Individually with Fe^{2+} and Fe^{3+}. The electrochemical behavior of the MFCs that were inoculated with sludge was compared to the electrochemical behavior of the abiotic MFCs in which acetate,

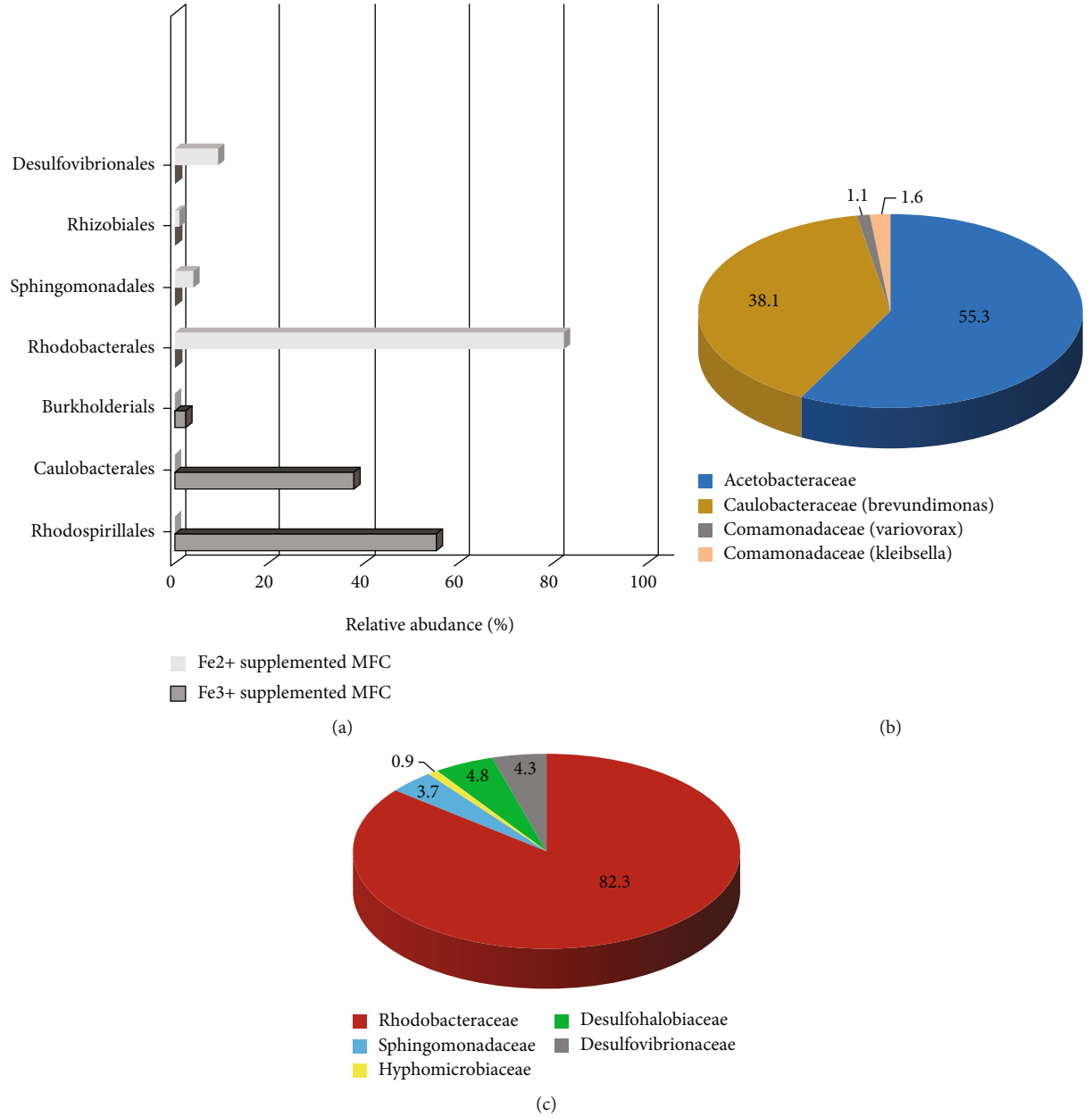

FIGURE 4: Microbial population distribution: (a) at the order level, comparison of the different microbial groups in both MFCs; (b) at the family level in the MFC supplemented with Fe^{3+}; (c) at the family level in the MFC supplemented with Fe^{2+}.

sulfate, and iron ions were added together. The results of the five cycles that were conducted in each MFC remained the same since the first cycle; this indicated that at the time of the measurements, the behavior of anode polarization with biofilm was stable. The results are shown in Figure 3 (current and current density vs. potential (V)). A comparison of the current of the biotic MFCs with the abiotic MFCs is shown in Figure 3(a) (abiotic and biotic MFCs with Fe^{2+}) and Figure 3(b) (abiotic and biotic MFCs with Fe^{3+}), in which it can be seen that the shift of the curves abiotic versus biotic is pronounced as a result of the redox reactions and that the current is higher in the biotic MFCs than in the abiotic. Two peaks are observed in the voltammogram obtained with biotic MFCs; the first one is located at a peak potential of – 0.75 V and the second one at a peak potential of +0.75 V; this is a proof of the catalytic activity of the biotic MFCs. The maximum current achieved was of 0.2 mA and 0.4 mA for abiotic and biotic MFCs with Fe^{2+} (twofold higher for biotic than abiotic), respectively. In the case of the abiotic and biotic MFCs with Fe^{3+}, the current achieved was of 0.05 mA and 0.3 mA (sixfold higher for biotic than abiotic), respectively (Figure 3(b)). In this case, no oxidation or reduction peaks were detected; however, a higher double layer capacitance was achieved with biotic than with abiotic

MFCs. The current densities were of 0.025 mA/cm^2 and 0.01 mA/cm^2 for the biotic and abiotic MFCs with Fe^{2+}, respectively (Figure 3(c)), and of 0.017 mA/cm^2 and 0.0020 mA/cm^2 for the biotic and abiotic MFCs with Fe^{3+}, respectively (Figure 3(d)). The overall results show that in the biotic MFCs supplemented with Fe^{2+}, a higher redox activity was developed than in the biotic MFCs supplemented with Fe^{3+}, and it can be attributed to the activity of the microorganisms on the anode and the role, still unknown, of the planktonic cells, since Fe^{2+} could not be detected in any of them on day 15 of the batches. This may have been due to several reasons, for example, a fast oxidation (Fe^{2+} that was taken as electron donor to the anode) in the case of the addition of Fe^{2+}, whereas in the case of the input of Fe^{3+} to the cell a fast reduction to Fe^{2+} occurred and then an immediate reoxidation of it again. Other reasons could be the formation of nanoparticles of Fe^{3+} with sulfide as it has been reported to occur in similar scenarios [20] and the composition of the microbial community [2], which will be presented here. It was also observed that in the MFCs amended with Fe^{3+} whose anode had developed a biofilm prior to the addition of Fe^{3+}, the current density remained within the same order of magnitude as in the MFC in which the biofilm was formed within the 15 days of the test. It has been reported that on a long-term basis (over 40 days of incubation at 30°C) in MFCs supplemented with glucose as electron donor in the anodic chamber, thiosulfate, and Fe^{3+}, using an inoculum taken from an anaerobic digester, the current densities may reach up to 0.12 mA/cm^2 while nanoparticles of biosulfides are synthetized [20]. On the other hand, an inoculum composed of marine sediments (a rich microbial community), supplemented with acetate, Fe^{3+}, sulfate, and sulfide, may reach current densities between 0.15 mA/cm^2 and 0.175 mA/cm^2 [7] in approximately 15 days of operation showing a slightly higher open circuit voltage and current density when the MFCs are supplemented with Fe^{3+} and acetate. The current densities reached in the present experiments are low; however, the electrogenic character, which will be discussed later in this document in the microbial community analysis, may be attributed to the bacteria in view that the medium components and possible side reactions in the MFCs contributed at lower extent to the generated current as was shown in the abiotic experiments. The contribution due to the medium components and side reactions is not always considered when presenting current or power densities, which at a glance may be much higher than the obtained in the present work.

Despite the reports on the role of iron, and particularly Fe^{3+} on the MFCs, the ferrous iron role has been documented to a lower extent. The present work evaluated the addition of Fe^{2+} as an important component in wastewater containing metals and sulfate (i.e., acid mine drainage) and its presence in natural environments in which IRB and SRB may coexist.

3.4. Microbial Community Composition of the MFCs Supplemented with Fe^{2+} and Fe^{3+}. The analysis of the microbial community was conducted in both biotic MFCs, the one supplemented with Fe^{3+} and the supplemented with Fe^{2+}. According to the sequencing results (Figure 4), it was clear that the addition of the ferric and ferrous iron exerted an influence on the composition of the microbial community and its metabolic activity, which was modified in a relatively short period of time (~20 days total). It was expected an influence, but it was higher than expected based on the cultivation period.

In both MFCs, taxonomic identity, as established through database, showed that Proteobacteria was the most dominant phylum, comprised of Alpha-, Beta-, and Delta-Proteobacteria. In the case of the MFCs supplemented with Fe^{3+}, the predominant microbial population belongs to the Alpha and Beta-Proteobacteria distributed in the orders of *Rhodospirillales, Caulobacterales*, and *Burkholderiales* as shown in Figure 4(a). The *Rhodospirillales* order comprised the family *Acetobacteraceae* (unclassified at genus level) with a predominance of 55.3% at family level out of the total microbial population. This represents the major taxa identified in this system (Figure 4(b)). The order *Caulobacterales* was categorized as *Caulobacteraceae* at family level, representing 38% of the microbial population; this family was identified as *Brevundimonas* sp. at genus level. At lower extent (around 2%), *Burkholderiales* were identified as *Comamonadaceae* family; this family was distributed between *Klebsiella* and *Variovorax* at genus level (Figure 4(b)). On the other hand, according to the sequencing data in the MFCs supplemented with Fe^{2+}, the dominant microbes belong to the Alpha-Proteobacteria (86%), and a lower proportion of Delta-Proteobacteria (8%) was also found. Alpha-bacteria were distributed in the orders of *Rhodobacterales, Sphingomonadales*, and *Rhizobiales*, together accounting for more than 86% out of the total microbial population (Figure 4(a)). The orders *Desulfohalobiaceae* and *Desulfovibrionaceae* belonging to the Delta-Proteobacteria were identified at lower extent (approximately 4% each). The predominant microbial population (82%) found in this MFC was recognized as *Rhodobacter* sp. at genus level (Figure 4(c)) belonging to the *Rhodobacteraceae* family.

The occurrence of the microbial population obtained is coherent with the microbial activity found in the Fe^{3+} and Fe^{2+} MFCs. In general, all the microbial groups identified in the MFCs belong to the phylum Proteobacteria, which has been shown to enclose several microorganisms presenting electrogenic activity. Previous studies have demonstrated that the Proteobacteria constitute electrochemically active bacteria (EAB) in MFC reactors [30]. Additionally, due to the remarkable diversity of electroactive microorganisms, Logan et al. [2] proposed that these microorganisms can be categorized according to the power densities produced as (a) microorganisms that cannot efficiently perform exogenous electron transfer and show low power production (10 mW/m^2), (b) microorganisms with intrinsic limited ability to transfer electrons or with difficulties to transfer electrons due to the reactor architecture (<100 mW/m^2), and (c) efficient exoelectrogenic microorganisms (>100 mW/m^2), enabling power production which in many cases exceeds >1,000 mWm^{-2}. According to this classification,

the microbial cultures evaluated in the MFCs were able of producing current from acetate in a range considered enough to demonstrate the activity of electrogenic microorganisms; this is ~0.025 mA/cm^2 (equivalent to 161.75 mW/m^2 calculated with the data of the experiment).

Some of the microbial groups found in each of the systems have specific metabolic roles in the experimental conditions evaluated in the Fe^{2+} and Fe^{3+} MFCs; the right microbial partners can perform complex processes in MFCs. In the MFC supplemented with Fe^{3+}, the families of *Caulobacteraceae* (represented by *Brevundimonas* with a high relative abundance of 38.1% at genus level) and *Comamonadaceae* could produce electricity as previously reported [2, 31, 32]. Additionally, *Comamonadaceae* constitute a remarkable phenotypic diversity which includes anaerobic denitrifiers and iron reducing bacteria as reported by Willems [33]. The microbial groups found in the Fe^{3+} MFC reactor are also similar to those reported as groundwater microbiome, particularly *Brevundimonas* and some others that could be associated with Fe^{3+} reduction and arsenic (As) mobilization in sediments and soil [34]. These have also been identified during iron and manganese (Mn) removal (through oxidation) from acid mine drainage in an iron–manganese oxidizing consortium [35]. Particularly, *Klebsiella* could be associated with the iron reduction. Lovley [36] proposed a sequence of cooperative metabolic activities between fermentative microorganisms and dissimilatory iron reducing microorganisms where acetate is oxidized to carbon dioxide with Fe^{3+} serving as the sole electron acceptor. Even if the most common iron reducing microbes as *Geobacter* sp. or *Shewanella* sp. have not been found as part of the microbiome in the MFC, the biological activity of some of the microorganisms identified could fit this model of microbially catalyzed oxidation of organic matter coupled to Fe^{3+} reduction. It is also known that *Bacillus subtilis* and *Klebsiella aerogenes* produce quite low current densities in pure cultures. Thus, it has been proposed that such low-power-producing microorganisms are classified as weak exoelectrogens and that production of low current densities is associated with unique roles in biofilm microbial ecology.

Regarding the microbial population found in the Fe^{2+} MFC reactor, the main taxa was *Rhodobacter* (82%) at genus level. These bacteria belong to the nonsulfur purple bacteria, which are microorganisms that have shown a versatile metabolic activity that allows them to grow in all known ways of life. There have been some reports in the literature that indicate that phototrophic purple nonsulfur bacteria as *Rhodopseudomonas palustris* and *Rhodobacter sphaeroides* can be used for electricity generation and substrate decomposition in a photobiological fuel cell [37, 38]. Xing et al. [38] reported that the *Rhodopseudomonas palustris* DX-1, isolated from a MFC, produced electricity at higher power densities (2720 ± 60 mW/m^2) than mixed cultures in the same device. More recently, Xu et al. [32] identified *Rhodobacter* at genus level in high relative abundance in the anode of a MFC treating wastewater under nonsaline conditions. Additionally, since the sulfate concentration decreased, the sulfate reduction was detected at larger extent in the MFC amended with Fe^{2+}, which is associated with the identification of sulfate reducing bacteria represented by *Desulfohalobiaceae* and *Desulfovibrionaceae*at family level, although these groups were detected at low proportion in the microbial population.

4. Conclusion

In this study, it was demonstrated that the selection of Fe^{2+} over Fe^{3+} in a sulfate reduction bioprocess promoted an improvement on the performance of the MFC, and also, it affected the microbial composition of the biofilms. Anode biofilms generated from a sulfate reducing sludge presented a higher electrogenic character in the presence of Fe^{2+} along with sulfate reduction and high COD removal. The presence of Fe^{3+} along with sulfate diminished the sulfate reduction, COD removal, and electrogenic activity. The current densities achieved in the presence of Fe^{2+} (0.025 mA/cm^2, equivalent to 161.75 mW/m^2) and Fe^{3+} (0.017 mA/cm^2) along with the microbial communities developed on the anodes suggest that the electroactive consortia developed on the biofilms were strongly influenced by the iron ions. *Rhodospirillales, Caulobacterales,* and *Burkholderiales* were the predominant orders of bacteria identified in the presence of Fe^{3+}, whereas *Rhodobacterales, Sphingomonadales*, and *Rhizobiales* were predominant in the presence of Fe^{2+} in combination with the orders *Desulfohalobiaceae* and *Desulfovibrionaceae*, which were not detected in the presence of Fe^{3+} in which case sulfate reduction was lower. The influence of iron ions on the composition of the microbial community was expected but at lower extent than the observed. The results are in agreement with previous observations on shifts of microbial population composition occurring in short periods of time, which suggests that electrochemical and microbial characterization should be conducted periodically in MFCs aimed to remove metals. Further work will be needed in order to elucidate the effect of COD composition on the MFC performance in the presence of iron ions and microbial composition. For instance, other carbon sources such as butyrate, propionate, or a mixture of both and even a complex feeding consisting of artificial or actual wastewater could be evaluated. Among them, the utilization of actual wastewater as a COD source is of paramount interest due to the scaling implications of this kind of systems.

Data Availability

Data were submitted (Project ID Mgp 99135) for online annotation using the quality control (QC) pipeline.

Conflicts of Interest

The authors declare that there are no conflict of interest.

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