Domestic wastewater hydrolysis and lipolysis during start-up in anaerobic digesters and microbial fuels cells at moderate temperatures

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ABSTRACT

Raw wastewater hydrolysis rates during start up in microbial fuel cells (MFC) and anaerobic digestion (AD) systems, seeded with a mesophilic inoculum from a digester, were compared at moderate temperatures (27.5℃ and 8℃). Temperature drop affected both the lipids and carbohydrates hydrolysis rates but not necessarily the protein removal rates (temperature-independent rates for MFC), which were significantly influenced from treatment alteration (AD to MFC). MFC showed robust proteolysis at low temperature compared to AD, the second seems to have a higher potential at warmer conditions. A lipases activity assay showed that although at 27.5℃ both AD and MFC are likely to hydrolyse lipids, the latter has a higher lipolysis potential at low temperatures. Preliminary community structure analysis showed that the switch from AD to MFC alters the bacterial community by 15% with the MFC showing higher diversification; temperature decrease though alters the community by 40%. Key organisms that appear to be favoured at the MFC setups are *Geobacteriaceae*, taxa likely related to the hydrolytic capacity of this setup.

KEYWORDS: Cold-adapted; Hydrolysis; Lipolysis; Low temperature wastewater treatment; Microbial Fuel Cells;

1. Introduction

Over the past century, energy generation from fossil fuels has been the predominant path for economic growth and industrialization (Rifkin, 2002) . Increase in energy demand and intensive release of carbon dioxide through rapid urban development have rendered the conventional wastewater treatment processes (mainly aerobic) unsustainable. Thus, carbon neutral/positive wastewater treatment is a crucial environmental challenge that needs to be addressed (Logan, 2008). As water becomes a more economically valuable resource, sustainable wastewater treatment plays a vital role in the water cycle and is essential for the preservation of life as it can be an additional resource for water supply.

Compared to conventional, energy-intensive biological wastewater treatment, anaerobic treatment provides with numerous benefits (e.g. less energy requirement, methane generation, less biological sludge production) (Malina and Pohland, 1992; Van Haandel and Lettinga, 1994) . Classical Anaerobic Digestion (AD) is based on the bioconversion of organic substrates to biogas (Pham et al., 2006) . The metabolic pathway in AD is a three-stage process consisting of hydrolysis, acidogenesis, and methanogenesis. These steps account for the reduction of carbon from complex organic matter into methane and carbon dioxide.

Microbial Fuel Cells (MFC) is a recently developed bio-electrochemical process that generates electricity by means of anaerobic oxidation of organic matter using microorganisms (Ahn and Logan, 2010) . The substrate degradation stages are similar to AD. However, volatile fatty acids (VFAs) that are produced by fermentation can be consumed by respiring bacteria resulting in a transfer of electrons to the MFC anode, and ultimately in inhibition of methanogenesis (Velasquez-Orta et al., 2011) to promote H_2 production and to render oxygen the ultimate electron acceptor.

The temperature in anaerobic treatment systems is the '*Achilles heel*' as it adversely affects substrate utilization rates, specific growth rates and biogas production rates (Kettunen and Rintala, 1997; Lawrence and McCarty, 1969; Sanz and Fdz-Polanco, 1990). Most anaerobic systems operate within the mesophilic temperature range with an optimum around 35-40℃ (Bohn et al., 2007). As in AD, the performance of MFC is likely dependent on temperature. The potentially larger microbial diversity in MFCs (non-electro-active, facultative organisms) though increases the likelihood of higher tolerance to low-temperature compared to AD. Larrosa-Guerrero et al., (2010) showed that MFC is a promising treatment at cold conditions but could not show what the advancement to hydrolysis was (the common rate limiting step at low temperatures). This was most likely attributed to the highly biodegradable fraction of the substrate (brewery) that was used in that study. The potential of such electrogenic systems to treat wastewater at a larger scale, at low temperatures, was firstly showed by Cotterill et al., (2017) using an MEC (Microbial Electrolysis Cells) setup. This MEC study, as well as all the above fuel cells-focused' ones could not clearly quantify and/or explain how and at what degree hydrolysis can be realized, the most common limitation for most systems operating at low temperature.

Hydrolysis is the initial degradation step where complex organic material is converted to simpler molecules (Velasquez-Orta et al. 2011). Wastewater rich in fat and protein content may result in limited treatment (Petropoulos et al., 2018; Petropoulos et al., 2019; Cammarota and Freire, 2006) as these compounds are less readily to be degraded compared to carbohydrates (Miron et al., 2000; Vidal et al., 2000).

A plethora of previous research studies have investigated the effect of temperature on the performance of MFC and AD (Ahn and Logan 2010; Velasquez-Orta et al. 2011), as well as the improvement of hydrolysis of rich in fat wastewater (Leal et al. 2002; Mobarak-Qamsari et al. 2012; Vidal et al. 2000) . Nevertheless, there is no comprehensive evaluation and comparison of the hydrolysis rates of lipids, proteins and carbohydrates of domestic wastewater between MFC and AD treatment systems operating at warm and cold temperatures. The importance of adequate hydrolysis during the start-up is of high importance, especially in projects that operate at low temperatures. The reason is that adaptation to temperature requires time and accumulation of un-hydrolysed material during acclimation is inevitable. Once acclimation is achieved fermentation and acidification is likely and threatening, especially in the case of slower adaptation rates of the cells participating in the later steps of biodegradation.

This study, that was carried out at the laboratories of the Environmental Eng. (Dept. of Engineering at Newcastle University, UK - finalized on 2018), compares the performance of hydrolysis of wastewater-originated lipids, proteins and carbohydrates, at low temperatures (8-27.5ºC), during the start-up of MFC and AD systems. It also preliminary describes how the microbial community structure in the two treatments and temperatures alters, and finally evaluates the kinetics of the lipases activity after the observation of the high lipid removal potential; lipids that account for the major hurdle at low temperature treatment (Petropoulos et al., 2018; Petropoulos et al., 2019).

2. Materials and Methods

Experimental set-up: The experiment was conducted to compare the hydrolysis performance between MFC and a conventional AD reactor at two different temperatures (27.5℃ and 8℃). Eight duplicate reactors were set up and run in parallel, incubated accordingly to the selected operational temperatures (i.e. two MFC and two AD reactors in a 27.5℃ incubator and two MFC and two AD reactors at 8℃).

The selected substrate was raw domestic wastewater (after screening) from Spennymoor wastewater treatment plant (Tudhoe Mill, Durham, UK). The inoculum had a mesophilic origin (anaerobic sludge from Bran Sands, Advanced Anaerobic Digestion Facility, Northumbrian Water Ltd (Teesside, UK)).

Reactor configuration: To retain a similar setup between MFC and AD a cation exchange membrane was introduced (Nafion 117, with an area of 12.6 cm^2) to separate all eight reactors into two chambers (operational & non (for the MFC and AD respectively)). The total volume of each chamber was 78 ml, with a length of 6 cm and a 4cm internal diameter. The material used for the anode and cathode was carbon fibre felt (2.7 cm × 2.7 cm × 1.2cm) with a surface of 21.5 cm^2 and a 2.5 cm^2 titanium, platinum coated mesh; the cathode surface area was 8.13 cm^2. Stainless steel wires were attached to both electrodes connected to a circuit with a 470Ω resistor (for the MFC reactors, no resistors were connected to the AD reactors).

Substrate sterilization: UV sterilization was introduced to eliminate any bacterial interference between the autochthonous cells from wastewater and those inhabiting the seed. A UV lamp (Hozelock Vorton, UK 11W) was selected for the sterilization of wastewater. Sterilization was carried out as per Petropoulos et al. (2017) and the efficiency was measured using agar plates containing nutrient medium (APHA 1995) . The experimental set-up included 6 feeding cycles with raw wastewater at HRT of 5 days; the substrate´s average COD was 505.4±44.4 with a peak at 675.5 and a low at 354.0 mg/L.

Reactor set up: Nitrogen gas was sparged in both sterile media and inoculum (99.98%) until Dissolved oxygen (DO) reached close to 0.0 mg L^{-1}. 30 ml of sparged inoculum (anaerobic sludge) was applied to the anode chamber in addition to 25 ml of sterile media (raw influent). The reactor was further sparged with N_2 for 2 minutes forming a headspace of 15ml. The MFC cathode chamber was filled with 50mM pH 7-phosphate buffer (autoclaved 15 mins at 121℃); for the AD, the corresponding chamber was filled with distilled water. The cathode compartments of all treatments' (including AD reactors) were left exposed to ambient air. The feeding strategy included the removal of the reactor´s supernatant liquid phase after settling, using a sterile syringe (VWR, UK) through a sampling port which was kept sealed over the operation. The sampling port was designed away from the anode to minimize the likelihood of disturbance of the communities developed on it. After preparation four out of eight reactors (two for each reactor type) were placed in an incubator (Stuart Scientific SI 50, UK) at 27.5℃, the remaining four were set at 8℃ (Sanyo MIR-254, USA incubator).

Voltage monitoring and biogas collection: The voltage produced from the four MFC was quantified using a data logger. The negative and positive leads of the multimeter were attached to the anode and cathode wires respectively. The cell voltage was recorded via Pico-log recording software for data acquisition, measurements were taken after every half an hour. The generated biogas of the anode compartment was measured on the basis of liquid displacement using a glass tube (vol: 12ml), sealed with rubber septa (for sampling purposes).

Lipid test: Total lipids were estimated with the (Bligh and Dyer 1959) method using a solution of methanol and chloroform (1:1 (v/v) of $CHCl_3$: MeOH). The sample (4 ml) after mixing and homogenization was separated into two layers of chloroform (with the dissolved lipids) and methanol. Further centrifugation in IEC table-top centrifuge at 1000 rounds per minute, at a 5 minute cycle, at ambient temperature assisted in a better

visualization of the two-phases with no particulate material in suspension. The bottom phase was recovered using a 1ml pipette that was gently inserted through the upper face. The lipid content was estimated by weighing the residual after a 3day chloroform evaporation in a fume cabinet.

Carbohydrate analysis: The total carbohydrate content was estimated via the anthrone method first described by (Hedje and Hofreitter, 1962). A solution of 75% H_2SO_4 solution, anthrone reagent and glucose standards were prepared and stored at 5℃. In detail, a standard glucose-based curve was prepared after serial dilutions of a 10% stock of 100 mg glucose dissolved in 100 ml de-ionized H_2O. Additionally, 0.5g of dry anthrone dissolved in 250 ml of H_2SO_4 (75%). Afterwards, chilled 75% H_2SO_4 (2 ml) was added into digestion tubes having 1ml of either diluted samples or standards. The tubes were then capped and vortexed, 4 ml of chilled anthrone solution was then added. After preparation, the tubes were positioned at a heating block for digestion at 100℃ for 15 min. The carbohydrates' content was quantified at a wavelength of 578 nm (Merck, UK, spectrophotometer).

Protein analysis: For the protein content measurement, a Protein Quantification Kit-Rapid was selected (Sigma Aldrich, UK) operating based on the interaction between Coomassie Brilliant Blue (G-250) and protein content. The maximum absorbance changes according to this interaction and is detected at 595 nm (Bradford 1976) . Specified standard protein concentrations of Bovine Serum Albumin (BSA) were prepared via serial dilutions of BSA (stock of 4000μg/ml, Sigma Aldrich, UK) with de-ionized water. A sample of 0.1 ml of BSA solution was pipetted to glass, 5 ml of CBB solution was then added, mixed for satisfactory interaction and left to rest for 2 minutes. The final solution was then transferred to semi-micro disposable cuvette (VWR, UK) and tested at 595 nm using spectrophotometer (Merck, UK).

Hydrolysis rate: The rates were calculated using the *Eq*.1 shown below; the conversion to COD was based on (Sanders et al. 1996):

$$Hydrolysis\ rate\ (mgCOD/L.d) = \frac{C_a - C_b}{b - a} \qquad Eq.1$$

Where：

C_a (mg COD L^{-1}) = substrate conc. in the mixed liquor (day 'a');

C_b (mg COD L^{-1}) = substrate conc. in the mixed liquor (day 'b').

Statistical analysis of the rates was carried out by Minitab 16 (Minitab, USA) statistical package, using ANOVA and Nested ANOVA (rates of equal variance) to compare hydrolysis efficiencies between same or different temperature/treatment.

The statistical test for the proteins, lipids and carbohydrates were carried out at the last days of each batch (for the 6^{th} batch this was carried out on day 31).

Gas chromatography: Gaseous CO_2 was measured using a Thermo scientific Trace GC Ultra gas chromatographer. The sample was taken from the glass tube headspace and transferred into a 3.0ml exetainer using a 5.0 ml gas tight syringe (SGE Analytical Science, Australia). Gas sample of 100 μl was injected to the GC injector port. Serial injections of standard gas (50% CO_2) were employed for the implementation of the standard curve.

The gaseous methane (CH_4) was also measured via gas chromatography; specifically, samples of 100 μl were abstracted from the head space using a gas tight syringe (SGE-Europe) and then they were injected to a Carlo Erba HRGC S160 GC-FID (HP-PLOTQ column).

Carbon dioxide and methane dissolved in the liquid phase were also taken into consideration as they were thermodynamically estimated using Henry coefficients (as per Dolfing and Janssen 1994).

Enzyme extraction: Lipase extraction was carried out according to (Gessesse et al. 2003) using 10 ml of complete mixed sample (wastewater and anaerobic sludge). The pH was adjusted to 8.0 using Trizma (Tris HCl) buffer (Sigma Aldrich, UK) achieving a final concentration of 10mM. Triton X-100 and EDTA solution was also added at the optimum concentration suggested (0.5 and 10 mM respectively). The sample was then

sonicated (Labsonic sonicator) with 13 kHz frequency for 30 min, with 2 min bursts followed by 5 min rest. During the rest time the samples were placed on ice. After sonication the samples were centrifuged at 4ºC for 15 min at 4200 RPM. The extracted enzymes were abstracted from the supernatant. The assay was carried out on the final experimental day (Day 34; 3 days after the 5 days HRT).

Lipase assay: *P*-Nitrophenyl palmitate (pNPP) (Sigma Aldrich, UK) was employed as assay substrate. 20 mM of stock were prepared with Isopropanol (Sigma Aldrich, UK). The solution was further diluted 1:20 in 20 mM Trizma (Tris HCl) Buffer (Sigma Aldrich, UK) containing 0.1% Gum Arabic and 0.4% Triton X-100 (Sigma Aldrich, UK). Mixture of 0.1 ml extracted enzyme and 0.9 ml of substrate was set at various temperatures (8℃, 27.5℃ and 37℃). The assay absorbance was measured at 410 nm at 30-40 min intervals via spectrophotometry (Merck, UK). The results were expressed in Units (U) of enzyme activity (lipases); 1U corresponds to the amount of enzyme that is required to catalyse 1 μmol of *para*-nitrophenyl per minute.

VFA analysis: Sterile syringes were used to get the samples from the liquid phase of the operating reactor. The samples were transferred to the micro-centrifuged tubes (2mL), centrifuged at 13000 g for 3 minute and supernatant was decanted for the analysis. The VFA were measured by ion exchange based modified standard method (Manning and Bewsher, 1997). The resulting samples were analyzed on a DIONEX ICS-1000 equipped with an Ionpac ICE-AS1, 4x250mm column using a 1.0 mM heptafluorobutyric acid eluent solution.

Molecular analysis: Denaturing gradient gel electrophoresis (DGGE) was employed to analyse the microbial community structure. Samples of biomass obtained from the pellets formed from the VFA preparation for the AD treatments. For the MFC trials the samples were abstracted from the anode after scratching it with a pre-autoclaved (121ºC for 15 minutes) scalpel. The remaining biomass from the electrode was resuspended in PBS. The scratched and resuspended samples were then combined in a 2ml eppendorf and centrifuged (3 min at 13,000 × *g*) to form the biomass pellet used for further analysis.

The DNA from the reactors' samples was extracted via FastDNA® SPIN for soil kit (Q-BIOgene, Cambridge, UK) following the method of supplier. Polymerase chain reaction (PCR) was used to generate amplicons prior DGGE. Primer2 and Primer3 were used for PCR (Muyzer et al., 1993). PCR products were analysed by DGGE on a D-gene DGGE system (Bio-Rad, Hercules, CA, US) using polyacrylamide gel (10%) containing a denaturant gradient of 30-60% (denaturant: 7 M urea + 40% $^{v}/_{v}$ formamide in TAE buffer). SYBR gold (Sigma, Poole, UK) stain was used to stain the gel that was finally viewed under UV using Bio-Rad Fluor-S ® Multi Imager (Bio-Rad, UK).

The microbial community diversity developed at different treatments and temperatures was analysed as per the band patterns obtained from DGGE after processing the final gel image using Bionumerics (Applied Maths, Austin, Texas, US). After band matching through the specific software the image was quantified and re-examined in Prime6 (Multivariate statistics, Lutton, UK). The data were transformed by 'presence or absence', the similarities between conditions were examined using the 'Bray-Curtis' similarity test. The outcomes were displayed as dendrogram and MDS plots.

Selected DNA bands were excised from the bacterial gel, amplified, purified and sequenced as per (Petropoulos et al., 2017) . Sequences were compared to the GenBank database using BLAST (Benson et al., 2008).

3. Results and Discussion

3.1. *Hydrolysis of particulate organic compounds*

The wastewater was characterized relatively rich in fats with average concentrations of 294.2±48.6, 110.6±10.7 and 29.7±6.2 mg COD L^{-1} for lipids, proteins and carbohydrates respectively; the average total COD of the substrate was 505.4±44.4. For both treatment systems and temperatures, the highest hydrolytic efficiency in terms of rates was achieved on the 6th cycle (Day 26-31; Figure 1; Figure 2), expected considering that time promotes microbial adaptation. The evolution of the rates for each individual hydrolysis process are given at Figure S1 (supplementary material); the same image shows a plateau in the rates achieved within 3 consecutive batches for the proteins and lipids (compounds that were in abundance and most challenging to be degraded).

Comparisons at the maximum rates achieved showed that at high temperature (27.5℃) lipids hydrolysis (Figure 1a) occurs negligibly (p: 0.311) faster at AD compared to at MFC, the difference in hydrolysis rates was equally

negligible for the carbohydrates (p: 0.311), (Figure 1c). At low temperature, the phenomenon becomes reversed giving an insignificant advantage to the MFC (P = 0.859 and 0.22 for lipids and carbohydrates respectively). Protein lysis (Figure 1b) clearly benefited from the AD at a high temperature (p = 0.005) whilst at low MFC hydrolysed proteins faster (p = 0.046) (Figure 1b). This is a promising result since low-temperature hydrolysis is the '*Achilles heel*' at such systems and un-hydrolysed matter tends to accumulate (Petropoulos et al., 2019).

According to the hydrolysis rates of lipids, proteins and carbohydrates, the last was surprisingly the slowest to be hydrolysed for both setups whereas lipids achieved the highest hydrolysis rate. This can be explained by Monod kinetics that manifest that degradation rate (μ_{max}) is related to the substrate concentration (k_s), thus, the increased lipids concentration in the influent assisted in the achievement of high rates. This also supports that the developed community has a proven ability to degrade lipids efficiently and effectively, even at low temperatures (8ºC). Such a scenario was expected considering the origin of the biomass, a mesophilic digester that treats a mixture of industrial/trade wastewaters containing fats, combined with municipal wastewater.

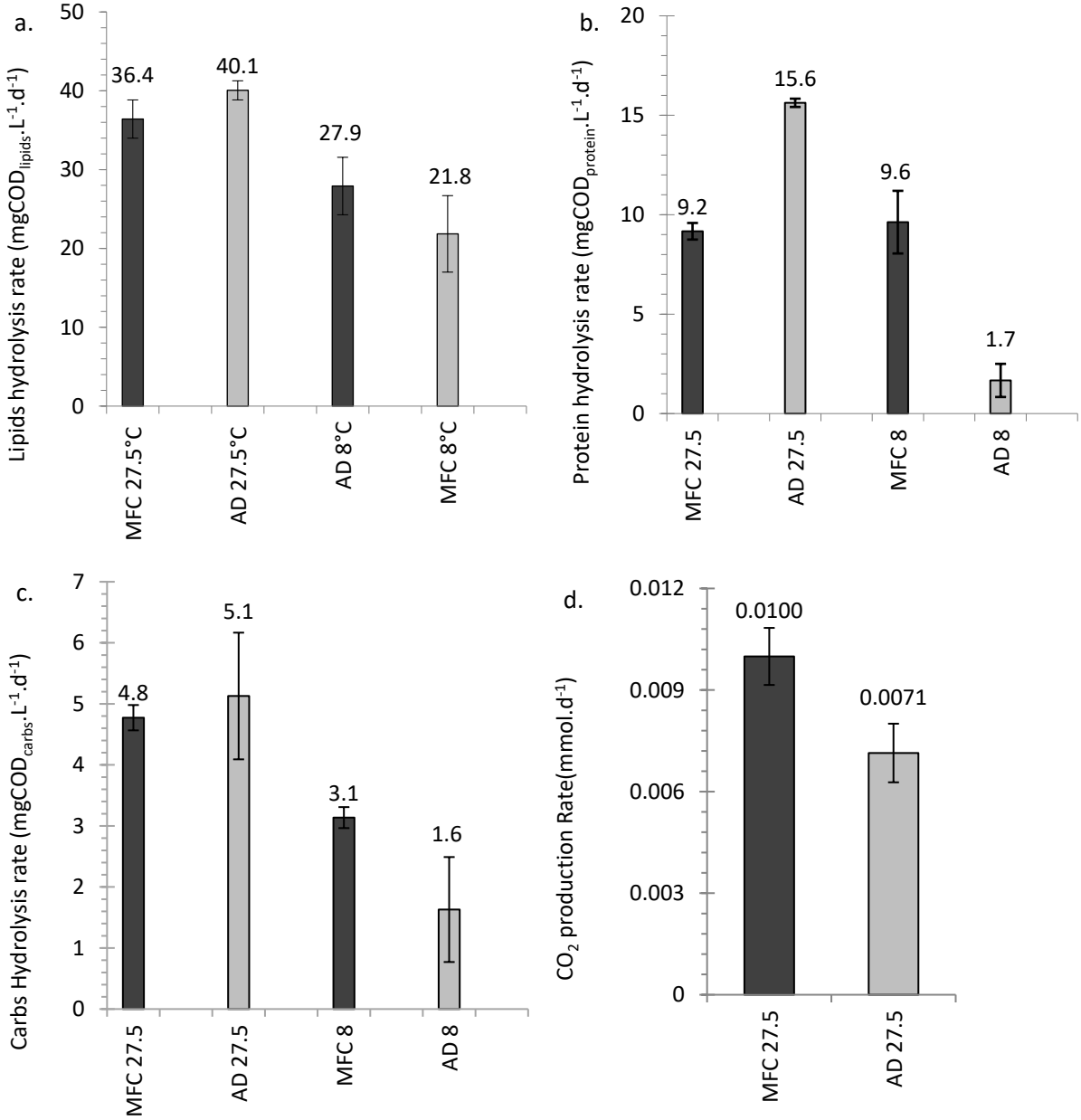

Figure 1 – Hydrolysis rates of a) lipids per treatment and temperature, b) proteins per treatment and temperature, c) carbohydrates per treatment per temperature; d) CO_2 production rate per treatment at 27,5ºC; all results presented were estimated on the 6th cycle where the highest performance achieved, 27.5 and 8 stands for to 27.5 and 8ºC.

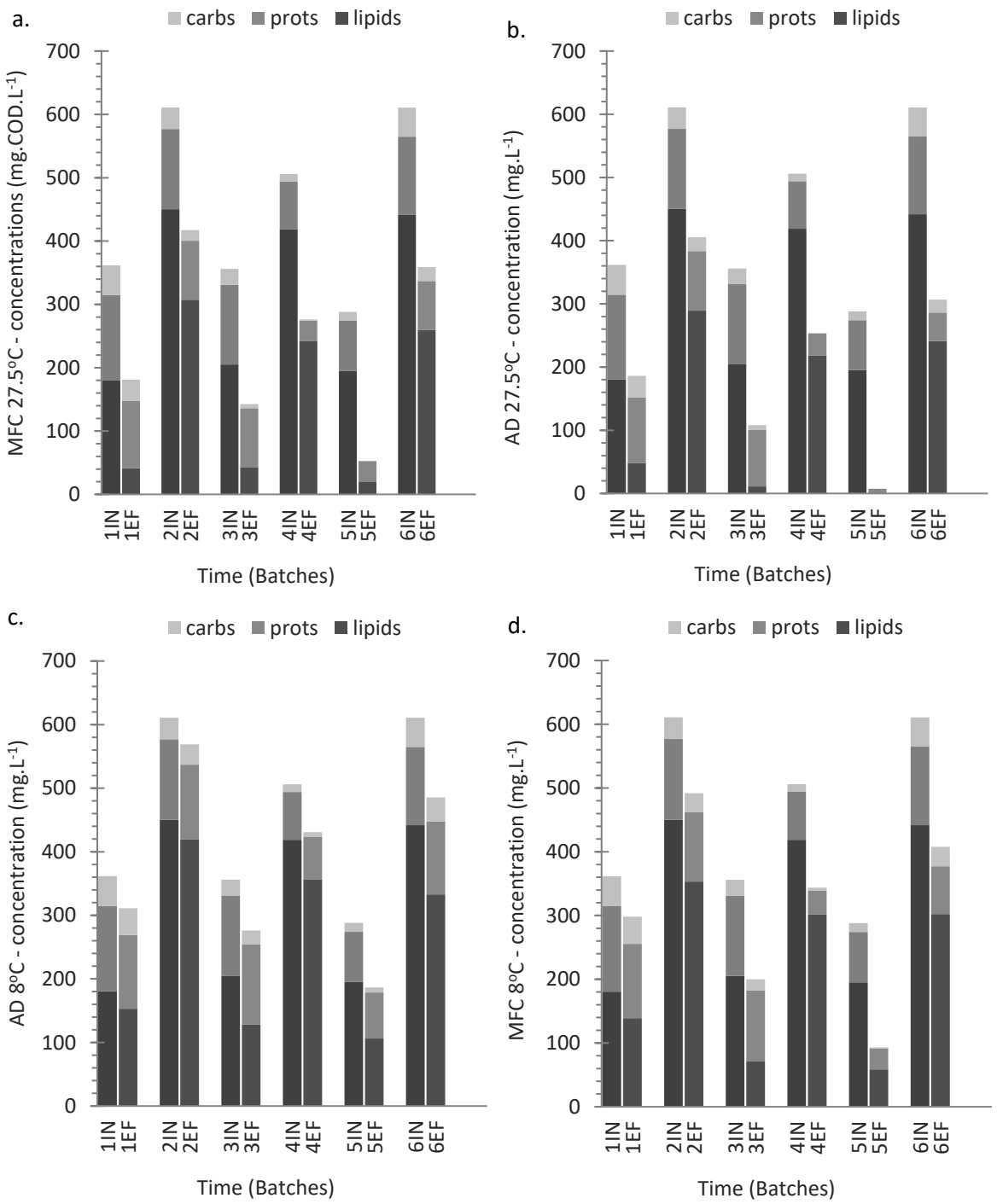

Figure 2 – concentration of lipids', proteins', and carbohydrates' COD in the influent and effluent of the reactors trialled, a) MFC at 27.5ºC; b) AD at 27.5ºC; c) AD at 8ºC; and d) MFC at 8ºC; the numbers at the X-axis stand for the consecutive batches, the IN and EF stand for the influent and effluent at each batch.

Table 1 – Average hydrolysis efficiency achieved for each of particulate matter (lipids, proteins, carbohydrates)

Treatment	MFC 27.5ºC	AD 27.5ºC	MFC 8ºC	AD 8ºC
Lipids (mg COD.L^{-1})	51.7±7.4%	57.3±11.4%	35.2±15.2%	20.9±13.2%
Proteins (mg COD.L^{-1})	34.6±3.2%	43.4±8.6%	27.5±6.3%	7.5±2.4%
Carbohydrates (mg COD.L^{-1})	51.6±2.0%	52.8±2.2%	28.4±1.8%	16.3±0.9%

3.2. *Lipid hydrolysis*

Average lipolysis efficiency was measured the highest for $AD_{27.5°C}$, and $MFC_{8°C}$ at warm and cold temperature respectively compared to $MFC_{27.5°C}$ and $AD_{8°C}$ (as per the difference between the lipids' COD in and out at all batches) (Figure 1a, 2, Supplementary S1a). As expected, temperature drop significantly affected the overall lipid removal rates (Figure 1a, 2, Supplementary S1a) in both systems (Nested ANOVA for both systems p = 0.05). The difference though was not that significant for the MFC where the rates of removal were only little or almost unaffected by temperature decrease. Removal of lipids was evident at all batches with the rates reaching the maximum on the last batch (Figure 2, Supplementary S1b) at all temperatures and treatments. Throughout operation it was the AD27.5℃, and MFC8℃ that consistently achieved the highest rates at all batches at 27.5 and 8ºC respectively after batch 1.

Regardless the reduced by temperature rates, both systems had an exceptional lipid hydrolysis capacity (Figure 2, Table 1 (average lipolysis efficiency achieved)) during this start-up. This is of major importance considering that numerous studies identified lipid hydrolysis as the rate-limiting step (Miron et al., 2000; Petropoulos et al., 2018, Petropoulos et al., 2019; Petruy and Lettinga, 1997 and other).

Lipid hydrolysis at 8ºC is an important observation knowing that fats due to hydrophobicity attach onto the surface of the anaerobic sludge, hindering the transport of soluble substrates to the biomass, decreasing the degradation rate (Rinzema et al. 1993). This phenomenon often leads to limited treatment and failure, event that could be then prohibited in MFC treatment setups operating at low temperatures.

3.3. *Protein hydrolysis*

The highest protein hydrolysis rates were also observed on the 6th cycle (Figure 1b, 2, Supplementary S1b). The average protein removal efficiency as per the feed batches at all treatments/temperatures is showed on Table 1; this highlights that MFC possesses a distinctly better capacity to operate at lower temperature over AD, with hydrolysis remaining unaffected by temperature as per the comparison from the removal rates achieved for this treatment (Figure 1b, p: 0.318; $MFC_{27.5C}$-MFC_{8C}). This supports that the bacteria developing at MFC may excrete/secrete proteases with a wider optimum temperature range than those formed from the communities developed at AD.

The phenomenon was reversed at higher temperatures with AD lysing proteins faster than MFC (p = 0.029; $AD_{27.5C}$-AD_{8C}). This suggests that proteolysis was accelerated in MFC compared to AD at low temperatures and vice versa at higher ones.

Overall, protein removal rates at higher temperature started increasing after batch 3 for both treatments; at lower ones, the MFC also accelerated at the same batch, the AD_{8oC} rates though remained consistently low (Figure 2).

In municipal wastewater the protein content is usually low; thus, the impact of MFC to the quality of treatment may become more significant at industrial wastewater streams that may contain high concentrations of proteins; especially after knowing that protein hydrolysis can be rate-limiting (Pavlostathis and Giraldo-Gomez 1991).

3.4. *Carbohydrates hydrolysis*

Carbohydrates: As for lipids and proteins, at the 6th batch hydrolysis reached the maximum in terms of carbohydrates degradation rates (Figure 1c; 2 Supplementary S1c). The average efficiency achieved is presented on Table 1; once again MFC performed hydrolysis similarly with AD at warm conditions whilst at cold MFC outcompeted AD. In terms of the maximum achieved rates, no significant differences was observed between the two systems at the same temperatures (P = 0.776; AD-$MFC_{27.5C}$, AD-MFC_{8C}). The ease in carbohydrates degradation (Pavlostathis and Giraldo-Gomez 1991) combined with the low initial concentrations explain why low rates were realized at all treatment and temperatures (< 5.1 mg $COD.L^{-1}.d^{-1}$) . As expected, nested ANOVA showed that temperature significantly affects the carbohydrate hydrolysis (P = 0.010) at both systems. Overall, carbohydrates' hydrolysis rates started accelerating at the last 2 batches at warm conditions; at cold the rates started increasing immediately, however, the improvement over time was slow compared to the increase achieved at warmer conditions.

3.5. *Gas formation during operation*

No gas was produced from both MFC and AD reactors at 8°C. It is likely that low temperature shocked both the electrogenic and methanogenic populations, hindering gas production at both systems considering the mesophilic origin of the seed. Another scenario is that gas remained dissolved in the aqueous phase and equilibrium between phases was not achieved, although assumed. Presence of dissolved gases, higher than what is originally expected by Henry coefficients in the liquid phase has been previously observed (Smith et al. 2013). Hence, bioconversion for both systems was terminated during fermentation, before gasification, fact that is supported from the accumulation of the VFAs in the mixed liquor (193.2 and 75.3 mg VFA.L^{-1} in MFC and AD at 8ºC respectively). The presence of VFA at 8°C as fermentation products proves the occurrence hydrolysis.

At 27.5ºC for AD, the produced CH_4 was the highest on the 6th batch with biogas content of high purity (90% CH_4 over CH_4-CO_2) (Figure 3 a, b). This amount corresponded to 42% of the theoretically expected methane from the COD added ($0.35L_{CH4}.gCOD_{removed}$). The rest of the organic matter is likely to have reacted with SO_4 (measured in the influent as 95 mg $SO_4.L^{-1}$) in the presence of sulphate reducing bacteria (SRB), a reaction that is thermodynamically more favourable than methanogenesis. Traces of methane also appeared at the MFC, especially during the start-up, but the produced volume declined over time (Figure 3b). This is expected considering the origin of the inoculum employed (digester). Velasquez-Orta et al. (2011) also showed this emerging competition for VFA between methanogenic archaea, secondary fermenters and respiring bacteria in MFCs, fact that could also explain the slightly higher CO_2 production rate achieved at the MFC compared to the AD (Figure 1d, 3a).

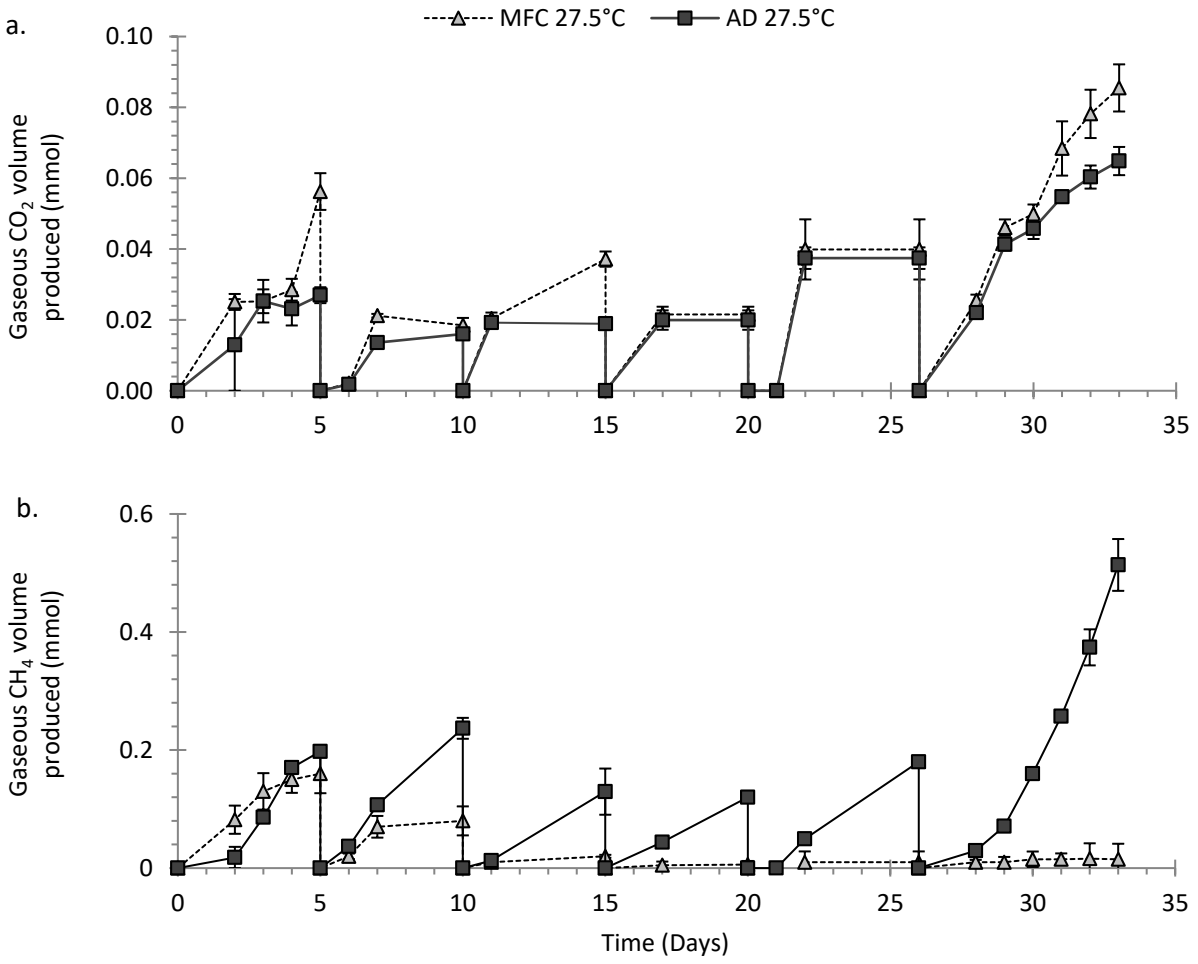

Figure 3 – Volume of the gases a) CO_2 and b) CH_4 formed in both MFC and AD at 27,5ºC; no gases were observed at 8ºC from all the samples taken from the headspace; error bars stand for standard error (n=3).

3.6. *Lipolysis Activity*:

The high lipolysis performance (Figure 1a) was further investigated to understand and quantify the capacity of lipolysis of this biomass knowing how challenging lipolysis at these temperatures is (Petropoulos et al., 2018; Petropoulos et al., 2019).

Between the MFC and AD, negligible difference was observed in terms of the lipolysis activity at 27.5ºC ($p = 0.85$); the systems at 8ºC though seemed to perform differently with the MFC being able to excrete significantly more active enzymes than AD ($P = 0.0025$) (Figure 4a). This comes to a disagreement with the lipid hydrolysis rates (Figure 1a) where lipolysis was not affected by treatment selection. This disagreement may be the result of the high error bars in the rates that led to precision biases. A different scenario is that lipases may have high specificity for specific fats and the assay's substrate (p-nitrophenyl palmitate) could have been a representative one. All in all, this observation shows that MFC forms communities that can promote lipid hydrolysis at low temperatures.

When the activity was measured at 37ºC the corresponding performance pattern remained the same. MFC setups could promote the development of communities that secrete enzymes that lipolyze wastewater-originated lipids faster than the AD communities when both operating at low temperatures. The phenomenon becomes reversed at higher temperatures where the enzymes secreted from AD cells perform negligibly higher.

The increase of lipolysis capacity at 37ºC suggests that the bacterial optimum is higher than 27.5ºC highlighting the mesophilic origin of the seed. The fact that the samples at 8ºC do not perform equally to those at 27.5ºC when at 37ºC shows that part of the bacterial population was shocked from temperature decrease and that the number of lipases secreted per cell is either not equal or not as active as those generated at higher temperature.

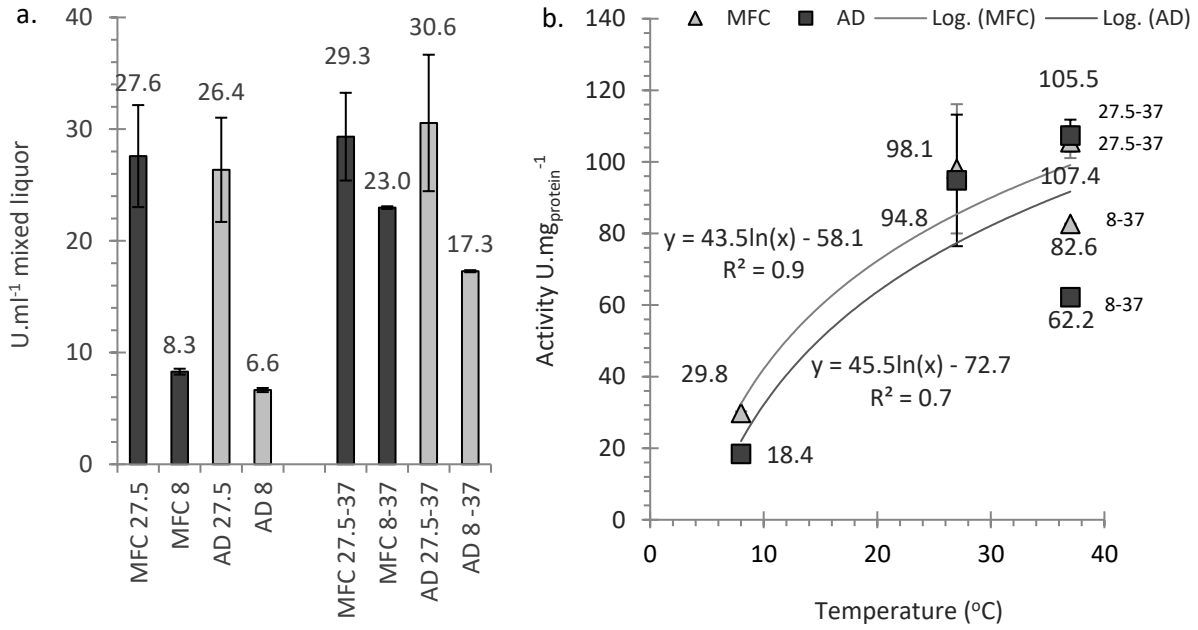

Figure 4 – Lipolysis activity at different conditions (temperature, treatment); a) lipolysis activity of enzymes for both systems and temperature (operational temperature on the left and at 37ºC on the right); b) Lipolysis activity per protein content at different temperature (8, 27.5, 37ºC).

The activity per mass of proteins (Figure 4b) also demonstrated that the lipases secreted by the MFC cells can be more active at lower temperature than those produced from AD cells. At higher temperatures, high error bars do not allow us to confidently conclude which setup performs better. In the overall, the achieved protein specific lipolysis rates are higher than those previously reported in literature (i.e. Petropoulos et al., 2018). This can be a bias resulted by the fact that not all proteins are lipases and that different lipases:proteins ratios may lead in differences between studies. Hence, the protein specific lipolysis activity from this study can only be a qualitative finding. This is enough though to support the trend between the two treatments employed (MFC and AD) since the same inoculum and feed was used, both incubated at the same temperature conditions.

Finally, the lipolysis pattern for both systems agrees with the 'activity-temperature' exponential trend indicating that for every 10ºC raise the activity doubles (Burgess and Pletschke 2008) .

3.7. *Voltage output*:

The voltage of the MFC system was recorded from Day 2 to Day 34. Figure 5a, b shows the two representative cycles at D2-5 when the start-up took place and the 1st voltage traces appeared at 27.5ºC (within 3 days), and D26-34 where the first signs of voltage at 8ºC appeared.

The enrichment stage lasted 2 days at 27.5℃ obtaining a voltage of 20mV (Figure 5a). After acclimation, the voltage reached up to 72.74 mV. Until batch 5 the voltage for MFC was fluctuating between 25-75 mV. The fluctuations could have been related to insufficiency of readily available carbon in the substrate. The maximum voltage production appeared on Cycle 6 (Figure 5a, b) with 7-day voltage stability (peak at 113.74 mV). Unfortunately, its replicate could not operate similarly in terms of voltage (constantly lower value of approximately 20mV); however, it also showed clear signs of stability.

Stable voltage was also produced in one of the MFCs operating at 8℃ after 30 days of operation ranging between 14-18 mV (Figure 5b) but only for one replicate. The inconsistency could have occurred due to several factors, e.g. un-favourability and lack of acclimation of the seed to temperature and substrate, which is likely due to the short exposure time. The sign of stability after that prolonged period, even to one of the two replicates, suggests that after long acclimation periods a functional operation can be achieved. Such behaviour was also observed by (Larrosa-Guerrero et al., 2010) who demonstrated the possibility of electrogenesis at low temperature stating that is likely in such systems the microorganisms generating electricity can potentially be more tolerant to low temperature compared to others developed at different treatment systems. Velasquez-Orta et al., (2011) managed to produce adequate voltage at low temperatures operation within 8 days, whilst Heidrich, (2012) in a similar study at 7.5℃ showed that acetate-fed reactors inoculated with wastewater cannot produce any electricity any earlier than 34 days. This highlights the importance of the 'correct' for the needs inoculation to accelerate treatment (i.e. in the case of low temperature operation the introduction of a cold-adapted arctic inoculum could accelerate the process). The use of cold-adapted inocula as alternative seeding strategy has been recently investigated by Petropoulos et al., (2017).

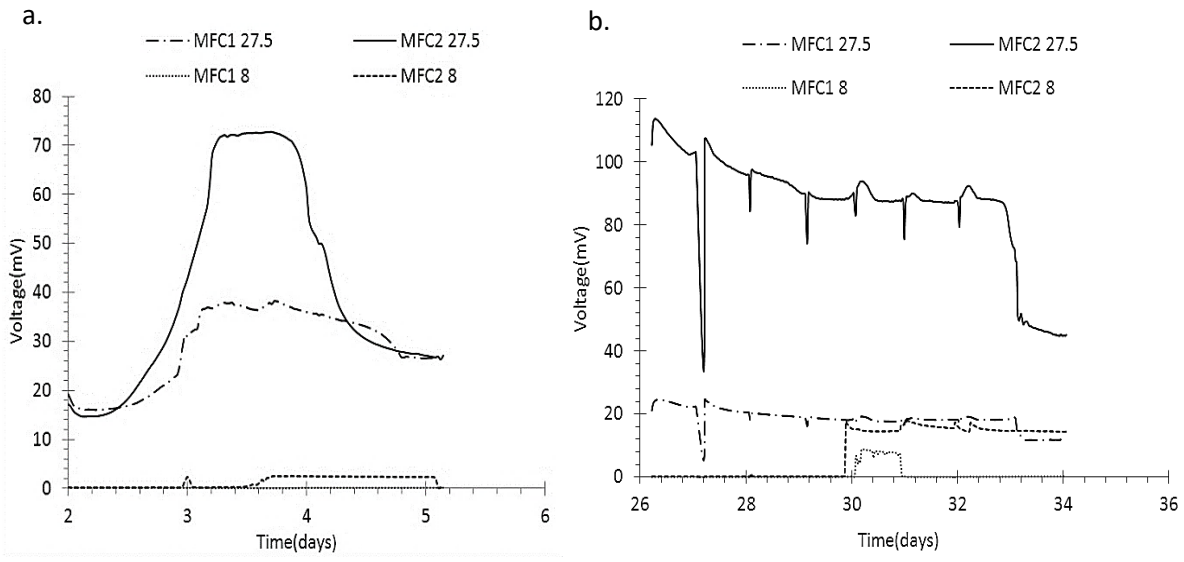

Figure 5 - a) MFC reactor voltage at 27.5 and 8ºC for 1st cycle (Day 2-5); similarly, for the 6th cycle (Day 26-34)

3.8. *Phylogenetic community structure*

Temperature decrease had a major effect on the bacterial communities for both systems (similarity >60%).This is less likely to have happened due to inhibition caused by VFA accumulation as the detected concentrations were relatively low leaving adaptation be the most profound reasoning.

Preliminary bacterial community analysis of the two systems showed that after 34 days communities for both systems slightly differ when at same temperature (similarity >85% at both systems). Thus, the differentiation in hydrolysis rates between the systems should be related to this minor 15%. The MFC phylogenetic microbial structure was previously studied by Velasquez-Orta et al. (2011) who revealed that MFC microbial communities utilizing complex compounds (e.g. starch, wastewater) tend to diversify more than those utilizing certain intermediates (e.g. acetate) due to the increased stage of biodegradation interactions that performed from different consortia. This is aligned with what is observed in this study with the replicates from MFC at 27ºC diversify at a wider range than for AD at similar conditions. This is explained by the potential development of respiratory microorganisms' necessary for electron transport purposes especially in the initial experimental days where exo-electrogens try to predominate over methanogens. Such scenario would promote MFC over AD rendering the 1st able to cope with a wider range of conditions.

Additionally, it is observed that the MFC community diversifies at lower temperatures more than at higher ones (Figure 6a, b). This is explained by the slow predominance of the exo-electrogenic bacterial communities at low temperatures due to decelerated growth and migration (Curtis and Sloan 2004) .

An important observation, although the degree of similarity is low, is the presence of the *Geobacteriaceae* family band at the MFC that was not apparent at the AD treatments. The importance of this family at MFC systems has been previously reported by (Lesnik and Liu, 2014) in the presence of acetate. Heidrich et al., (2018) and Heidrich et al., (2014) confirmed the importance of this consortium at low-temperature operational MFC using actual wastewater as substrate. It is likely that this can be the microbial consortium responsible for the high lipolysis capacity of the MFC at low temperature considering that *Geobacter* has a proven lipolysis capacity (Hu et al., 2010).

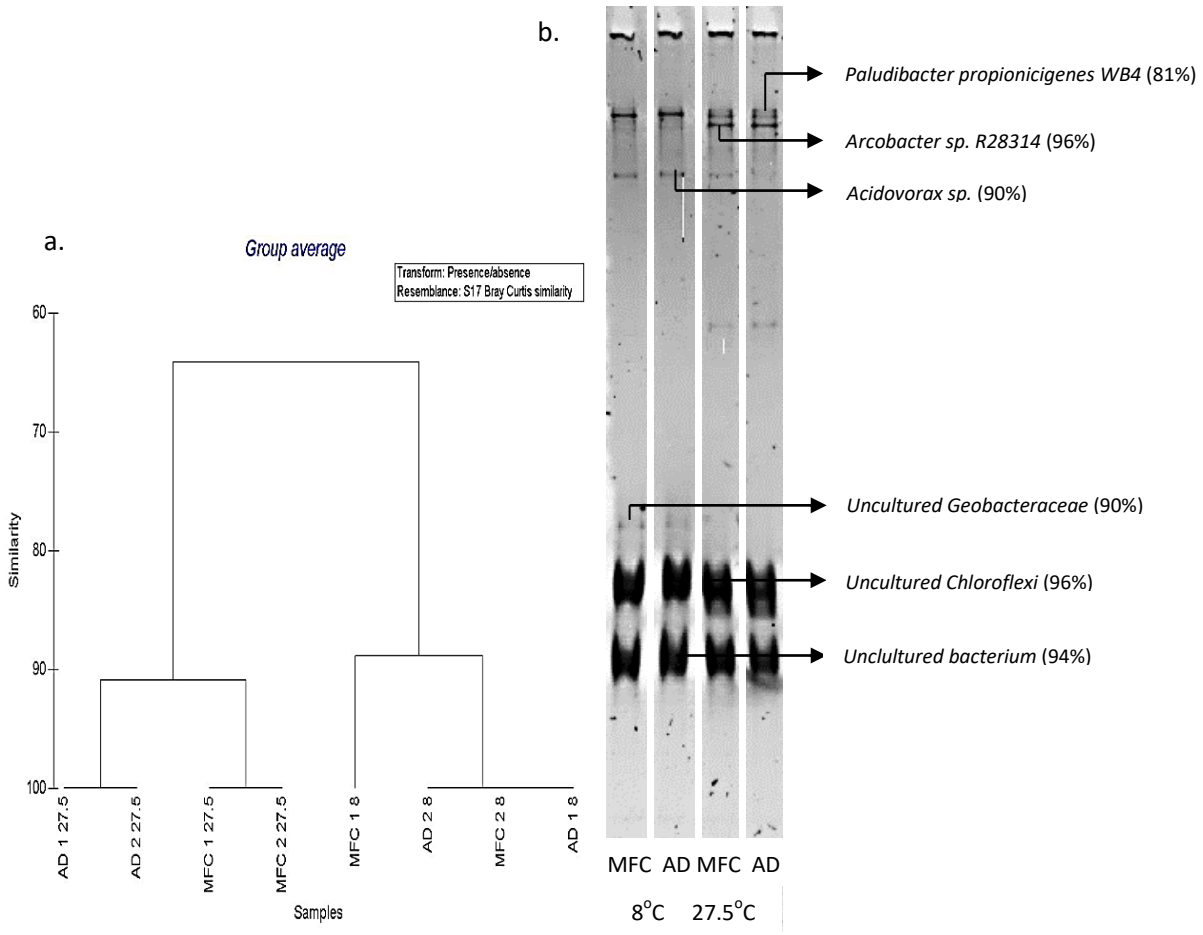

Figure 6 – a) Bacterial phylogenetic structure as a dendrogram plot; b) DGGE pattern with the corresponding most abundant bacterial taxa detected on the 34th experimental day for both temperatures and treatments

(PDF) Domestic wastewater hydrolysis and lipolysis during start-up in anaerobic digesters and microbial fuel cells at moderate temperatures. Available from: https://www.researchgate.net/publication/333421810_Domestic_wastewater_hydrolysis_and_lipolysis_during_start-up_in_anaerobic_digesters_and_microbial_fuel_cells_at_moderate_temperatures [accessed Jul 24 2019].

3.9. *The potential of the MFC community at low temperatures*

Minor changes in the community structure developed at MFC setups can promote treatment and overcome limitations that previously were complicated tasks at low-temperature operation (lipid and protein hydrolysis at AD), especially during start-ups. The molecular tools that were employed in this study (DGGE) though were old and almost outdated to further understand the microbiology behind this differentiation in treatment. This study could enable further research using more modern approaches (2^{nd} and 3^{rd} generation sequencing) to into-detail describe what the key cells and mechanisms are in these electrogenic systems operating at low temperatures.

In the overall, it seems that MFC develops communities with a wider temperature range, able to realize hydrolysis even at lower temperatures, one of the major bottlenecks at operation in cold.

Conclusions

AD is likely to hydrolyse domestic wastewater faster at warm temperatures compared to MFC where the second has a higher potential at cold environments. Switching from AD to MFC slightly alters (<15%) the bacterial community; this resulted to the development of cells able to effectively hydrolyse proteins and lipids at lower temperature. In general, temperature drop may affect hydrolysis of lipids and carbohydrates but protein lysis especially in MFC remains unaffected. Preliminary molecular work showed that minor differences in the community, most likely related to the family of *Geobacteraceae*, is one of the key features that renders hydrolysis (and especially lipolysis and proteolysis) at MFC accelerated compared to AD for operation at low-temperatures.

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