In the current global energy scenario, it is essential to reduce our dependence on fossil fuels. Over the last few decades, the prices of these energy sources have undergone oscillations that have threatened most worldwide economies, especially those of oil-importing countries. Furthermore, the burning of fossil fuels significantly promotes climate change due to the large emission of greenhouse gases (CO2).

These growing environmental concerns have accelerated the development of sustainable energy sources with minimal carbon footprints. Within the field of liquid fuels, biofuels (mainly bioethanol and biodiesel) have emerged as an eco-friendly alternative. Bioethanol can replace gasoline engines, whilst biodiesel can take the place of petrol diesel engines. The strategy for progressive implementation of biofuels has been applied in different parts of the world, especially in Europe, the United States and Latin America. Brazil is the market leader in the production of biodiesel and bioethanol, with an incessant growth supported by government incentives. Biodiesel, formed by the transesterification of triglyceride and methanol, is receiving more attention due to the exponential growth experienced in Brazil over the last ten years. The growth of this sector has brought a concomitant increase in glycerol stock (1 kg of glycerol for each 10 kg of biodiesel). In such a situation, traditional industries (pharmaceutical, agricultural, food, cosmetics and so on) are no longer able to manage the surplus of glycerol.

Glycerol is a biodegradable substrate that can be processed by different microorganisms, such as Propionibacterium, Escherichia, Anaerobiospirillum, Klebsiella, Citrobacter, Clostridium, Lactobacillus and Bacillus, from which more added value products can be obtained (propane-1,3-diol, pyruvate, ethanol, 1-butanol and short chain organic acids). These applications have stimulated the utilization of glycerol as an organic substrate in biological-based valorization processes, such as the Microbial Fuel Cells (MFC).

An intermediate mode is the cycling feed of the MFC (intermittent fed-batch (IFB)), in which the cell operates as a discontinuous reactor between the feed cycles, with the microorganisms contained in the anode being timely fed. Such a configuration might be applied to small-size industries where the waste can be accumulated and treated all at once in periodic cycles. This might permit the design of larger MFCs that could provide higher power densities. Several parameters influence MFC performance, such as the operating conditions, cell configuration, membrane nature, electrode materials and the external resistance. Among the first type of parameters, the organic loading rate (OLR) and sludge age (SA) are the most influential parameters. The OLR can be regulated through the substrate concentration and/or the feed influent flowrate. The latter parameter defines the hydraulic retention time (HRT) for a defined volume of the reactor in a continuous biological reactor (average time that a fraction of fluid remains within the reactor). This parameter has been demonstrated to importantly influence the performance of MFCs, with the existence of an optimum HRT for electricity production and organic matter removal. In cycling-fed setups, the HRT can be also applied, being defined as the quotient between the reactor volume and timely feed volume in each cycle. As far as the authors are aware, only Jayashree et al. have studied the influence of the HRT in an IFB system. They concluded that the HRT impacts on the MFC power generation, the Chemical Oxygen Demand (COD) removal and the bacterial communities involved in the MFC.

A previous study from this research group demonstrated the influence of glycerol concentration on the performance of a glycerol-fed MFC. Continuing the research theme of glycerol utilization for energy recovery in a MFC, this work focuses on the influence of the HRT on the performance of an IFB glycerol-fed MFC, with emphasis on the electrochemical activity (polarization and electrode curves and Coulombic efficiency (CE)), COD and ammonium and phosphate removal, along with the products derived from the biological activity upon the synthetic effluent.
Materials and Methods

Glycerol purification.—The glycerol used in this study was obtained from a biodiesel synthesis with soybean oil and methanol, using KOH as the catalyst.\textsuperscript{23} The glycerol obtained was purified according to the following sequence.\textsuperscript{24,25} First, the excess methanol was removed by vacuum evaporation. Next, the remaining glycerol phase was acidified with phosphoric acid (5% w/w solution in water) until pH 3 was reached. This gave rise to the appearance of three phases, an uppermost phase, containing the non-reacted fatty acids and the rest of the biodiesel, a lowermost phase, containing the solid salts of potassium phosphate (primarily KH\textsubscript{2}PO\textsubscript{4}), and an intermediate phase, transparent with a faint yellowish tonality. The fatty acid and biodiesel upper phase was carefully removed, whereas the glycerol and inorganic salt solid fraction was centrifuged in order to better separate the two phases (CELM, Mod. COMBATE, Brazil). The supernatant glycerol phase was carefully extracted and refined by vacuum filtration.

A final treatment comprised the use of an anionic exchange resin (Dowex–1, Sigma Aldrich). Before applying it to the glycerol phase, the resin was pretreated in a 0.1 mol L\textsuperscript{−1} NaOH solution in order to leave all the exchange sites in the OH\textsuperscript{−} form. The ionic exchange treatment allows us to control the phosphate (excessive phosphorous may be detrimental for microbiological systems) and neutralize the excess acid added. In a typical procedure, 8 g of resin was added to 200 mL of glycerol solution. The system was left for 24 h under stirring. The removal of phosphorous and other elements was confirmed by inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo Fisher Scientific, ICAP 6000 model, USA). The final refined glycerol solutions were stored in amber recipients in order to avoid any degradation associated with light.

The glycerol concentration was determined by high performance liquid chromatography (HPLC, Flexar LC, Perkin-Elmer, USA) with a refractive index (RI) detector. The stationary phase used was an ion exchange PolyPore-H column (Perkin-Elmer, dimensions of 220 × 4.6 mm, particle size of 10 μm, hydrogen form) and the mobile phase was a 25 mmol L\textsuperscript{−1} H\textsubscript{2}SO\textsubscript{4} solution. The column temperature was set at 25 °C with a flow rate of 0.5 mL min\textsuperscript{−1}. The calibration curve was built with 0.2, 0.4, 0.6, 0.8 and 1 mol L\textsuperscript{−1} glycerol solutions (from glycerol ACS, Vetec). The corresponding final glycerol concentration was 852 g L\textsuperscript{−1}. Original glycerol solutions were 1:20 diluted in order to render a solution within the calibration curve.

MFC setup.—The MFC experimental setup consisted of a two-chamber cell with an “H” configuration,\textsuperscript{3} made of PVC (Figure 1). In order to separate the anodic and cathodic compartments, a proton exchange Nafion NR211 (25 μm, IonPower, USA) membrane was placed between them. Two graphite rods were used as the anode and cathode (geometric area of 27.75 cm\textsuperscript{2}). In the case of the anode, the graphite rod was sanded with sandpaper in order to increase the surface roughness with the purpose of favoring the anchorage of the microbes. In the case of the cathode, a catalytic layer containing 0.5 mg cm\textsuperscript{−2} Pt/C (from commercial 20% Pt/C, Premetek, USA) and 10% wt of Nafion as the ionic carrier and binder (from a commercial 5% wt Nafion emulsion in a mixture of aliphatic alcohols, Ion Power, USA) was deposited onto the graphite rod by brushing. Air was bubbled in the cathodic compartment by a fishbowl compressor. Copper wires were used as electrical connections within the MFC, which were carefully isolated with epoxy resin to prevent any interference associated with exposure of Cu to the biological environment.

In order to start up the MFC, the anodic compartment was filled with 140 mL of a synthetic wastewater, whose composition is collected in Table I, and 100 mL of activated sludge donated by Brasilia Wastewater Treatment Company (CAESB, Asa Norte Wastewater Treatment Plant WWTP), totalling a volume of 240 mL. In order to favor the growth of anaerobic bacterial communities, the sludge was sealed for 7 d before the cell mounting. The cathode solution was filled with 240 mL of a phosphate buffer solution, whose composition is also shown in Table I, in order to maintain the pH of the solution at around 7 and avoid the appearance of a pH gradient between the anode and cathode that may be detrimental to the operation of the MFC.\textsuperscript{26}

MFC operation.—The first necessary step in any MFC is the acclimatization process. The cell voltage was followed under closed circuit conditions with a 100 Ω resistance with the aid of a potentiostat/galvanostat (AUTOLAB III, Metrohm Autolab BV, Netherlands). Such a low resistance was chosen in order to favor the growth and activity of the exoelectrogenic microorganisms.\textsuperscript{27} The operating cycles lasted for 24 h, after which a certain volume of the anolyte solution was taken out and used for COD and total suspended solids (TSS) monitoring. In the initial acclimatization stage, in order to keep the same HRT and SA, the anolyte was gently stirred while suspending the sludge contained in the anode reservoir. The same volume of fresh feed solution was added, corresponding to a feeding rate of 10 mL d\textsuperscript{−1} (HRT and SA of 24 d).

After observing a stable closed circuit voltage, the electrochemical measurements were carried out. Polarization curves (in triplicate) were recorded for consecutive days until a reproducible electrochemical response was obtained. The voltage was varied from the open circuit voltage (OCV) to a nearly zero voltage at a scan rate of 1 mV s\textsuperscript{−1} with the aid of the potentiostat/galvanostat. In order to better interpret the electrochemical results, the anodic and cathodic polarization curves were recorded by the use of a Ag/AgCl reference electrode (+0.198 V vs. normal hydrogen electrode). The counter electrode was the other electrode in the MFC. The CE was also quantified as described in the literature.\textsuperscript{28} The procedure for the estimation of the ohmic resistance is detailed elsewhere.\textsuperscript{8} Briefly, the cathode potential was measured and compared in the anode and cathode reservoirs with the aid of the reference electrode. The difference between both potentials was plotted versus the current density, observing for all the cases a

| Compound | Concentration/ mg L\textsuperscript{−1} | Compound | Concentration/ g L\textsuperscript{−1} |
|----------|--------------------------------------|----------|--------------------------------------|
| Glycerol | 1080 | Na\textsubscript{2}HPO\textsubscript{4} | 2.75 |
| (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} | 74.2 | NaH\textsubscript{2}PO\textsubscript{4} | 3.67 |
| KH\textsubscript{2}PO\textsubscript{4} | 44.5 |
| NaHCO\textsubscript{3} | 111.0 |
| MgCl\textsubscript{2} | 37.1 |
| CaCl\textsubscript{2} | 30.7 |
| (NH\textsubscript{4})\textsubscript{2}Fe(SO\textsubscript{4})\textsubscript{2} | 3.1 |

*optimized from Guimarães and Linares.\textsuperscript{8}
linear dependence, which is believed to be mostly due to the ohmic resistance. The specific resistance is calculated from the slope of the aforementioned plot and the ohmic resistance from the quotient of this value and the area used in order to normalize the current (electrode area).

Biochemical parameters of interest were monitored along with the electrochemical polarization curves throughout the experiments. The VSS were quantified according to ASTM D5907.29 The COD was monitored right after feeding with fresh feed solution (initial COD at the operating cycle) and at the end of the 24 h cycle (final COD at the operating cycle, sample taken from the extracted daily volume of the anolyte), allowing the estimation of the COD percentage removal for each cycle at the different HRT. Ammonium and phosphates were monitored with the aid of commercial kits supplied by Alfakit (Brazil) and determined spectrophotometrically on a photo colorimeter (AT 10P II model, Alfakit, Brazil). In these cases, the values were only monitored at the end of the 24 h cycle (samples also taken from the extracted daily volume of the anolyte). The parameters were monitored in, at least, three consecutive days after stabilization of the cell voltage. These values were averaged and the confidence intervals were calculated.

The study HRTs are listed in Table II. The sequence followed for the HRTs was from the highest to the lowest value. In order to maintain constant the sludge and exclusively modify the HRT, the volume of homogenized anolyte liquor extracted was always 10 mL. It is important to note that the composition of the anodic feed solution is always the same regardless of the HRT. This was taken out after extracting the differential volume between the total and the 10 mL of homogenized liquor from the supernatant. The aforementioned protocol of measurements was always repeated after any change in the HRT. The measurements were carried from longest to shortest HRT.

A final study carried out on the MFC was the analysis of the kinetics of the glycerol before modifying the HRT. Upon an operating cycle (24 h), several aliquots of the supernatant were collected at different times. Before the extraction of each sample, the anolyte was softly stirred (in order to homogenize the solution and avoid the extraction times. Before the extraction of each sample, the anolyte was softly stirred (in order to homogenize the solution and avoid the extraction times.

Table II. HRTs and the corresponding volumes of homogenized liquor, supernatant and total volumes used.

| HRT/d | Supernatant volume extracted/mL | Homogenized liquor extracted/mL | Total volume extracted/mL |
|-------|--------------------------------|--------------------------------|--------------------------|
| 24    | 0                              | 10                             | 10                       |
| 9.6   | 15                             | 25                             | 35                       |
| 7.5   | 22                             | 32                             | 54                       |
| 6.4   | 27.5                           | 37.5                           | 65                       |
| 4.8   | 40                             | 50                             | 90                       |

The final COD presents a minimum for the HRT of 7.5 d, corresponding to a daily feeding volume of 32 mL. Therefore, the maximum COD removal capacity of the MFC is attained for this HRT, as shown in the inset of Figure 2. This behavior evidences the presence of an optimum HRT in which the whole microbial community present in the anodic compartment possesses the maximum capacity for abating the substrate present in the feed. Longer HRTs lead to a notable decay in the COD removal capacity, whereas shorter HRTs also depress the percentage of removed COD.

In order to assist in interpreting these results, Figure 3 displays the volatile suspended solids (VSS) as a parameter that can give an indirect idea (due to the counting of extracellular polymer produced by bacteria, secreted enzymes, cell debris, etc.) of the amount of microorganism presents.16 As can be seen, the VSS increases with the HRT, attaining a maximum value at an HRT of 7.5 d. Based on this, the larger amount of microorganisms are the primary explanation for the better COD removal performance achieved at the HRT of 7.5 d. Long HRTs result in an insufficient organic loading rate, disfavoring the bacterial activity and growth. At short HRTs, the organic matter availability may no longer be the limiting factor, but the limited activity and growth rate of the microbes lead to the observed plateau in the VSS and the increase in the final COD at the HRT of 6.4 and 4.8 d.

Other nutrients, such as phosphates and ammonium are necessary for the microbe activity. Attention must be paid to these nutrients since an unbalance can be detrimental for the aquatic environment.30 With the purpose of exploring the MFC capacity for managing ammonium and phosphate, Figure 4 displays the concentrations of ammonium and phosphate at the end of the daily operating cycle (final concentrations, measured from the extracted volume of the anolyte), which are compared with that of the fresh feed solution as a parameter to infer the MFC nutrients removal capacity. As can be observed, ammonium and phosphate concentrations are reduced compared to the values in the influent, as a result of the microbiological activity. Ammonium presents
aminium concentrations, a minimum concentration for the HRT of 7.5 d, whereas the minimum phosphate is achieved at an HRT of 9.6 d, conditions in which N and P are effectively removed. Larger HRTs imply a smaller microbial population with the consequently less efficient N and P removal. At shorter HRTs, the microorganisms are not capable of removing the larger N and P feeding rates, leading to the observed increase in the influent ammonium and phosphate concentrations. The reported HRT for the maximum N and P removal are in consonance with the HRT for which the maximum COD is removed. This confirms that for an effective treatment, the three basic nutrients (organic carbon, nitrogen and phosphorous) are required and their respective loadings, through the HRT, must be controlled for an appropriate wastewater treatment.

Bearing in mind that the primary aim of MFCs is electricity generation, Figure 5 displays the temporal evolution of the cell voltage (circuit closed with a 100 Ω resistance) and the average stationary cell voltages (collected in the 22nd hour of the daily voltage monitoring) for each HRT. In order to check the system reversibility, the system was returned to the HRT of 7.5 d in the last days of operation. As stated in previous studies, the MFC requires a certain acclimatization time in order to achieve stable cell voltages. From Figure 5a, it can be inferred that, at least, one initial month is necessary in order to acclimatize the electricity-generating microorganisms (EGMs) to the new substrate and conditions in the MFC. Afterwards, subsequent variations in the HRT do not require such a long time, indicating that the EGMs have already adapted to the glycerol substrate and can respond more rapidly to any change in the operating parameters. Regarding the HRT, it can be seen that there exists an optimum HRT for the electricity generation, with a maximum cell voltage at an HRT of 7.5 d. The smaller bacterial population at longer HRTs may be responsible for the lower cell voltage due to the concomitant smaller EGM population. On the other hand, shorter HRTs also diminish the cell voltage. On the basis of the constancy of the microbiological population, the reduced electrochemical performance could be explained in terms of the more favorable development of the non-EGM moiety at larger OLRs. The existence of optimum retention times have been widely observed in the literature and are attributed to the balance of sufficient time for the organic matter degradation and subsequent conversion into electricity, and a suitable amount of substrate feed for the activity and growth of the EGM fraction.

In order to quantify the percentage of substrate transformed into electricity, which can give us an idea of the activity development of the EGM, Figure 6 shows the values of the CE for each HRT. As can be observed, the CE is strongly influenced by the HRT. A drop in the CE is observed as the HRT decrease, indicative that a lower fraction of substrate degradation is utilized by the EGM for the generation of electricity. This result confirms that at shorter HRT more non-EGM (most likely fermentative) proliferate among the overall microbial community, leading to the decay in the CE to minimum values, especially at the two shortest HRTs, where a steeper decay is observed. Long HRTs favor the activity of the EGM in terms of degrading a large percentage of substrate and transforming it into electricity, although the electrochemical performance is low due to the reduced substrate and EGM availability.

The combination of the results displayed in Figures 5b and 6 further evidence the existence of an optimum HRT, trade-off between the substrate availability and the activity and development of the EGM.

**Figure 4.** a) Average effluent ammonium and b) effluent phosphate concentrations for different HRTs.

**Figure 5.** a) Temporal evolution of the cell voltage and b) average cell voltage for different HRTs (1: 24 d, 2: 9.6 d, 3: 7.5 d, 4: 6.4 d and 5: 4.8 d).

**Figure 6.** CE of the MFC for different HRTs.
Further electrochemical results shown in Figure 7 further confirm the existence of an optimum HRT. Figure 7a shows the polarization curves obtained after the closed circuit cell voltage stabilization. As can be observed, the HRT notably impacts on the polarization curves. In accordance with the results presented in Figure 5b, the OCV and, by extension, the entire polarization curve, achieve a maximum for the HRT of 7.5 d. The combination of sufficient organic matter loading with a suitable EGM performance gives rise to the observed best electrochemical performance, with a maximum power density of 38 mW cm\(^{-2}\) (Figure 7b).

To further assist in explaining the polarization curves, Figure 7c displays the anode and cathode potentials. The HRT noticeably impacts the anode performance. The anode potential reduces with the HRT to the minimum values achieved for the HRTs of 7.5 and 6.4 d. This can be explained in terms of the larger OLR and EGM population. At the HRT of 4.8 d, the anode performance is also close to that of 7.5 and 6.4 d. This result is very interesting and shows that, in spite of the reduced CE attributed to the smaller EGM fraction, the exoelectrogenous microorganisms present possess a high intrinsic activity that conducts to the low anode potential even under an intense competition with the fermentative bacteria. The cathode potential curves reveal that at short HRTs, the cathode performance deteriorates, explaining the poorer cell performance of the 6.4 and 4.8 d HRT cell performance. Shorter HRTs might imply higher substrate (and intermediates) concentrations within the anode reservoir, leading to a more prominent crossover of these compounds through the membrane. This phenomenon would depolarize the MFC oxygen reduction performance,\(^{24}\) leading to the observed drop in the cathode performance.

Finally, Figure 7d displays the ohmic drop observed in the MFC, from which the ohmic resistance of the MFC can be estimated (values collected in Table III). As can be seen, the ohmic resistance initially decreases with the shorter HRT until a minimum after which the ohmic resistance again augments. Larger feed volumes imply a larger amount of nutrients, most of them in the form of salts, which contribute to the decrease of the ohmic resistance. However, an excess of salts can indeed result in an increase of the ohmic resistance by the large occupation of the sulfonated groups by cations such as Na\(^+\), NH\(_4\)+, Ca\(^{2+}\) and Mg\(^{2+}\), whose mobility is lower than that of protons.\(^{35}\) Furthermore, a higher nutrient concentration is known the undesired membrane biofouling phenomenon due to the larger growth of microorganisms adhered to the cationic exchange membrane.\(^{36}\)

A final study consisted of the degradation kinetics of the glycerol substrate used and the identification of the metabolites formed. Figure 8a shows the evolution of the glycerol concentration for different HRTs, whereas Figure 8b shows the evolution of the intermediates detected for the HRT that gave rise to the best electrochemical performance (7.5 d). As can be seen, the glycerol degradation kinetics are influenced by the HRT. In overall terms, the shorter the HRT, the more rapidly the glycerol is degraded. A more detailed analysis reveals that, at the two longest HRTs, glycerol is degraded more sluggishly, which can be explained by taking into account the smaller and less active (Figure 3b) microbial population. At the other HRT, the larger SSV firstly favors more rapid glycerol degradation (Figure 3a), more intensely for shorter HRTs, in spite of the relative constancy of the microbial population. This behavior might be explained by the eventual changes in the distribution of microorganisms. At short HRTs, more fermentative bacteria may be present, which, might be more active for degrading glycerol than the EGM.

Figure 8b shows the products detected by HPLC during the kinetics studies (substrate glycerol also plotted as reference). As can be observed, glycerol degrades rapidly, leading to the formation of different products. Formate is the most abundant detected one. The presence of formic acid is an indicative of the presence of Escherichia Coli, whose anaerobic fermentation is known to produce it (which

| HRT/d | Specific resistance/\(\Omega\) m\(^2\) | Ohmic resistance/\(\Omega\) |
|-------|-------------------------------|---------------------|
| 4.8   | 3.36                          | 1.210               |
| 6.4   | 2.47                          | 0.890               |
| 7.5   | 2.41                          | 0.868               |
| 9.6   | 2.86                          | 1.031               |
| 24    | 3.26                          | 1.175               |
Figure 8. a) Glycerol degradation process during a cycle day for the different HRTs and b) products formed during the glycerol degradation at the HRT of 7.5 d.

This study demonstrates the influence of the retention time on the performance of an IFB glycerol-fed MFC. An optimum HRT of 7.5 d. At this value, the EGM acts most effectively, due to the performance of an IFB glycerol-fed MFC. An optimum HRT of 7.5 d. At this value, the EGM acts most effectively, due to the presence of acetate derives from the metabolism of the intermediate acetyl-CoA. This can be further used as a substrate by the microorganisms, leading to the lower concentrations compared to formate observed. Propionate can be an indication of the presence of Propionibacterium, whereas butyrate is an indication of some Clostridium. These compounds are a confirmation of the intense anaerobic activity occurring in the anode compartment. Finally, some traces of glycerate were detected in the first hours of the test, as a possible indication of some Gluconobacter bacteria in the medium. The presence of these species corroborates the complex microbiota (mixed culture) present in the anode of the complex microbiota used.

Conclusions

This study demonstrates the influence of the retention time on the performance of an IFB glycerol-fed MFC. An optimum HRT of 7.5 d has been found for effective removal of COD, ammonium and phosphate. Longer HRTs suppose a poor substrate loading which diminishes the microbial population, whereas shorter ones do not allow the microorganisms effectively degrade the nutrients. The HRTs also impact on the electrochemical performance of the MFC, with the best electrochemical performance (maximum closed circuit cell voltage, power output, anode and cathode potential) obtained for the same HRT of 7.5 d. At this value, the EGM acts most effectively, due to a sufficient amount of substrate fed combined with the largest activity of the EGM, in addition to a not excessive fraction of fermentative microorganisms. Finally, the monitoring of the glycerol degradation process has evidenced the larger activity of the microbes at short HRT and the formation of several derivatives of short-chain organic acid as intermediate of this process, in special formate. The variety and complexity of the products matches with the mixed nature of the microbiological culture used.

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