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Start-up phase of an anaerobic full-scale farm reactor – Appearance of mesophilic anaerobic conditions and establishment of the methanogenic microbial community

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HIGHLIGHTS

- Mesophilic biogas farm reactor can be initiated using psychrophilic seeding material.
- pH, alkalinity, free-NH_3, TS and O_2 were the main drivers of the microbial dynamics.
- *Firmicutes* and *Methanosarcina* were the dominant groups established at steady state.
- Interactions between eukaryotes, bacteria and especially archaea were evidenced.

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ABSTRACT

The goal of this study was to investigate how the microbial community structure establishes during the start-up phase of a full-scale farm anaerobic reactor inoculated with stale and cold cattle slurry. The 16S/18S high-throughput amplicon sequencing results showed an increase of the bacterial, archaeal and eukaryotic diversity, evenness and richness during the settlement of the mesophilic anaerobic conditions. When a steady performing digestion process was reached, the microbial diversity, evenness and richness decreased, indicating the establishment of a few dominant microbial populations, best adapted to biogas production. Interestingly, among the environmental parameters, the temperature, alkalinity, free-NH_3, total solids and O_2 content were found to be the main drivers of microbial dynamics. Interactions between eukaryotes, characterized by a high number of unknown organisms, and the bacterial and archaeal communities were also evidenced, suggesting that eukaryotes might play important roles in the anaerobic digestion process.

1. Introduction

In view of a holistic approach towards organic waste management, renewable energy production, environment conservation and nutrients recovery, an increasing number of farmers invest in anaerobic treatment of organic leftovers available on their farms such as wastewater, manure, animal slurries, and crop residues. Furthermore, contrary to solar and wind energies, biomass is regarded as one of the future most prominent renewable energy resources, since a continuous energy generation and storage can be guaranteed (Appels et al., 2011).

Anaerobic digestion (AD, syn. biomethanation) of biomass is regarded as a promising source of green energy because it also generates additional environmental benefits to the society, *e.g.* reduction of greenhouse gas emissions, nutrients recovery, and organic soil conditioners. Indeed, the digestion residue, called digestate, is used in agriculture as nutrient-rich fertilizer and/or organic amendment. Furthermore, the AD process generates other non-profit benefits such as odour reduction and inactivation of microbial pathogens and weed seeds (Engeli et al., 1993; Yiridoe et al., 2009).

The AD process involves different microbial groups that interact together in the absence of oxygen to decompose organic matter into biogas, which is a mixture of methane (CH_4), carbon dioxide (CO_2) and trace gases such as hydrogen sulphide (H_2S), ammoniac (NH_3) and hydrogen (H_2). Biogas can be valorised in a combined

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heat and power (CHP) unit to produce electricity and heat. Alternatively, biogas can be upgraded to biomethane to reach the purity of natural gas and be injected into the municipal gas grid or be used as transportation fuel. The AD process is divided into four main stages (hydrolysis, acidogenesis, acetogenesis and methanogenesis), each involving different microbial communities (Weiland, 2010). These microbial groups are in perpetual interactions and the complexity of their associations and functioning is far from being well understood (Carballa et al., 2015). Moreover, according to some authors, the performance of an AD reactor is closely linked to the structure and dynamics of its microbial community (Demirel and Scherer, 2008). For this purpose, the start-up phase of an anaerobic reactor, aiming at developing an active microbial biomass that reaches a satisfactory treatment performance (Escudié et al., 2011), is considered as a critical point (Ike et al., 2010). Especially methanogenic archaea are considered a rate-limiting key-players of the AD process due to their slow growth rates and high sensitivity to different environmental conditions (Weiland, 2010). Thus, process inhibition can often be encountered due to an imbalance between the VFAs and other precursor-producing bacteria and methanogenic archaea (Ahring et al., 1995). Moreover, many environmental parameters such as the digestion temperature (Luo et al., 2015), the organic loading rate (OLR) (Goux et al., 2015) or even the substrate type (Westerholm et al., 2016) greatly influence the microbial community development and structure. In consequence, knowledge on the dynamics of microbial communities is required to prevent process imbalance, to better understand performance and stability of a reactor, but also to shorten the start-up phase of a new reactor and thus to improve the economic competitiveness of the AD process.

Even though the start-up phase of anaerobic reactors has been studied for lab-scale reactors (Ziganshina et al., 2014; Goberna et al., 2015), only a few studies have dealt with the microbial community dynamics at this stage in full-scale biogas units (Angenent et al., 2002; Ike et al., 2010). Furthermore, none of these studies used the high-throughput sequencing approach to characterize the microbial populations, and to the best knowledge of the authors, the studied reactors were all inoculated with warm and acclimated anaerobic sludge coming from another running reactor, such as a secondary anaerobic digester (Angenent et al., 2002), or thermophilic and mesophilic reactors from cow and pig manure-treating plants (Ike et al., 2010), but never with psychrophilic non-anaerobic materials.

In the present study, the performance and dynamics of microbial consortia, including small anaerobic eukaryotes, were monitored during the start-up phase of a full-scale farm AD reactor inoculated with psychrophilic non-anaerobic materials (run-off water and stale and cold cattle slurry), and the progressive warming and establishment of anaerobic conditions till full operational status was reached.

2. Methods

2.1. Full-scale farm reactor design and operation

The construction of a farm anaerobic reactor of around 500 m^3 working volume, located in the Northeast France, was completed at the end of 2013. The reactor is a completely stirred tank reactor type and consists of a concrete tank of 12 m in diameter and 5 m high. It is equipped with a heating system to reach the mesophilic temperature range (defined as 37–42 °C in this study, temperature commonly used in AD) and a mixer (Fig. 1).

Even though the seeding microorganisms and the inoculum size are considered important factors for the start-up of an AD process (Ike et al., 2010), due to the lack of an adequate inoculum, the reactor was initially partly filled with 250 m^3 of a mixture of run-off water collected on the farm premises and courtyard and cattle slurry available on the farm, and previously stored for a few months in an outdoor open storage tank. This inoculation method is common when no anaerobic seeding sludge is available. Following the reactor filling, the heating system was initiated with the heat provided by the combined heat and power unit (CHP) operating with propane. During 30 days, the reactor was regularly filled with a mix of run-off water, cattle slurry and manure to initiate the digestion process (Supplementary Fig. S1). Following this period, feeding was carefully initiated with plant biomass, including grass, immature rye and maize silages, hay, straw, green grass, and animal effluents, such as solid meat- and dairy-cattle manure and slurry available on the farm at an average loading rate (LR) calculated over the entire monitoring period of 5420 ± 2821 kg d^{-1}. Additional details on the input materials and loading rate are specified in Supplementary Fig. S1. Over a period lasting 152 days, the sludge temperature increased progressively inside the anaerobic reactor from 5.6 °C to around 42 °C, which was the final temperature desired by the plant owner.

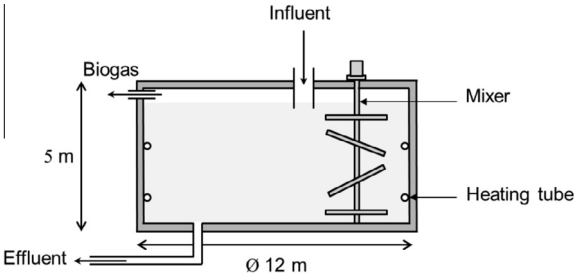

Fig. 1. Schematic of the full-scale anaerobic reactor.

2.2. Samples collection and metabolic parameters analysed

Establishment of anaerobic conditions inside the reactor during the start-up phase was monitored during 175 days. For this purpose, a sludge volume of around 1 L was collected following a thorough reactor mixing. The first sampling was done on the day the heating was initiated (day 1). During the study time, a total of 12 sludge samples were collected according to the biogas plant operator availability at day 1, 6, 14, 21, 30, 35, 42, 57, 70, 96, 152 and 175 and named hereinafter from d1 to d175 in the figures. For each sludge sample, different aliquots were frozen on-site in liquid nitrogen. Back to the lab, aliquots of 200 µL were preserved at −80 °C prior the DNA extraction while aliquots of around 15 mL were stored at −20 °C prior the VFAs concentration measurements. The remaining unfrozen sludge was used to measure the pH, total solids (TS, %) and volatile solids (VS, %) contents, total alkalinity (mg $CaCO_3$ L^{-1}), and ammonium-nitrogen concentration (kg NH_4-N m^{-3}), as described in Goux et al. (2015). In accordance to Emerson et al. (1975) and Körner et al. (2001) the free ammonia content (free-NH_3, kg m^{-3} of sludge) was calculated, taking into account the temperature, pH, and the measured NH_4-N content of the sludge in the reactor at the moment of sampling. The detailed protocol for the VFAs concentration (mg kg^{-1}) measurement is as described in Goux et al. (2015). Total VFAs concentration was expressed in terms of the sum of measured acetate, propionate, isobutyrate, butyrate, isovalerate, valerate and caproate concentrations. The estimated methane production (m^3 CH_4 m^{-3} reactor day^{-1}) was calculated starting from day 54 (*i.e.* when the produced biogas started to be used in the CHP unit to generate heat and electricity), based on an energy content of methane of 35 MJ m^{-3} and the monthly amount of electricity produced by the plant, and the CHP unit electrical efficiency as stated by the

manufacturer (38.75%). The biogas composition inside the reactor (CH_4, CO_2, O_2 (%), H_2S (ppm)) was monitored using a Multitec® 540 apparatus (Hermann Sewerin GmbH, Gütersloh, DE).

2.3. DNA extraction, 16S/18S rRNA amplicon libraries preparation and high-throughput sequencing

Altogether, bacteria, archaea and eukaryotes were targeted. The DNA extraction of the 12 collected samples was performed with the PowerSoil DNA Isolation Kit (Mobio Laboratories Inc., Carlsbad, CA, USA), according to the manufacturer's protocol. The quantity and quality of the extracted DNA were assessed using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and electrophoresis migration on a 1% agarose gel. Modified primer pair S-D-Bact-0909-a-S-18 (5′-ACT CAA AKG AAT WGA CGG-3′) and S-*-Univ-1392-a-A-15 (5′-ACG GGC GGT GTG TRC-3′), targeting around 484 bp of bacterial V6–V8 region of the 16S rRNA gene, and an archaeal 16S rRNA gene-specific primer pair S-D-Arch-0519a-S-15 (5′-CAG CMG CCG CGG TAA-3′) and S-D-Arch-1041-a-A-18 (5′-GGC CAT GCA CCW CCT CTC-3′) targeting a fragment of V4–V6 region of around 526 bp, were selected after the study of Klindworth et al. (2013). Eukaryotic primer pair Ek7F (5′-ACC TGG TTG ATC CTG CCA G-3′) and EK516R (5′-ACC AGA CTT GCC CTC C-3′), targeting an 18S rRNA V1–V5 fragment of around 509 bp was selected according to Santos et al., 2010. As modification, the Nextera XT® transposase sequence (Illumina Inc., San Diego, USA) was included in the 5′ end of the forward and reverse primer, and additional four N (*i.e.* four random nucleotides) were added in the forward primer to increase the nucleotide diversity. Amplicons were generated using the Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs Inc., Ipswich, USA), were purified with the AMpure magnetic beads (Agencourt, Beckman Coulter Inc., Fullerton, USA) and quantified with the Qubit® dsDNA HS assay kit (Life Technologies, Carlsbad, USA). Purified amplicons were diluted to the concentration of 1 ng μL^{-1}, and the Nextera XT® barcodes and the Illumina adapters necessary for hybridization to the flow cell, were added to each amplicon during the cycle-limited PCR using the Nextera XT Index kit. Purification of the resulting PCR products was carried out with the AMpure magnetic beads (Agencourt). Obtained and purified libraries were then pooled in equimolar concentrations, and the final concentration of the library pool was determined with the KAPA SYBR® FAST Universal qPCR Kit (Kapa Biosystems, Wilmington, USA). Libraries were mixed with Illumina-generated PhiX gDNA control libraries (5%) and sequenced on the Illumina MiSeq system with the MiSeq Reagent Kit V3-600 cycles.

2.4. Data analysis

The CLC Genomics Workbench 7 and Usearch (v7.0.1090_win64) (Edgar, 2010) software were used to demultiplex, quality trim and assign the obtained sequence reads to operational taxonomic units (OTUs) at 97% of similarity. For eukaryotes, only the forward read was used for further analysis as the final amplicon length exceeded the calculated *in silico* 509 bp, what prevented an efficient forward and reverse read assembly, due to either too small or not existing sequence overlap. Taxonomic classification was done using the Greengenes database (http://greengenes.lbl.gov/) with a confidence threshold of 80% and the resulting nucleotides sequences were deposited in GenBank database under accession numbers KT251256 to KT252397 for bacteria, KT252398 to KT252430 for archaea and KT386389 to KT386961 for eukaryotes. Based on the high-throughput sequencing results, richness (numbers of OTUs), Shannon–Weaver (H'), Simpson (D), Pielou (J), Sørensen and Bray–Curtis indices were calculated to evaluate respectively the diversity (H' and D), evenness (J) and the dissimilarity in terms of membership (Sørensen) and community structure (Bray–Curtis) between the microbial communities at different time-points.

The influence of the environmental parameters on bacterial, archaeal and eukaryotic community dynamics (H', D, J and R, as well as the ten most abundant OTUs of each domain) was analysed using Spearman's rank correlations. Spearman's rank correlation coefficient and their corresponding p-values (considered statistically significant if under 0.05) were calculated using R (Version 3.1.2) (Core Team, 2008).

3. Results and discussion

3.1. Reactor performance during the start-up phase

The temperature inside the reactor reached 37 °C at day 42 (Fig. 2C). Following the temperature increase, and due to the feeding regime rich in manure and immature rye silage (Supplementary Fig. S1), the concentration of CH_4 and CO_2 in the reactor headspace increased and stabilised after day 42 (Fig. 2B). In relation to the increasing production of biogas, the O_2 concentration decreased from around 18% at day 1 to less than 3% at day 42. Thus, the mesophilic anaerobic conditions were considered to be reached inside the studied reactor at day 42.

All along the monitoring of the farm reactor, the pH fluctuated between 7.3 and 8.0, and the total VFAs content in the sludge remained low, with less than 450 mg kg^{-1} of sludge (Fig. 2C and F). Acetate was the dominant VFA and represented on average more than 90% ± 12 of the total VFAs. Propionate, which is commonly used as an indicator of process perturbation (Marchaim and Krause, 1993), never exceeded a concentration of 68 mg $kg^{-1}$of sludge. In the studied reactor during the start-up phase, propionate and other longer VFAs were gradually and effectively consumed by the establishing microbial community.

During the monitoring phase, increasing alkalinity from around 4400 at day 1 to more than 16,000 mg $CaCO_3$ L^{-1} at day 175 (Fig. 2E), was related to the incorporation of manure as input material starting from day 21 (Supplementary Fig. S1). Even though manure is rich in recalcitrant lignocellulose, it is considered as an excellent co-substrate for co-digestion as it is characterized by a high buffering capacity (Goberna et al., 2015). Further increase in sludge alkalinity could be partially explained by the new feeding regime rich in various plant biomasses initiated at day 31 (Supplementary Fig. S1). Feeding the reactor with solid substrates resulted in an increase of the TS and VS content from respectively around 1.0% and 42.0% at day 1 to around 13.0% and 64.0% at day 175 (Fig. 2D).

Based on the monthly amounts of electricity produced from day 54, when propane was replaced by the produced biogas to feed the CHP unit, the estimated methane production showed a steady increase (Fig 2A). Around 0.13 m^3 CH_4 m^{-3} reactor day^{-1} was produced by the biogas unit between days 54 and 62, 0.30 m^3 CH_4 m^{-3} reactor day^{-1} between days 63 and 92, and finally 0.53 and 0.57 m^3 CH_4 m^{-3} reactor day^{-1} between days 93 and 123, and days 124 and 153, respectively. These last two values (0.53 and 0.57 m^3 CH_4 m^{-3} reactor day^{-1}, Fig. 2A) showed that in around 120 days the reactor reached a good CH_4 productivity level which was similar to values quoted in the literature for a CSTR fed with cattle manure (Sakar et al., 2009). As a conclusion, a mesophilic AD process can be efficiently initiated with psychrophilic non-anaerobic material such as run-off water and stale cold cattle slurry.

3.2. Microbial community monitoring

The high-throughput 16S/18S rRNA gene amplicon sequencing approach applied in this study enabled to monitor the establishment

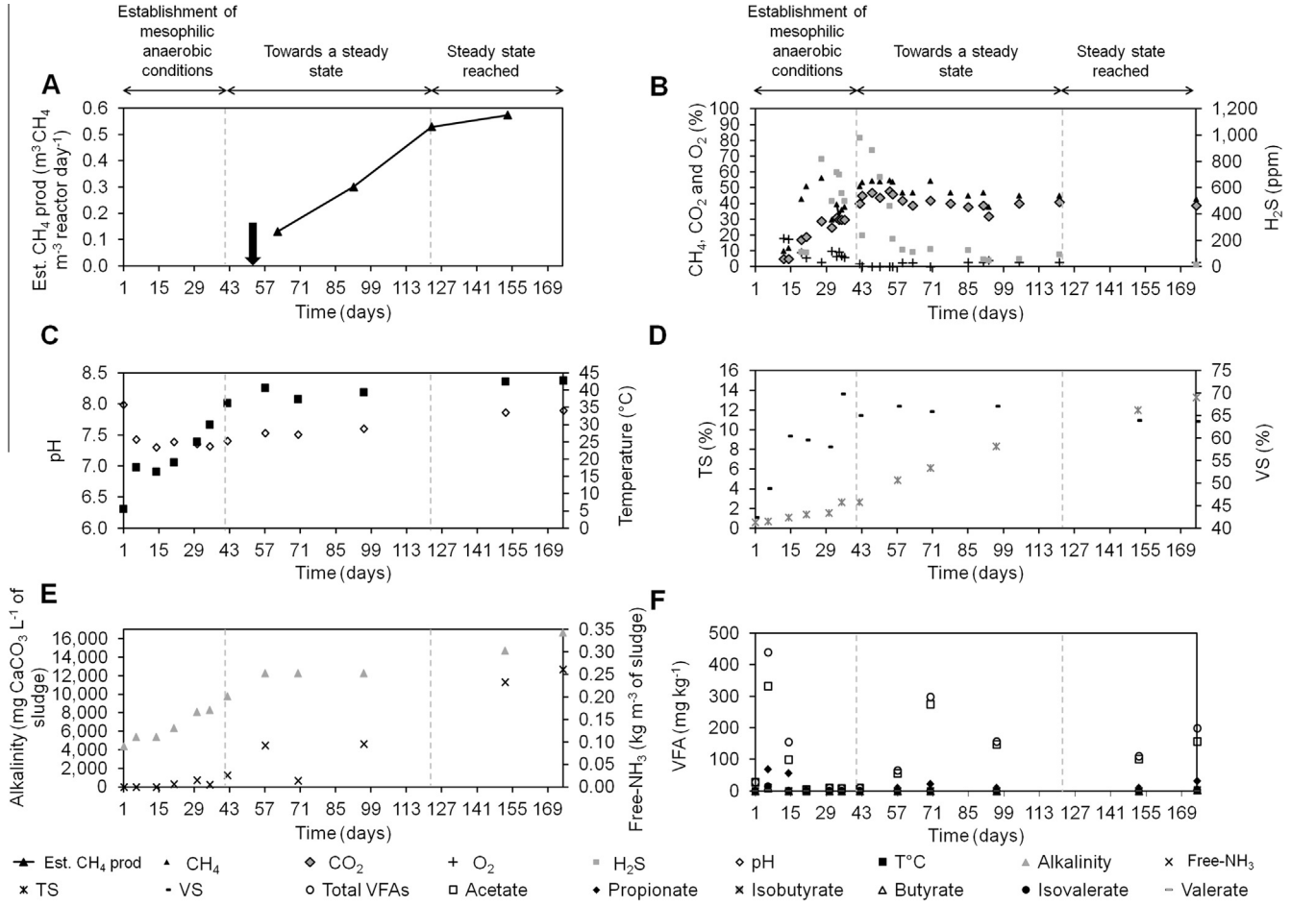

Fig. 2. Dynamics of the reactor performances and metabolic parameters during the start-up phase: (A) estimated methane production, (B) reactor headspace composition, (C) pH and temperature, (*D*) total and volatile solids, (E) alkalinity and free-NH_3, and (F) volatile fatty acids concentrations; the *black arrow* indicates day 54, when propane was replaced by the produced biogas to feed the CHP unit; *the grey vertical dotted lines* indicate the different phases defined during the monitoring.

over time of a mesophilic anaerobic microbial community initiated from a seeding with psychrophilic farm effluents. For the 12 samples analysed, a total of 2,284,500 raw reads were obtained and assigned after processing to 1142; 33; and 573 OTUs for the bacterial, archaeal and eukaryotic communities, respectively. The dynamics of microbial community structure over time was presented at the phylum level for bacteria, at the genus level for archaea, and for the three first classification levels in case of eukaryotes according to the complexity of each domain (Fig. 3). A direct comparison of the ratio bacteria:archaea:eukaryotes is not possible because specific primers for each domain of life were used for the high-throughput sequencing. While the Chao1 estimator rarefaction curves started to level off for the majority of the samples analysed for the bacterial community (Supplementary Fig. S2A), clear asymptotes were reached for the archaeal and eukaryotic communities (Supplementary Fig. S2B and C). These observations point out that most of the diversity for these two last communities was indeed characterized by high-throughput sequencing, whereas an additional sequencing effort would have allowed to describe on average 110 ± 19 bacterial OTUs more, *i.e.* around 10% of the observed OTUs.

3.2.1. Microbial community composition in psychrophilic seeding materials

At the beginning of monitoring (day 1), the microbial community was dominated by a few organisms (Fig. 3 and Supplementary Fig. S3). The phylum *Proteobacteria*, accounted for around 40% of the whole bacterial population (Fig. 3A). This phylum was represented mainly by two families *Campylobacteraceae* and *Comamonadaceae* (OTU_18, 41 and 1637, Supplementary Table S1 and Fig. S3A). Other bacterial phyla such as *Bacteroidetes*, *Firmicutes* and *Tenericutes*, with respectively *Porphyromonadaceae* (OTU_146), an unclassified family from the class OPB54 (OTU_17), and *Acholeplasmataceae* (OTU_11) being the dominant families, were also well represented, with abundance levels of around 20%, 18% and 10%, respectively (Supplementary Fig. S3A). Two genera constituted most of the archaeal community, with the genus *Methanocorpusculum* and the archaeon candidate division *vadinCA11* representing respectively around 70% and 25% of the whole archaeal community (Fig. 3B and Supplementary Table S2). These archaea most probably originated from the cattle manure and slurry. Indeed, the presence of bacterial phyla similar to those cited above, and the dominance of *Methanocorpusculum*-like archaea has been shown in cattle manure used for composting and in influent of a mixed plug-flow loop reactor converting cattle manure to biogas (Yamamoto et al., 2011; Li et al., 2014). However, other archaeal genera including *Methanosarcina* and *Methanobrevibacter*, not detected in the studied reactor, have also been found as co-dominant organisms in manure used as inoculum for another CSTR of 75 dm^3 working volume (Goberna et al., 2015). Regarding eukaryotes, organisms belonging to the division *Chlorophyta* represented more than 95% of the community at day 1 with OTU_2 and OTU _457 identified as members of the class *Chlorophyceae*

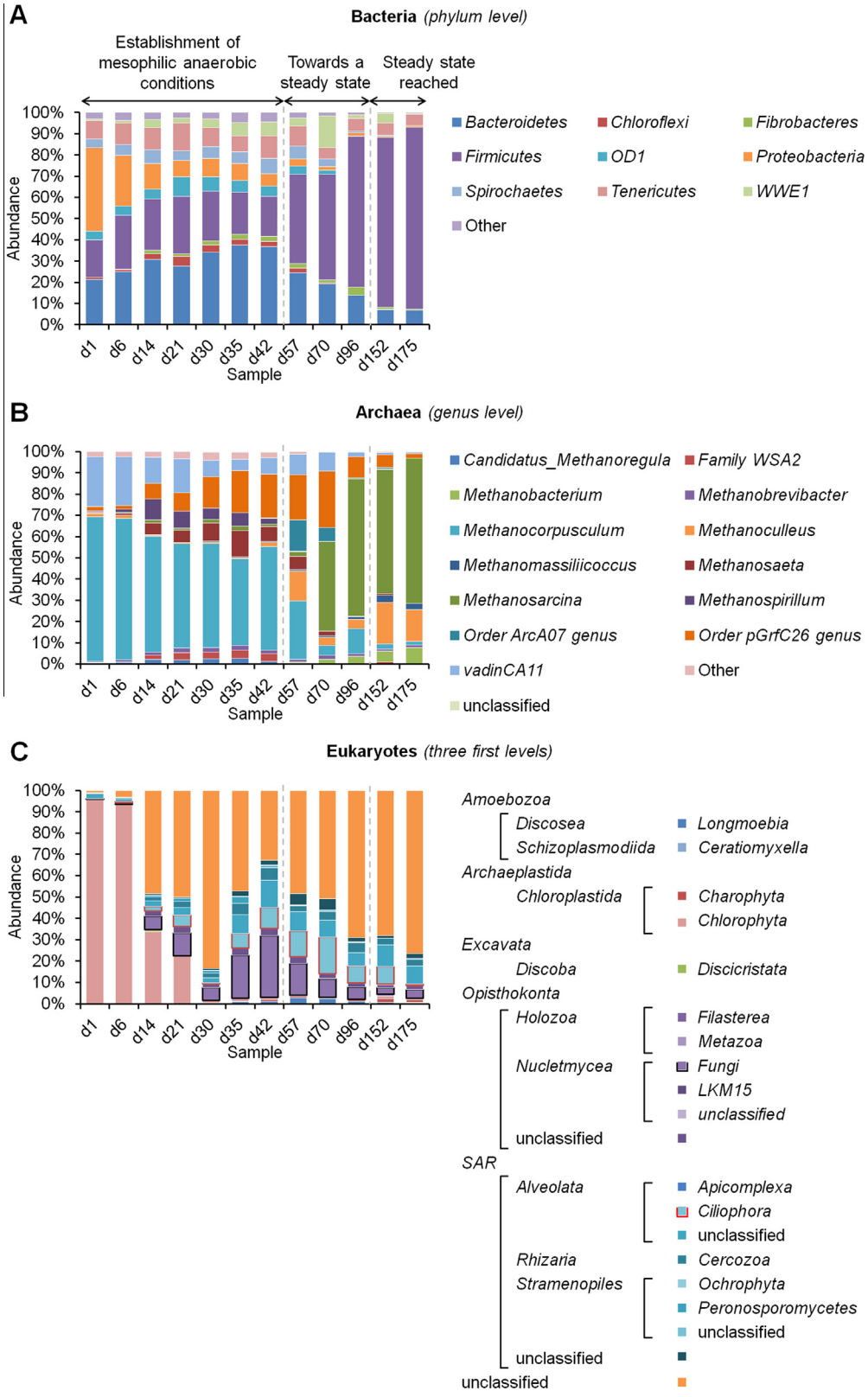

Fig. 3. Bacterial, archaeal and eukaryotic structure dynamics over time: results are presented at the phylum level for bacteria (A), at the genus level for archaea (B), and at the three first classification levels (C) for eukaryotes; *the grey vertical dotted lines* indicate the different phases defined during the monitoring.

(Fig. 3C, Supplementary Fig. S3B and Table S3). The high abundance of these green microalgae at the beginning of the monitoring was most probably a consequence of filling the reactor with run-off water that was stored in an outdoor open tank.

3.2.2. Microbial community dynamics towards the establishment of mesophilic anaerobic conditions

Interesting changes over time inside the microbial community could be underlined during the temperature increase and the

appearance of anaerobic conditions inside the reactor (Figs. 2–4). Indeed, between day 1 and 42 the previously dominant *Proteobacteria* phylum decreased in abundance from around 40% to 5% of the total bacterial community (Fig. 3A). At the same time, the abundance level of the *Bacteroidetes* phylum increased from around 20% to almost 40% with a dominance of the *SB-1* family (*e.g.* OTU_19 and 355). Concerning the other dominant bacterial phyla detected at the beginning of the start-up phase, their abundance levels remained relatively stable during the initiation of the mesophilic anaerobic conditions inside the farm reactor. Interestingly, whereas the bacterial community was only dominated by a few OTUs at day 1 (Supplementary Fig. S3A), and in spite of the changes at the phylum level and the appearance of new, yet rare OTUs, the diversity (H' and D) and evenness (J) indices remained quite stable as evidenced by H', D and J close to 7.5, 0.0 and 0.9, respectively, during the initiation of the mesophilic anaerobic conditions (samples from day 6 to day 42) (Fig. 4A–C). Bacterial richness R also remained unchanged between day 1 and day 42, with an average value close to 462 ± 45 identified OTUs (Fig. 4D).

Inside the archaeal community, the abundance of the two previously dominant organisms, the genus *Methanocorpusculum* and the archaeon candidate division *vadinCA11*, decreased from around 70% to 50% and from 25% to 6% of the whole archaeal community, respectively (Fig. 3B). Simultaneously, the abundance of the *Methanosaeta* genus, detected at a very low level at day 1, increased to approximately 20% of the total archaeal community towards the end of the establishment of the mesophilic anaerobic conditions (day 42). This genus was reported as highly competitive in anaerobic environment with low acetate concentration (Angenent et al., 2002; Li et al., 2014). Therefore, it was assumed that the overall low acetate concentration (70 ± 120 mg kg^{-1}) in the studied reactor during the monitoring period (Fig. 2F), favoured the increase in abundance of *Methanosaeta*. At day 42, other archaea representing the family *WSA2* and the genera *Methanosarcina* and *Methanospirillum* were detected with abundance levels of less than 10% each, whereas *Crenarchaeotal* order *pGrfC26*-related archaea represented around 20% of archaeal community. In contrast to bacteria, the diversity and evenness of the archaeal community increased from day 1 to day 42, as assessed by the values of the H', D and J indices changing over time from 1.7 to 2.7, 0.5 to 0.1 and 0.4 to 0.7, respectively (Fig. 4A–C).

The ecological microbial parameters (*i.e.* diversity, evenness and richness indices) were fluctuating much more for the eukaryotic community than for the bacterial and archaeal ones. Based on the two calculated diversity indices (H' and D), eukaryotic diversity globally increased during the establishment of the mesophilic anaerobic conditions in the studied farm reactor (Fig. 4A and C). At the same time, eukaryotic evenness and richness also increased (Fig. 4B and D). Community membership and structure changed as well during that time (Fig. 4E and F). The change of these ecological microbial parameters reflects the disappearance of the division *Chlorophyta* following day 6, and the appearance of specific anaerobic eukaryotes probably introduced with the cattle manure and slurry used to feed the reactor, such as *Fungi* and *Ciliophora* (Kittelmann et al., 2013) (Fig. 3C). However, concerning the taxonomic classification of eukaryotes, incompleteness of publicly available databases resulted in a high proportion of unclassified OTUs. Indeed, following the disappearance of *Chlorophyta* division from the reactor (day 14), unclassified sequences represented an average of 57.5% ± 15.8 of the whole eukaryotic community. Surprisingly, the majority of these unclassified eukaryotes was represented by a single OTU_1 (Supplementary Fig. S3B and Table S3), covering around 45% of the eukaryotic community at day 14 and day 21, and up to more than 90% at day 30. This over-dominance of OTU_1 (showing 95% of 18S rRNA gene identity to a free free-living phagotrophic flagellate *Rictus lutensis* gen. et sp. nov., isolated from low oxygen environments (Yubuki et al., 2010)) resulted in a drop of diversity (Fig. 4A and D) and evenness (Fig. 4B) of the eukaryotic community present in the farm reactor. Towards the end of the establishment of mesophilic anaerobic conditions inside the reactor (temperature above 37 °C and O_2 content in the reactor headspace under 3% reached at day 42), the relative abundance of OTU_1 decreased from around 35% to less than 1%. At the same time the abundance levels of *Fungi*, *Ciliophora*, and other organisms belonging to the *Opisthokonta* and *SAR* super-groups, increased. These changes were linked to an increase of the community diversity, evenness and difference in terms of community structure (Fig. 4A–C and F), whereas the global richness and similarity in terms of community membership remained stable (Fig. 4D and E).

3.2.3. Microbial community dynamics towards a steady state anaerobic digestion process

Although mesophilic anaerobic conditions established in the farm reactor after day 42, and while Luo et al. reported in 2015 that a microbial community of an anaerobic reactor could remained stable over time in the absence of disturbance, major shifts inside the microbial community were still evidenced afterwards indicating that the reactor did not reach yet a steady state microbial composition at this stage. Indeed, a gradual disappearance of *Bacteroidetes* was underlined, whereas at the same time the abundance of *Firmicutes* increased (Fig. 3A). This observation was corroborated by the Sørensen and Bray–Curtis indices, revealing major changes inside the bacterial community membership and structure respectively, between sampling days 42 and 57 (Fig. 4E and F). Indeed, the increase of the Sørensen index indicated the appearance of new OTUs (Supplementary Fig. S3A and Table S1) and the Bray–Curtis index indicated that only 39% of the community structure was shared between days 42 and 57, while it was 75% between days 35 and 42. Similarly to the bacterial community, changes inside the archaeal community after the establishment of mesophilic anaerobic conditions inside the studied reactor were also detected. Indeed, the high d57–d70 Bray–Curtis index of 0.50 (Fig. 4F) shows that 50% of the archaeal community structure was different between these two time-points. This change was best illustrated at the genus level with the appearance of *Methanosarcina*. Indeed, whereas *Methanosarcina* was detected at a low abundance of 3% at day 57, this genus characterized by a high growth rates and tolerance toward high total ammonium and acetate concentrations (De Vrieze et al., 2012), increased in abundance to around 45% of the whole archaeal community at day 70. Moreover, it seemed to compete with *Methanocorpusculum* and *Methanosaeta* which strongly decreased in terms of relative abundance at this time in the reactor (Fig. 3B).

Regarding the eukaryotic community, no change inside the community membership and structure could be pointed out based on the Sørensen and Bray–Curtis indices after the establishment of the mesophilic anaerobic conditions in the reactor. However, starting from this period, the abundance of *Fungi* and *Ciliophora* decreased while at the same time the abundance of other unclassified eukaryotes increased (Fig. 3C).

3.2.4. Establishment of microbial communities during the steady state anaerobic digestion process

Following the main changes in the microbial communities (especially for the bacterial and archaeal communities), the whole microbial (bacterial, archaeal and eukaryotic) community dynamics was then characterized by a global decrease in diversity, evenness and richness towards the end of the monitoring period. Indeed, while around 411 bacterial OTUs were identified at day 57 (respectively 30 archaeal OTUs and 209 eukaryotic OTUs), only 169 bacterial OTUs were detected at the last time-point of

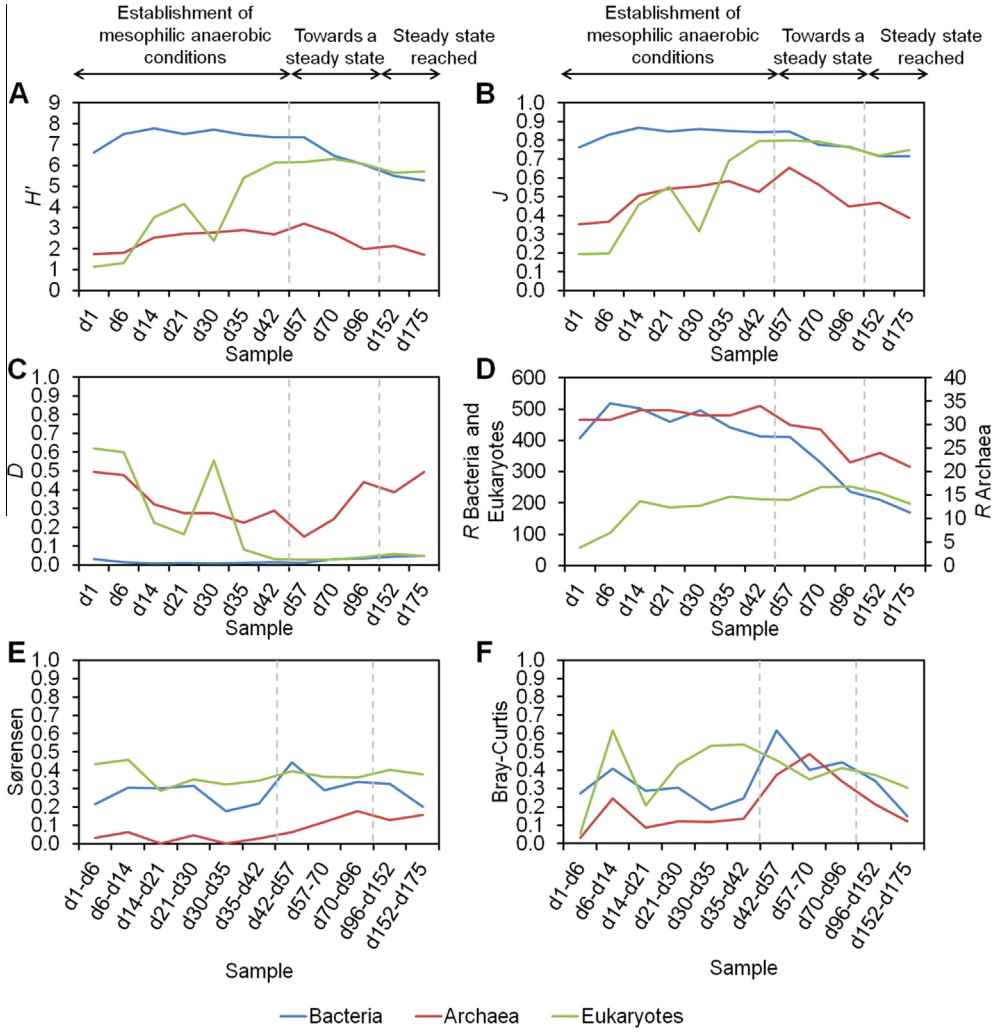

Fig. 4. Microbial ecology parameters dynamics for the bacterial, archaeal and eukaryotic communities: (A) Shannon–Weaver index (*H'*), (B) Pielou index (*J*), (C) Simpson index (*D*), (D) richness (*R*), (E) Sørensen index and (F) Bray–Curtis index; *the grey vertical dotted lines* indicate the different phases defined during the monitoring.

the monitoring (respectively 21 archaeal and 198 eukaryotic OTUs) (Fig. 4D). Similarly, the diversity index *H'* was reduced between days 57 and 175 for the bacterial, archaeal and eukaryotic communities (Fig. 4A). The evenness also decreased at the end of the monitoring for the three microbial communities (Fig. 4B). The diminution of these microbial ecological parameters reflects the installation of a specific microbial community, best adapted to biogas production from the used substrates, as following the day 96 these parameters remained rather unchanged over time. Moreover, typical microbes found in mesophilic anaerobic reactor fed with cow manure and slurry (Li et al., 2014) were detected in the studied reactor as well. Indeed, towards the end of the monitoring (days 96–175) *Firmicutes* became the dominant phylum, with an abundance level fluctuating from around 70% to 90% of the whole bacterial community. This phylum was mainly represented by taxa belonging to unclassified families of the orders *MBA08* and *SHA-98* and the *Ruminococcaceae* family. *Bacteroidetes* and *Tenericutes* shared the large part of the remaining community.

With regards to the archaeal community, establishment of stable operational conditions in the reactor towards the end of the start-up phase, continued to promote the increase in abundance of *Methanosarcina*, which represented around 65% of the population at the end of the monitoring period (day 175). The two other dominant taxa, *Methanoculleus* and *Methanobacterium* represented respectively 20% and 10% of the archaeal population (Fig. 3B). An over-dominance of *Methanosarcina* in other full-scale mesophilic anaerobic digesters have also been reported (St-Pierre and Wright, 2013). Finally, it is interesting to point out that the genus of the *Crenarchaeotal* order *pGrfC26*, previously characterized by an increasing abundance during the initiation of the mesophilic anaerobic conditions (days 1–42), represented less than 3% of the archaeal community at the end of the monitoring. While Carballa et al. (2015) proposed that a functionally diverse microbial community provides a suite of parallel pathways and in consequence that a higher microbial diversity is often correlated with good-performing anaerobic reactors, the results from this study point out that the loss of microbial diversity did not necessarily correspond to a decreased functional diversity in stable AD process. This loss in diversity can be potentially due to the fact that the microbial flora present in the inoculum used was non-anaerobic, and aerobic microbes became extinct as anaerobic conditions established overtime in the reactor. At the end of monitoring, the dominance of *Methanosarcina* in AD reactors can be considered beneficial, since this quite robust archaeon is able to use different substrates and thus multiple pathways for methane production, being at the same time an acetoclastic and hydrogenotrophic methanogen (De Vrieze et al., 2012). Further metagenomic and metatranscriptomic studies could confirm or infirm this speculation by studying the different potential and active metabolic pathways used by the microorganisms involved in the AD process.

Interestingly, and in contrast to the bacterial and archaeal community, the diversity, evenness and richness of the eukaryotic community reached higher values by the end of the monitoring compared to the seeding stage (day 1) (Fig. 4). Thus, the establishment of the mesophilic anaerobic conditions inside the reactor and/or the beginning of the feeding with plant materials resulted in an increase in the eukaryotic community richness and diversity. The role of this eukaryotic community in the AD process has yet to be determined as discussed below.

3.3. Correlation analysis within and between environmental parameters and microbial community structure

The influence of the reactor environmental parameters on the microbial community structure was evaluated by means of Spearman's Rank correlations (Fig. 5). All the correlation coefficients (r) and their corresponding significance levels are presented in Supplementary Table S4. With regards to the environmental parameters, temperature showed the strongest correlation with the free-NH_3 content. While free-NH_3 content is pH dependent (Emerson et al., 1975), any correlation between these two parameters could be evidenced, probably due to a very narrow pH range (7.3–8.0). The CH_4 content in the reactor headspace was positively correlated to temperature and alkalinity; and negatively correlated with the O_2 content. Furthermore, a strong correlation between alkalinity and TS content was also evidenced and could be potentially attributed to feed characteristics. Indeed, the latter result corroborates the assumption concerning an increased sludge alkalinity being a consequence of solid manure and plant materials added to the feeding regime of the reactor, respectively after day 21 and day 31.

Even though pH remained quite stable in a neutral range during the monitoring period, its small fluctuations negatively correlated with the bacterial diversity (HB and DB), evenness (JB) and richness (RB). pH was also negatively correlated with the archaeal diversity (HA) and richness (RA). This last result is in accordance with the sensitivity to pH of each archaeal species and with previous study indicating the appearance of new species (and a transition from acetoclastic toward hydrogenotrophic methanogenesis) following a pH decrease in anaerobic reactors (Goux et al., 2015). Other environmental parameters such as temperature, alkalinity, or the free-NH_3 and TS content also influenced bacterial diversity (HB) and richness (RB), and archaeal richness (RA) (Fig. 5 and Supplementary Table S4). Various environmental parameters, including temperature, pH, alkalinity, free-NH_3, TS, VS, as well as the O_2 content, appeared to strongly influence the abundance of many bacterial, archaeal and eukaryotic OTUs studied here. As all these parameters are well known to be crucial for the AD process and the establishment of an active anaerobic microbiome (Escudié et al., 2011; Goberna et al., 2015), it is reassuring to find such correlations in the dataset from this study as well.

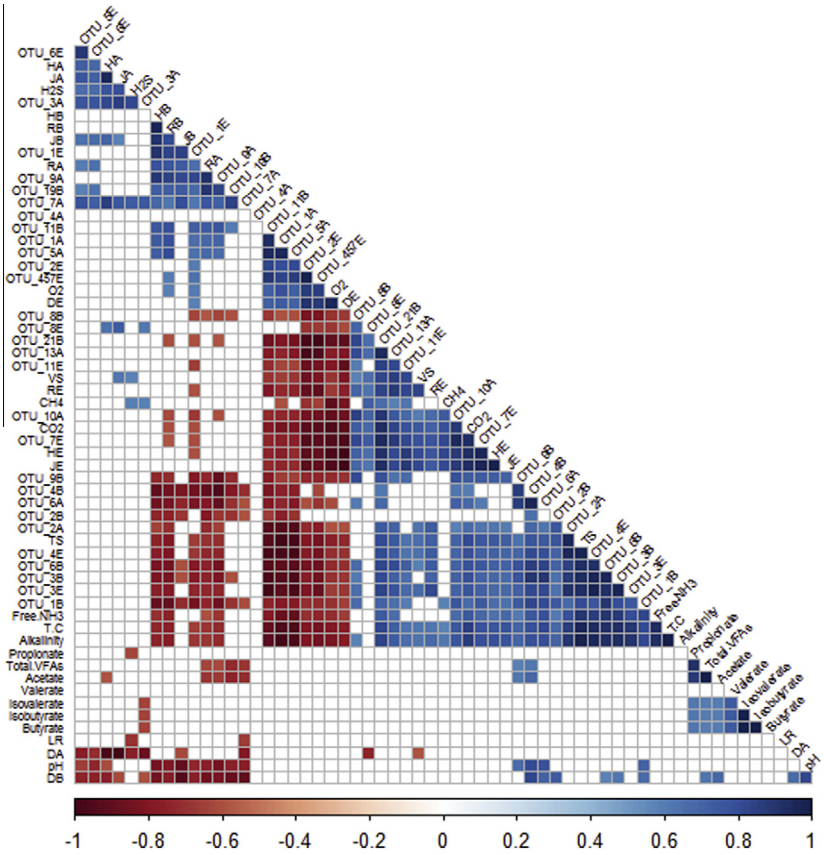

Fig. 5. Spearman correlation matrix of the relationships between the process parameters and the microbial community during the appearance of mesophilic anaerobic conditions: environmental parameters: pH, T.C: temperature, LR: loading rate; TS: total solids; VS: volatile solids; Alkalinity: alkalinity; Free.NH3: free NH_3; CH_4, CO_2, O_2 and H_2S: CH_4, CO_2, O_2 and H_2S contents in the reactor headspace; Total.VFAs, acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate: respective VFAs contents in the sludge. Microbial parameters: *H*, *D*, *J* and *R*: respective Shannon–Weaver, Simpson, Pielou and richness indices for the bacterial (B), archaeal (A) and eukaryotic (E) community; numbered OTUs referrers to their respective OTU for the bacterial (B), archaeal (A) and eukaryotic (E) community. Only the ten most abundant OTUs detected along the monitoring for each microbial community were used for the calculation. Only significant correlations (p-value < 0.05) are represented. The colours of scale bar indicates the nature of the correlation with *darkest blue* indicating a perfect positive correlation ($r = 1$), *darkest red* indicating a perfect negative correlation ($r = -1$), and fading colours a gradual loss in correlation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Significant correlations between bacterial diversity and archaeal richness (*i.e.* HB and RA), bacterial evenness (JB) and archaeal microbial parameters (*i.e.* HA, JA and RA) and bacterial richness (RB) with the archaeal one (RA), were evidenced and underlined the interrelations of these two communities involved in the AD process. Regarding eukaryotes, to the best knowledge of the authors, little is known about the involvement of eukaryotes in the anaerobic digestion process and their influence on bacteria and archaea. In large herbivore intestine tract, eukaryotic microorganisms such as ciliates and anaerobic fungi play a significant role in the degradation of plant material by the degradation of large substrate particles, which are then more accessible to bacteria (Flint and Bayer, 2008; Kittelmann et al., 2013). Ciliate are also known to host methanogenic archaea in their cytoplasm in a form of endosymbiosis or externally in an episymbiotic relationship (Hackstein, 2010). Furthermore, evidence of gene acquisition by horizontal transfer between prokaryotes and eukaryotes, suggested the role of eukaryotes as regulators of bacterial and archaeal communities by engulfment and predation (Devillard et al., 1999; Flint and Bayer, 2008). Even though the involvement of protozoa in anaerobic wastewater treatment process has been revealed thanks to a correlation between ciliate count and enhanced methane production (Priya et al., 2007), up to now no information concerning the eukaryotic dynamics during the start-up phase of a full-scale farm reactor and its potential interaction with prokaryotes is available.

Based on the results of this study, the diversity, evenness and richness of the eukaryotic community (*i.e.* HE, JE and RE, Fig. 5 and Supplementary Table S4) were positively correlated with temperature, alkalinity, TS, and VS content of the reactor. Furthermore, eukaryotic diversity and evenness were negatively correlated with the O_2 content in the reactor headspace. Interestingly, and similarly to results from Priya et al. (2007), the abundance level of the eukaryotic OTU_11 belonging to the *Ciliophora* division showed a positive correlation with the CH_4 content in the reactor headspace (OTU_11E, Fig. 5 Supplementary Tables S3 and S4).

No correlation could be shown between bacterial and archaeal ecological parameters (diversity, evenness and richness) and the eukaryotic ones. Nevertheless, many eukaryotic OTUs were significantly positively or negatively correlated with the bacterial and archaeal diversity, evenness and richness (Fig. 5 and Supplementary Table S4). For example, eukaryotic OTU_1 (Supplementary Table S3) potentially affiliated with *R. lutensis* gen. et sp. nov. which is characterized as a raptorial feeder that preyes on bacteria (Yubuki et al., 2010), showed strong positive correlations with bacterial community diversity (HB and DB), evenness (JB) and richness (RB). Whereas another unclassified eukaryotic OTU_3 showed negative correlations with the bacterial diversity (HB) and bacterial and archaeal richness (RB and RA). Furthermore, during the establishment of the mesophilic anaerobic conditions in the studied full-scale farm reactor, eukaryotic microbial community parameters such as the diversity, evenness and richness were strongly influenced by many bacterial and archaeal OTUs belonging *e.g.* to the order *Bacteroidales* (OTU_21B), archaeal order *ArcA07* (OTU_10A) or the *Crenarchaeotal* order *pGrfC26* (OTU_13A) (Fig. 5, Supplementary Tables S1, S2 and S4).

Based on the results of this study, it is clear that strong relationship between the eukaryotes and the remaining part of the microbial community in the AD reactor exists, as it was suggested in the rumen by Kittelmann et al. (2013). Nevertheless, eukaryotes are far from being well studied and known in AD reactors. Thus, their putative role inside the AD process, and especially here in the case of the establishment of mesophilic anaerobic conditions during the start-up phase of a full-scale anaerobic reactor, remains unclear. Indeed, most of the studies neglect their existence as they are rarely mentioned in the context of anaerobic digestion. Therefore, future works on the microbial monitoring of the AD process should also include eukaryotes, as they could be important regulators of the bacterial and archaeal community dynamics. Further metagenomic and metatranscriptomic studies of the eukaryotic fraction involved in anaerobic digestion could bring also valuable information about their potential action and role in the AD process such as predator of bacteria and archaea or on the contrary as symbiotic hosts in case of some archaea.

4. Conclusions

This study showed that biogas production can be initiated in a full-scale farm reactor using cold run-off water and stale cattle slurry as seeding material. The microbial transition from psychrophilic communities naturally present in stored cattle slurry and the one producing methane at mesophilic conditions, was evidenced by fluctuations in the microbial community structure in terms of diversity, evenness and richness. Significant correlations between some eukaryotic organisms and bacteria, archaea and studied ecological parameters indicated that this domain of life should be better investigated to reveal their potential interactions with other microbes having crucial functions during the anaerobic biomass degradation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.04.040.

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