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Organic overloading affects the microbial interactions during anaerobic digestion in sewage sludge reactors

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Abstract

There is still a lack of information about microbial interactions of anaerobic digestion microbiome during process disturbance what impedes to predict the mechanisms that drive community dynamics on these events. This paper aims to determine how an organic overloading affects these interactions and to characterize in detail the microbiome structure and diversity in sewage sludge anaerobic reactors during an acidosis event. Two identical sewage sludge anaerobic reactors were subjected to an organic loading shock by adding glycerol waste. As consequence, volatile fatty acids accumulated after only 24 hours (up to 2.5 g/L) while *Bacteroidales* and *Methanomicrobiales* became displaced by *Firmicutes* and *Methanosaeta* sp, showing that reactor acidosis can occur without an immediate decline of this methanogen. Network analysis revealed 9 clusters of co-occurring microorganisms with different behaviors during overloading. At first, *Veillonellaceae* family, the main glycerol degrading, associated with *Candidatus* Cloacimonetes, volatile fatty acids fermenters, increased their relative abundance in detriment of the syntrophic bacteria; although as conditions become more acidic, these groups were displaced by other fermenters like *Porphyromonadaceae* and *Chitinophagaceae*. Eventually, the methanogenesis failed 72 h after organic overloading, when pH reached values lower than 6. Overall, our results showed a succession of functionally redundant microorganisms, most likely because of niche specialization during organic overloading. The detailed temporal analysis elucidated the processes governing the dynamics anaerobic digestion microbiome, a knowledge required to develop anaerobic digestion management strategies based on its microbiome during process disturbances.

Keywords

Anaerobic microbiome; glycerol; *Methanosaeta;* sludge digestion; *Veillonellaceae*.

1. Introduction

Anaerobic digestion (AD) is a biological process governed by a complex consortium of microorganisms that works symbiotically to degrade organic matter into methane, water and carbon dioxide. The variability of AD microbial community is often related with AD performance (De Vrieze et al., 2013). Hence, knowledge about the structural organization and functioning of these communities, especially during AD imbalance events, is essential to improve the AD efficiency and allows an appropriate process management (Carballa et al., 2015).

In recent years, an increasing body of literature has assessed the influence of environmental selectors, like substrate type, ammonia concentration or temperature, in the composition of AD microbiome (Regueiro et al., 2016; Vanwonterghem et al., 2015; Zhang et al., 2014). Organic loading rate (OLR) is a critical AD operational parameter that must be controlled to avoid disturbances in the process (Abendroth et al., 2015; Rétfalvi et al., 2011). An OLR shock usually causes an imbalance between hydrolysis/acidogenesis and methanogenesis steps. Consequently, volatile fatty acids (VFA) accumulate, causing a pH drop and leading to methanogenesis failure (He et al., 2017).

Under high VFA concentrations and low pH values, hydrogenotrophic methanogens, that are more tolerant to stresses, dominate the metabolic pathways (Fotidis et al., 2014; Goux et al., 2015). However, the behavior of bacterial communities during organic overloading disturbances is more complex due to the high number of species and their functional redundancy (Carballa et al., 2015; De Vrieze et al., 2013). Even when the overloading is caused by the same co-substrate results of different studies are contradictory. For example, Regueiro et al. (2015) used glycerol to induce OLR shocks in pig-manure reactors observing the rise-up of *Bacteroidetes* and *Actinobacteria phyla*

and the overall decrease of *Firmicutes* despite the growth of *Tissierallaceae* family. In contrast, in sewage sludge digesters under glycerol-induced OLR shocks, *Bacteroidetes* decreased while *Firmicutes* increased due to the rise of *Veillonellaceae; Ruminococcaceae* and *Clostridiales* Family X taxa (Ferguson et al. 2018; Braz et al. 2018; Ferguson et al. 2016). Similarly, the community response is not predictable by the feedstock or the inoculum used in the reactors. For instance, in sewage sludge digesters under overloadings induced with sugar beet pulp, *Bacteroidetes phylum* did not changed in contrast with previous studies (Goux et al. 2015).

These conflicting results indicate that the knowledge about mechanisms controlling changes in AD microbiome composition is still insufficient to predict the dynamics of microbial community in AD process. Microbial community assembly processes might include stochastic and deterministic mechanisms (Zhou et al., 2013, Ofiteru et al., 2010). However, a growing body of literature indicates that deterministic processes, particularly environmental selectors and species interactions, guide long-term population changes in AD steady-state reactors (Peces et al. 2018; Ju et al. 2017; Lucas et al. 2015; Vanwonterghem et al 2014; Fernández et al. 1999). During OLR shocks, AD microbial community is affected by the external stressors (Ferguson et al. 2018; Xu et al. 2018; Goux et al. 2015). Nevertheless, the importance of biotic interactions during overloadings remains obscured as both synchronised and divergent changes in composition has been observed (Ferguson et al. 2018; Braz. et al., 2018; Goux et al. 2015).

Co-occurrence networks can reveal the relationships between different microbial groups in complex systems (Deng et al., 2012; Faust et al., 2012), but only few studies have applied them to disentangle microbial interactions in AD. Xu et al. 2018, used this analysis to find the connections between microorganisms and operational parameters

during and organic overloading event in anaerobic digesters, observing that the *Firmicutes* phyla was the group most connected with biogas production. Rui et al. (2015) investigated the microbiome of 43 anaerobic reactors using co-occurrence networks finding that, despite high variations among microbial communities, the AD core-community is organized into functional groups of microorganisms.

Understanding the mechanisms that govern the microbial species interactions during AD imbalance events is needed to develop adequate process management strategies and to avoid process failure (Widder et al., 2016). The importance of microbial interactions in AD and other microbial systems during perturbations, has long being acknowledged (Koch et al. 2013; Fernández et al. 1999) but remains scarcely explored. Therefore, this study aimed to investigate the biotic interactions and dynamics that are stablished during an OLR shock in sewage sludge digesters microbiomes in a thorough temporal scale. To achieve this goal, the microbial communities of two parallel anaerobic digesters feed on sewage sludge and operated identically, were exhaustively studied with high-throughput amplicon sequencing during steady-state and acidosis induced with glycerol as co-substrate.

2. Materials and methods

2.1. Anaerobic reactors and experimental periods

Two identical (R1 and R2) anaerobic continuous stirred (160 rpm, Heidolph RZR 2041) tank reactors with a working volume of 14 L were operated in mesophilic range (37°C) at a hydraulic retention time (HRT) of 20 days. They were inoculated with approximately 14.0 g VSS/L (14.5 g VS/L) of anaerobic sludge from a full-scale sewage sludge mesophilic anaerobic digester. The reactor feeding was prepared every other week mixing primary and secondary sludge (70:30, v/v) and kept at 4 °C until

needed. During stabilization period (day 0 to 94), both reactors were operated at an OLR of 1.8 g COD/ L d. Then, two periods were defined to evaluate the effect of organic overloading on the AD microbiome: pre-shock (period 1) and shock (period 2). During period 1 (day 95 to 102), the reactor biomass was sampled twice a day (at 10 and 16 h) to determine the microbiome fluctuation inherent to steady-state operation. On day 102, the OLR was increased to 5 g COD/L d by adding glycerol waste, a residue from a biodiesel production plant, and it was maintained until the end of the experiment (day 105). The glycerol waste was selected based in its substantial worldwide production (more than two million tons per year) and because it improves the methanization efficiency, making it a great co-substrate for AD process (Ciriminna et al. 2014; Regueiro et al. 2012). The characterization of feedstock used during stabilization, period 1 and 2 are shown in table 1. During this period (day 102 to 105), the AD microbiome was examined by an intensive sampling scheme, namely biomass was sampled 2-4 times per day (at 10, 13, 16 and 19 h). The detailed sampling scheme is presented in Table A1 (supplementary information). Biomass samples consisted of well-homogenized 2 mL triplicate aliquots that were stored at -80°C until the DNA extraction. Additionally, biogas production was measured online; pH, VFAs, total (TA) and partial (PA) alkalinity and total chemical oxygen demand (CODt) were measured each time that biomass samples were collected; and biogas composition was measured once a day.

2.2. Analytical methods

Biogas production was measured with gas flow meter (µ flow - Bioprocess control) and its composition was analyzed by gas chromatography (HP5890 Series II, thermal conductivity detector, stainless steel column and helium as carrier gas) (García-

126 Gen et al., 2015). Physico-chemical parameters, such as pH, CODt, total solids (TS),
127 volatile solids (VS), TA and PA were measured according to standard methods (APHA,
128 2012). VFA concentrations were determined by gas chromatography using a Hewlett
129 Packard 5890A device equipped with a flame ionization detector (García-Gen et al.,
130 2015). Reactor performance was compared between duplicates using a T-test,
131 considering p-value ≤ 0.01, in IMB® SPSS® Statistics Version 22.
132
133 *2.3. DNA extraction and sequence processing*
134 Biomass samples were thawed and homogenized thoroughly in a vortex. For
135 each sample, DNA was extracted from 1 mL biomass for which the supernatant was
136 removed after 3 min of centrifugation at 20 000 g using the Stool DNA Isolation Kit
137 (Norgen, Thorold, Canada) according to manufacturer instructions. Total DNA
138 concentrations were quantified in a Qubit fluorometer (Thermo Fisher Scientific,
139 Waltham, MA, USA) and checked for size integrity by standard electrophoresis.
140 Fragments of the 16S rRNA gene were amplified for both *Bacteria* and *Archaea*
141 domains with primers including Illumina adaptors and barcodes. The V3V4 region of
142 the bacterial 16S rRNA gene was amplified with the primer pair S-D-Bact-0341-b-S-17
143 and S-D-Bact-0785-a-A (Klindworth et al. 2013). For *Archaea*, the V2V3 region was
144 amplified with the primer set Arch1F and Arch1R (Cruaud et al., 2014). An initial
145 polymerase chain reaction (PCR) was carried out with 3 ng of extracted DNA, 100 nM
146 for bacterial primers or 200 nM for archaeal primers and 1X Q5® High Fidelity Master
147 Mix (New England BioLabs) which contains the Q5® High Fidelity DNA polymerase,
148 2mM $MgCl_2$ and 200 µM dNTPs. The PCR was performed as follows: an initial
149 denaturation at 98 ºC for 30 s, followed by 20 or 22 cycles for *Bacteria* and *Archaea*
150 primers respectively that consisted on denaturation (98 ºC) for 10 s, annealing for 20 s

and extension (72 ºC) for 20 s; finishing with a final extension step at 72 ºC during 2 min. The annealing temperature was 50 and 48 ºC for bacterial and archaeal primers respectively. These PCRs were followed by a second PCR to add the Illumina adapters and barcodes to the amplicons that was run under similar conditions but only for 15 cycles and with an annealing temperature of 60 ºC. Once constructed, DNA libraries were checked for size and concentration using a Bioanalyzer (Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). After library preparation, samples were quantified by qPCR, pooled and sequenced in a MiSeq (Unidad de Genómica, Parque Científico de Madrid). Paired-end reads (2 × 300) were generated according to the manufacturer instructions (Illumina, Inc.).

The obtained reads were de-multiplexed and trimmed to remove Illumina adapters, barcodes and sequencing primers. Then, paired reads were merged as previously described (Eren et al., 2013) removing any read containing bases with a quality score under 30, and using a minimum overlapping length 50 basepairs. Any sequence with indeterminations was also removed from the analysis. Quality-filtered sequences were analyzed for chimeras with VSEARCH (Rognes et al., 2016). After quality filtering, 3.1 million bacterial and 1.4 million archaeal sequences were clustered into 23,703 and 711 different OTUs, respectively (Table A2) using an open-reference approach into Operational Taxonomic Units (OTU) at a 97% cutoff for sequence similarity in the QIIME pipeline v.1.9.1 (Caporaso et al., 2010). The taxonomic affiliations of OTUs were determined using the RDP classifier (Wang et al., 2007) against the SILVA v123 database (Quast et al., 2013).

2.4. Diversity and network analysis

Richness and evenness indices were used to measure the within-sample bacterial and archaeal community diversity due to organic overloading. Community richness was estimated as the number of distinct OTUs, while community evenness, that measures the equitability between the different species present in a community, was determined as the Simpson evenness (E) index. Differences in community structure were assessed using the Bray-Cutis dissimilarities. This index was used to determine the community turnover through time of each reactor using a fixed window analysis to assess the degree of community change every three days (Read et al. 2011). For this analysis, we calculated the community structure change between the beginning and end of a period of time as the Bray-Curtis dissimilarity within the same reactor. To make possible the comparison of community turnover between the two experimental periods, period 1 was subdivided into windows of the same temporal length than period 2 (3 consecutive days). For period 2 dissimilarities were calculated between the initial and last day (from 102 to 105 days). Also, differences in community structure were visualized by transformed-based Principal Component Analysis (tbPCA) after applying Hellinger standardization to community data. Then community structure relationship with operational parameters was analyzed by calculating the multiple regression of the environmental variables with the ordination axes and the significance tested by a permutation test. Next, significantly correlated (p-value ≤ 0.01) operational parameters were projected onto the ordination. These analyses were performed using the R statistical environment with the Vegan package (Oksanen et al., 2016).

To find potential associations among microorganisms, a co-occurrence network analysis was performed based on the abundant taxonomic groups at genus level. Microbial networks were constructed with CoNet v.1.1.1. (Faust et al., 2012). Prior to analysis, OTUs were grouped by taxonomy affiliations at genus level, and then the 323

genera with relative abundances over 0.1% in any of the samples were included in the analysis. Compositional effects were avoided using bootstrap-renormalization procedure (Faust et al., 2012). Two correlations (Spearman and Pearson) and two distances (Bray Curtis and Kullback-Liblener dissimilarities) were used to infer relationships (edges) between microorganisms (nodes), discarding any linkage not supported by at least two measurements. Benjamini-Hochberg procedure was used to correct for multiple comparisons, discarding edges over the threshold of $q \leq 0.05$. Resulting network was then analyzed to detect highly interacting groups of nodes (clusters) using GLay algorithm (Su et al., 2010).

3. Results and discussion

3.1. Rapid change of operational parameters to organic overloading

Two replicate anaerobic reactors were seeded with the same inoculum and operated for 105 days, that were divided into three operational periods: stabilization period (day 0 to 94), pre-shock phase or period 1 (95-102 d) and overloading phase or period 2 (102-105 d). All measured operational parameters were statistically similar in both reactors throughout their operation (p-value ≤ 0.01). In stabilization period, the CH_4 production was 0.9 g COD/L d, resulting in a methanization efficiency of around 45% during a very stable operation (Table 2). The same steady trend was observed for pH, TA, PA and VFAs values that did not vary over the stabilization period. Likewise, in period 1, reactors performance remained constant and comparable to the stabilization period (Fig. 1). These results are similar to previous data in sewage sludge anaerobic digestion literature (Razaviarani and Buchanan, 2014; Rétfalvi et al., 2011).

During OLR shock (period 2), the operational parameters showed three different phases (Fig. 1). First, in the initial 9h of overloading, no significant changes were

observed in reactors functioning, except for the methanization efficiency, which dropped to 22%. Second, between 9 and 33 h large changes were observed when the VFA concentration rose to 2.4 g/L, and consequently, pH and alkalinities decreased. Biogas composition changed from 61% to 30% CH_4 and from 30% to 60% CO_2 (Fig. 1A) in response to a pH decrease and consumption of PA (Fig. 1B). Finally, after 33 h of overloading to the end of the experiment (72 h); the TA, PA and total VFA concentrations stabilized (VFAs > 5 g/L) while the CH_4 fraction in the biogas increased to 42% (48h), probably due to the decline of CO_2 production. The experiment was stopped after 72 h of OLR shock, when pH was below 6 and CH_4 production decreased to 0.49 COD/L d. Regardless the changes in some operational parameters, the TS and VS remained stable during all experiment period.

Although butyric, acetic and valeric acids also accumulated, propionic acid had the highest concentration among VFAs (Fig. 1C) as previously observed by authors that used glycerol to induce the organic overloading (Ferguson et al., 2016; Regueiro et al., 2015; Rétfalvi et al., 2011). However, the observed shifts of operational parameters in these studies occurred later (after 2 to 5 days of overloading) than in study (only 24h). Differences in reactor operation could explain this observation, like more TA available or prior acclimatization of the community to glycerol as co-substrate.

3.2. Microbial community diversity and structure reflected the OLR shock

The OLR shock decreased archaeal but not bacterial richness. It also affected the uniformity of both communities although in an opposite trend: *Bacteria* became more uneven, while *Archaea* became more even (Fig 2). A reduction on microbial diversity has been observed in other studies of OLR shocks in anaerobic reactors (Fitamo et al., 2017; Kampmann et al., 2014; Xu et al., 2018). It has been proposed that the increase of

VFAs induced by the OLR shock inhibits the growth of some microorganisms (Fitamo et al., 2017). In this sense, our results suggest that while several *Bacteria* become inhibited resulting in the dominance of less organisms, some *Archaea* might perish under period 2 conditions causing a loss of species.

Community structure was also modified during the organic overloading following similar changing trends in both R1 and R2 that were correlated with the increase of OLR, VFAs accumulation and the decrease of pH (p-value ≤ 0.01; Fig 3); in concordance with other observations of structure shift during overloading influenced by those operational parameters (Goux et al., 2015; Hori et al., 2006; Xu et al., 2018).

In addition, community structure was different between R1 and R2 even though they were inoculated from the same source. Communities with diverging taxonomic compositions under stable environments have been repeatedly observed in bioreactors and other environments (Fernandez-Gonzalez et al., 2016; Louca et al., 2018) which can be the result of stochastic immigration and ecological drift (Zhou and Ning, 2017); although, as recently discussed by Louca et al. (2018), taxonomic turnover is generally not explained by ecological drift and can be driven by intrinsic, mostly biological, deterministic processes.

The temporal change of the AD microbiome or community turnover, became accelerated during overloading. In period 1, community turnover were 19±2 and 8±4% for *Bacteria* and *Archaea* respectively, likewise to other observations on bioreactors during steady-state periods (Hai et al., 2014). In contrast, during period 2 microbial community turnover largely increased (*Bacteria*: 48±2% and *Archaea* 39±1%) indicating the strong influence of the sudden OLR shock provoked by the addition of glycerol as a new co-substrate on the microbial community organization and its low resistance to change in face of a perturbation. Microbial communities of wastewater

treatment systems can have short reaction times toward sudden external perturbations (Braz et al. 2018; Koch et al. 2013). In other studies, when the microbial community was gradually adapted to a new co-substrate, for example, by applying a gradual OLR increase or pulse feeding; the AD microbiome structure was more resistant to organic overloading or other process imbalances (Ferguson et al. 2016; De Vrieze et al. 2013; Ghasimi et al. 2015).

3.3. Organic overloading changed community composition and induced a temporal succession of co-occurring microorganisms.

Both *Bacteria* and *Archaea* community compositions were similar between replicated reactors despite the differences in community structure (Fig. 4). Community composition was quite stable in period 1, when over 70% of *Bacteria* belonged to phyla *Bacteroidetes, Firmicutes, Proteobacteria* and *Chloroflexi* (Fig. A1) and the most abundant genera were *Sedimentibacter* sp. and two unknown *Bacteroidales* taxa (Fig 4A, Fig. A1). Most *Archaea* taxa were methanogens, dominated by *Methanosaeta* sp. and an unknown *Methanomicrobial* genus (Fig. 4B, Fig. A1). These groups of organisms are commonly found in sewage sludge anaerobic digesters and many have been described as part of the core community of full-scale mesophilic sludge digesters (Mei et al., 2017).

In period 2, community composition was altered and a temporal succession of the dominant microorganisms occurred (Fig 4). The changes included initial increments of *Veillonellaceae* and *Treponema* bacterial groups as well as *Methanosaeta* and *Methanoculleus* methanogenic genera; microorganisms that decreased towards the end of the experiment. It is widely known that AD microbial community composition is commonly affected by organic overloading (Goux et al., 2015; Regueiro et al., 2015).

AD process is possible due to the activity of several microorganisms that form a highly-interconnected community in which several species establish different types of biological associations, such as mutualism, symbiosis or competence. The temporal succession of organisms and their potential associations during the organic overloading was explored by a network analysis on the dominant fraction of the microbial community (relative abundances > 0.1% in any of the samples). Nine highly interconnected groups or clusters of co-occurring microorganisms could be identified (Fig 5, Fig A2), that altogether contained 63.7 ± 6.9 and 95.5 ± 1.9 % of the bacterial and archaeal communities, respectively. These clusters were characterized by distinct temporal trends, indicating the occurrence of a temporal succession of related groups of microorganisms during the OLR shock (Fig. 6, Fig. A3). Clusters also had different taxonomic compositions (Fig. 7, Table A3). While clusters 2, 4, 6 and 7 contained both archaeal and bacterial taxa; clusters 1, 3, 5, 8 and 9 were solely composed by *Bacteria*. Only abundant clusters (total accumulated relative abundance by domain > 5% on any sample, Fig. 6) are further discussed.

During the first 9 hours of overloading and before operational variables showed relevant shifts, the relative abundances of several clusters changed respect to the baseline observed during period 1. While clusters 3 (only *Bacteria*) and 7 increased, clusters 4, 5, 6 and 9 decreased (Fig. 6); trends that overall, were maintained during the increment of propionate concentration between 9 and 48 h of overloading (Fig. 1). The most abundant organisms in cluster 3 were unknown members of *Veillonellaceae* family (Fig 7, Table A3), that is a group of fermentative organisms commonly detected in anaerobic environments rich in organic matter (Marchandin and Jumas-Bilak, 2014), which

reached a maximum value of 21.9 % of bacteria relative abundance. In these reactors, this group likely drove the glycerol fermentation into propionate, a process described for many genera of this family such as *Anaerosinus* (Strömpl et al., 1999). The second most abundant organisms in cluster 3 was *Cloacamonaceae* uncultured genus W22, which is the main responsible for the rise of *Candidatus* Cloacimonetes (WWE1) phylum observed during period 2 (Fig. A1.A). The increment of *Candidatus* Cloacimonetes (WWE1) have been already reported during OLR shocks in anaerobic digesters related to the accumulation of propionate as these organisms might ferment aminoacids and syntrophically oxidize propionate into H_2, CO_2 and acetate (Goux et al., 2015; Hagen et al., 2014; Pelletier et al., 2008). Therefore, the increase of the uncultured W22 genera is likely linked to the production of propionate by *Veillonellaceae*, suggesting a commensal relationship between both organisms. Many other organisms of cluster 3 belonged to *Spirochaetes* phylum that might be able to perform syntrophic acetate oxidation (Lee et al., 2013).

In *Bacteria*, cluster 3 displaced microorganisms mostly from clusters 4, 6 and 9, which are rich in different types of hydrolytic and fermentative bacteria. Cluster 4, that was the dominant bacterial group in period 1, was mostly composed by an unknown *Bacteoidales* family SB-1 (Fig 7, Table A3). *Bacteroidales* is a broad order containing mainly anaerobic saccharolytic organisms, although other substrates might be used as well (Krieg et al., 2010). Other groups found within cluster 4 were also hydrolytic or fermentative bacteria like *Ruminoccoccaceae*, *Anaerolinaceae* T78 and *Synergistaceae* vadinCA02 group, from *Synergistetes* phylum, that exhibits obligatory anaerobic aminolytic metabolism. Cluster 9 was also dominated by fermenters, mainly *Sedimentibacter*, that only ferments pyruvate and amino acids, accompanied by other fermentative *Firmicutes* like *Anaerovorax* (Fig 7, Table A3). Most syntrophic bacteria

(i.e.: *Syntrophomonas* or *Syntrophus*, Fig 7, Table A3) located in Cluster 6, also diminished in abundance during the overloading. The latter can be related to the high propionate concentrations, which quickly reached values over 2 g/L (24h) and can inhibits syntrophic bacteria (Ban Q et al., 2013).

Archaeal cluster 7 also incremented during the initial 48 hours of overloading, displacing archaea within clusters 4 and 6 which only contains hydrogenotrophic methanogens (Fig 7, Table A3). Cluster 7 contained the largest archaeal diversity, with 9 taxa, representing on average the 58.3 ± 15.7 % of archaeal community. The principal organisms in the cluster was *Methanosaeta* (up to 68.5 % of Archaea), which was accompanied by *Methanoculleus*, *Methanobrevibacter* or *Methanobacterium*, all hydrogenotrophic methanogens. *Methanosaeta* became the most dominant methanogen during most of the overloading period. It is a well-known acetoclastic methanogen that grows in low levels of acetate. It has been suggested that *Methanosaeta* could survive only in low VFA concentrations (Griffin et al., 1998). However, our data showed that *Methanosaeta* can temporally withstand VFAs concentrations of mostly propionic acid, between 2.5 – 5.0 g/L. Franke-Whittle et al. (2014) also reported a single observation of an AD reactor with high VFA concentration (3.9 g/L of propionate and 1.25 g/L of acetate) dominated by *Methanosaeta*. These data suggest that the shift between hydrogenotrophic (*Methanoculleus* sp.) and aceticlastic (*Methanosaeta* sp.) methanogens as a warning indicator of acidosis during high OLR (Goux et al., 2015) is not universal.

After 48h of overloading, trends of the most dominant clusters changed. By then, more than 5.5 g/L of VFAs, mostly propionic and butyric acids, were already accumulated, lowering the pH (6.2) in the reactors. pH decreased even further after 72 h (5.8), when valeric acid concentrations also increased. In parallel, a succession between

different types of fermentative bacteria was observed. After 48h of overload, *Veillonellaceae* and its cohort (cluster 3) become replaced by *Bacteroidales* and *Synergistetes* of cluster 4, including a transitional increment of *Porphyromonadaceae* (cluster 5) at 54 h and increment of *Chitinophagaceae* (cluster 2) at the end of the experiment (Fig. 7, Table A3). Simultaneously, *Methanosaeta* (cluster 7) drastically dropped in abundance to finally be replaced by the putative hydrogenotrophic methanogen, *Methanobacteriales*_WSA2 (cluster 2, Fig. 7, Table A3); although the methane production strongly decreased.

The intense timeframe of this study allowed to partially unveil fast-changing biotic interactions in the AD microbiome. Acidogenic bacteria are known to react immediately when exposed to high substrate amounts by increasing their metabolic activity and cell numbers (Schnürer et al. 1999). The acidogenic that reacted faster and more intensely to the OLR shock was *Veillonellaceae* family. The rise of this taxon during glycerol OLR shocks seems to be common (Braz et al. 2018; Ferguson et al. 2018), probably due to its ability to degrade glycerol, but it is not universal (Regueiro et al. 2015). Similarly, a succession of microorganisms seems to be a common feature after an OLR shock, but the specific populations that are involved in the temporal succession are far from common among AD digesters, even in the case of using the same type feedstock or co-substrate (Braz et al 2018; Fitamo et al 2017; Goux et al. 2015; Regueiro et al. 2015).

Functional redundancy has been proven in long-term AD reactors (Theuerl et al 2015) and it is known that other microorganisms can overtake missing functions during perturbations (Fernández et al. 1999). The observed temporal succession among different taxa of the same functional groups (i.e.: fermenters, methanogens) during the changing conditions of the overloading event is a strong indication of the importance of

399 temporal metabolic niche effects (mechanisms selecting organisms able to exploit
400 specific methabolic pathways, Louca et al. 2018) in AD microbiome under OLR shocks.
401 In these events, several factors are selecting between functionally redundant
402 microorganisms. The introduction of a new substrate, the availability of its products of
403 degradation that promotes commensalism relationships, the changing pH conditions and
404 other biotic interactions like competition, are mechanisms creating new niches. Co-
405 occurring microorganisms likely established positive biological interactions, that in
406 some cases could be inferred (i.e. cluster 3). In other cases, co-occurring organisms are
407 limited by similar factors (i.e. cluster 6). However, the lack of taxonomic resolution and
408 the large fraction of microbial diversity still undescribed obscure the detailed nature of
409 the biotic interactions. As our knowledge about uncultured species and their capabilities
410 advances, we would be able to uncover the detailed nature of the biological processes.
411
412 **4. Conclusion**
413 The effect of a sudden increase of OLR by the addition of glycerol as a co-
414 substrate affected AD process performance similarly in both replicated reactors,
415 producing a fast VFA accumulation, reactor acidification and final AD process failure.
416 The overloading induced a redistribution in the equitability of microorganisms and a
417 loss of *Archaea* species, but also an increment in community temporal turnover,
418 indicating the lack of resilience of these microbiomes that were not previously exposed
419 to the added co-substrate. AD microbiome turnover seems to be driven by community
420 internal dynamics during steady-state AD operation whereas, an OLR shock imposes
421 environmental selecting criteria. The changing environment during overloading caused
422 a temporal succession of functionally redundant microorganisms suggesting niche
423 specialization. Several mutualistic and competitive relationships among groups of

microorganisms occurred, although most of the details of those mechanisms remain obscured due to the lack of information about genetic and physiological capabilities of many microbial clades of AD microbiome. Increments in VFAs are not always adverse for *Methanosaeta*, and reactor acidosis can occur, at least transitorily, without a decline of this methanogen. Overall, the observed succession of functionally redundant microorganisms influenced by changes on environment and species interrelationships highlights that deterministic processes drive AD community dynamics during OLR shocks.

Conflicts of interest

None

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version

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FIGURE CAPTIONS

Fig 1. Reactors performance during periods 1 and 2 (average values from R1 and R2 and standard deviation). A) Organic loading rate (OLR), methane production and biogas composition; B) pH, total and partial alkalinity, C) acetic, propionic, butyric and valeric acid concentrations. In panel C, time is expressed as day - hour of the day.

Fig 2. Boxplot of community richness and evenness for *Bacteria* and *Archaea* domains during periods 1 and 2. The graphics show the values of the minimum, first quartile, median, third quartile and maximum. The box comprises 50% of the observations and the points are the outlier values.

Fig 3. Transformed-based Principal Component Analysis (tbPCA) ordination diplot for the bacterial (A) and archaeal (B) communities. The tbPCAs show the microbial community structure at the OTU level for the two anaerobic reactors during period 1 and period 2. Labels indicate the time in hours after the beginning of the OLR shock. Labels of samples taken during period 1 have been omitted for clarity. Blue vectors indicate incrementing values of the operational variables that are significantly correlated with community structure (p-value ≤ 0.01).

Fig.4. Community composition profile at the most detailed taxonomic level available. Relative abundance of the most prevalent taxa (those exceeding 1% in average) in bacterial (A) and archaeal (B) communities in R1 and R2. For period 1, the averaged relative abundances of each taxon are shown (details included in Fig. A1). Period 2 is organized by hours after OLR shock.

Fig 5. Co-occurrence network of abundant genera (relative abundances > 0.1%) connected by positive interactions ($q \leq 0.05$). The 9 clusters of highly interconnected microorganisms are shown. Node color indicates the cluster affiliation while node shape refers to the domain: circle for *Bacteria* and square for *Archaea*. For taxonomy assignment of nodes see Fig A2.

Fig.6. Temporal trends during organic overloading of the abundant clusters of co-occurring microorganisms divided by domain. Accumulated relative abundances of bacterial (A), and archaeal (B) organisms. For comparison, average values during period 1 are also shown and denoted as time 0 where error bars indicate standard deviations. Clusters were considered as abundant if the total accumulated relative abundance by domain was larger than 5% on any sample.

Fig. 7. Variation through time of the dominant microorganisms (relative abundances >1% in any sample) in AD reactors. Microorganisms best taxonomic resolution is

723 indicated on the right, were *(A)* indicates *Archaea*, *(B) Bacteria* and (un) undetermined
724 when it was not possible to identify the genus. Cluster affiliation of each microorganism
725 is showed on the left column. Time is expressed as day - hour of the day.

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Table 1. Total COD, OLR, TS and VS values of feedstock used during the stabilization period, period 1 and 2.

Feedstock characteristics	Stabilization period	Period 1	Period 2
Total COD (g O_2/kg)	36.8 ± 0.9	43.1 ± 4.2	111.1 ± 5.3
OLR (g COD/ L d)	1.8 ± 0.1	2.2 ± 0.2	5.6 ± 0.3
TS (g TS/kg)	32.1 ± 0.9	26.6 ± 2.0	74.3 ± 2.1
VS (g TS/kg)	22.8 ± 0.5	21.7 ± 1.6	65.2 ± 1.9

COD: Chemical oxygen demand; OLR: Organic loading rate; TS; Total Solids; VS: volatile solids

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Table 2. Anaerobic reactors performance during stabilization period (days 0-94). Results correspond to averaged values and standard deviations from the two reactors (n = 42).

Operational parameters	
OLR (g COD/L d)	1.8 ± 0.1
CH_4 (g COD/L d)	0.9 ± 0.2
pH	7.3 ± 0.1
TA (g $CaCO_3$/L)	3.6 ± 0.1
PA (g $CaCO_3$/L)	2.8 ± 0.1
TS (g/L)	20.1 ± 3.2
VS (g/L)	10.1 ± 1.1
Acetic acid (mg/L)	< 60
Propionic acid (mg/L)	< 30
Methanization efficiency (%)	45 ± 0.1

OLR: Organic loading rate; TA: Total alkalinity; PA: Partial alkalinity; TS: Total solids; VS: Volatile solids.

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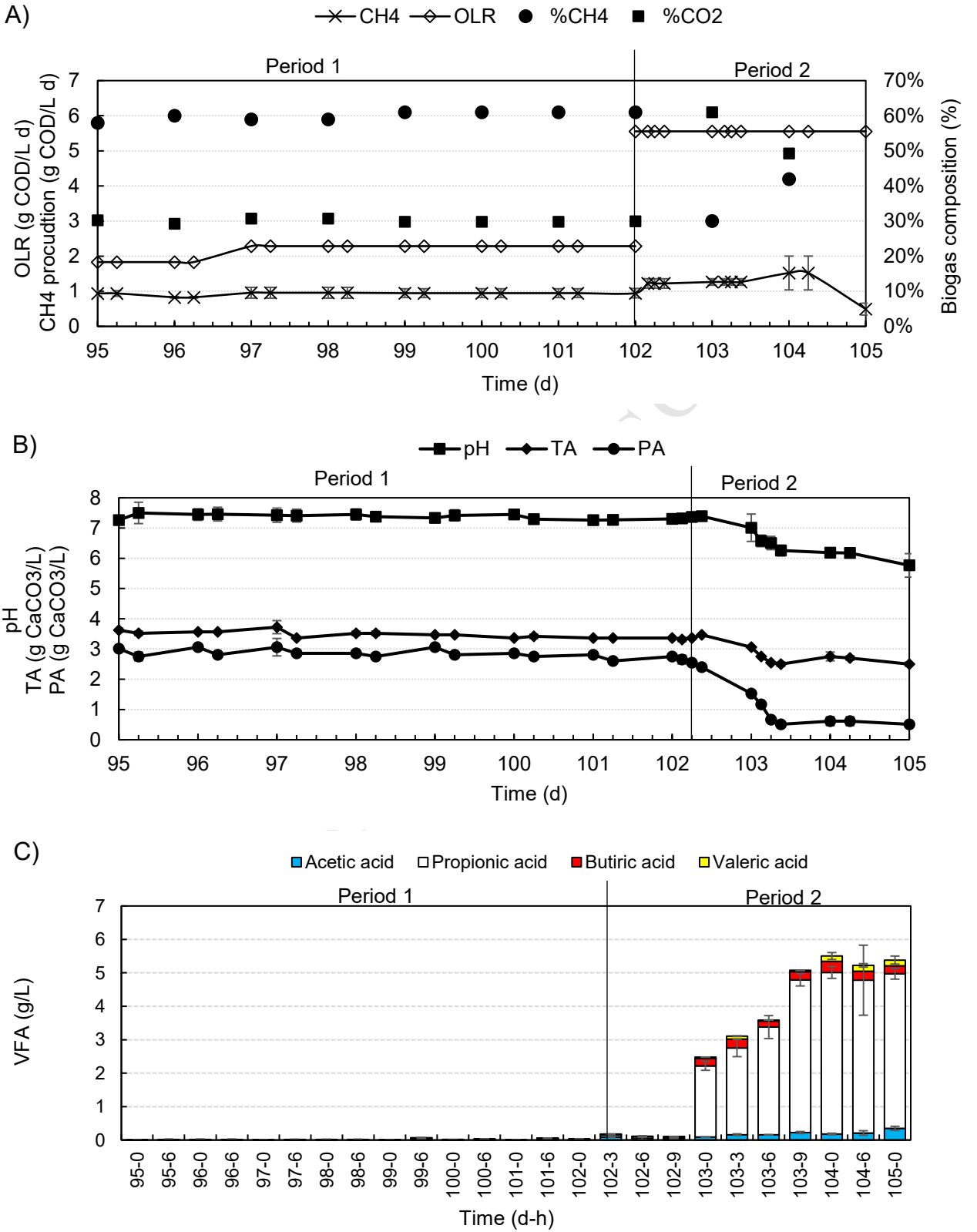
A)
CH4
OLR
%CH4
%CO2
Period 1
Period 2
OLR (g COD/L d)
CH4 procudtion (g COD/L d)
Biogas composition (%)
Time (d)
B)
pH
TA
PA
Period 1
Period 2
pH
TA (g CaCO3/L)
PA (g CaCO3/L)
Time (d)
C)
Acetic acid
Propionic acid
Butiric acid
Valeric acid
Period 1
Period 2
VFA (g/L)
95-0
95-6
96-0
96-6
97-0
97-6
98-0
98-6
99-0
99-6
100-0
100-6
101-0
101-6
102-0
102-3
102-6
102-9
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103-3
103-6
103-9
104-0
104-6
105-0
Time (d-h)

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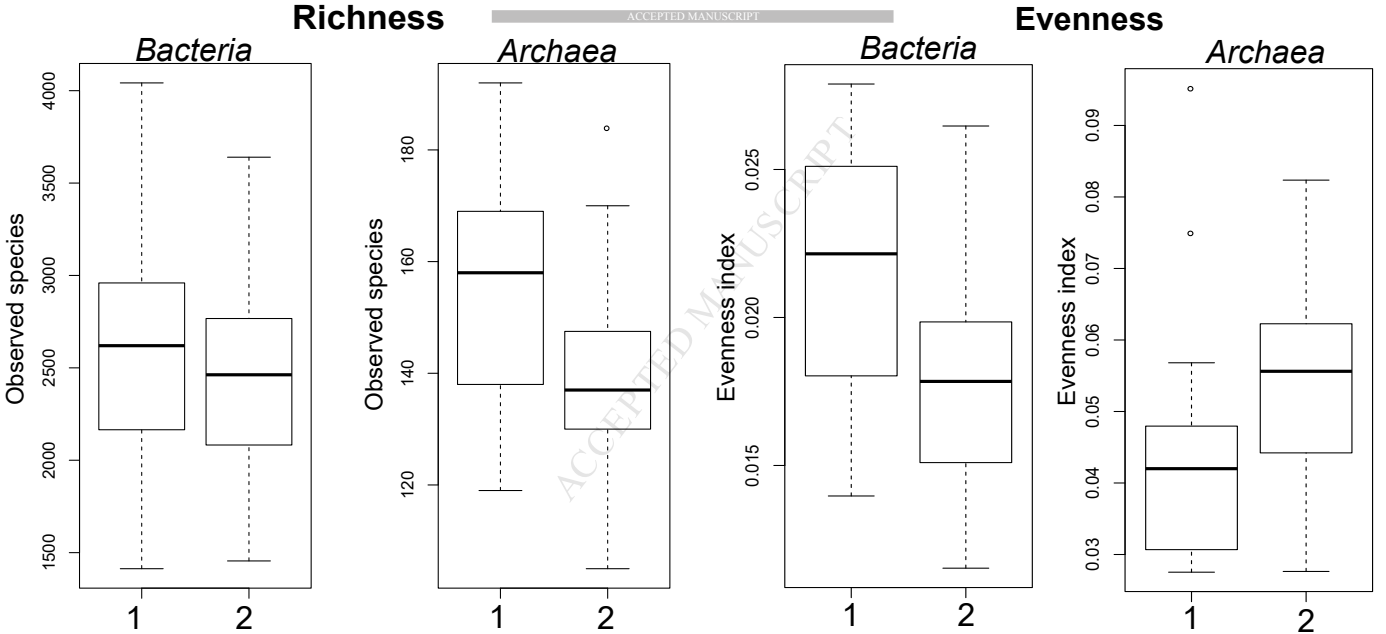

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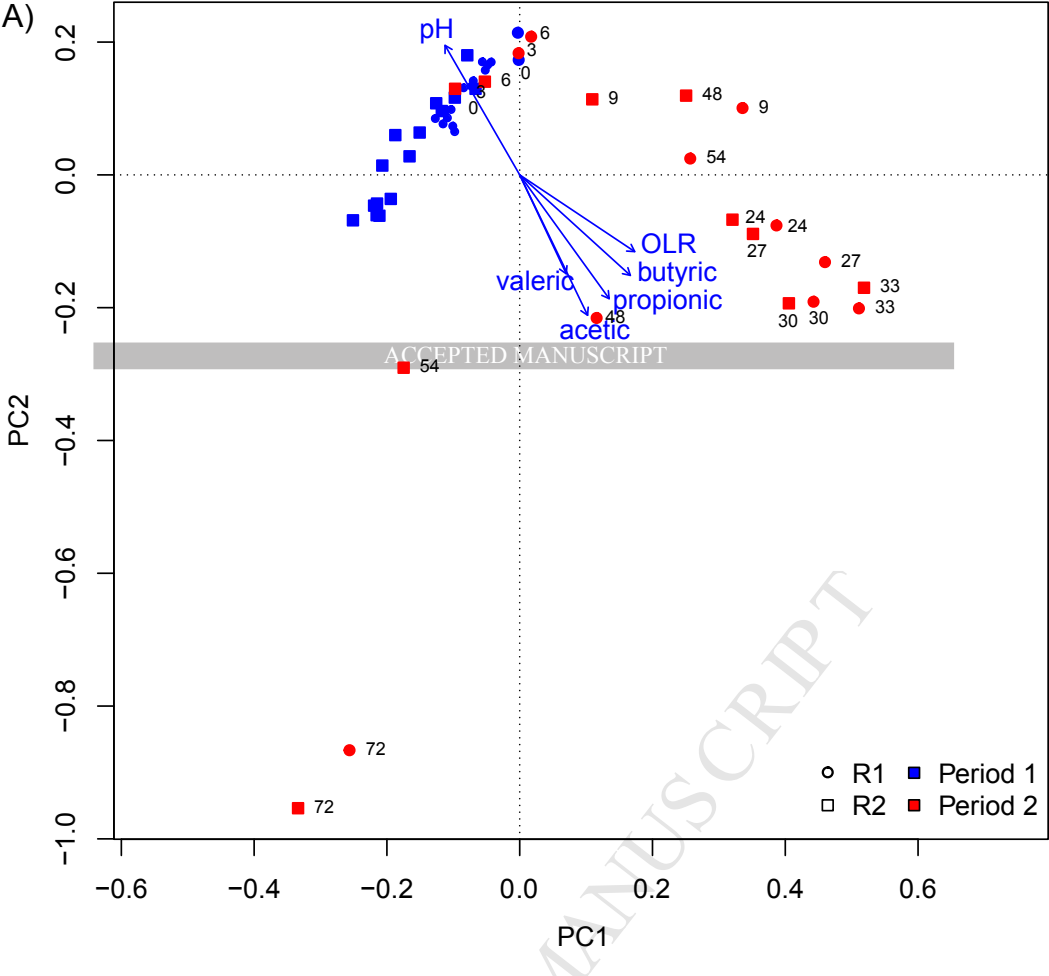

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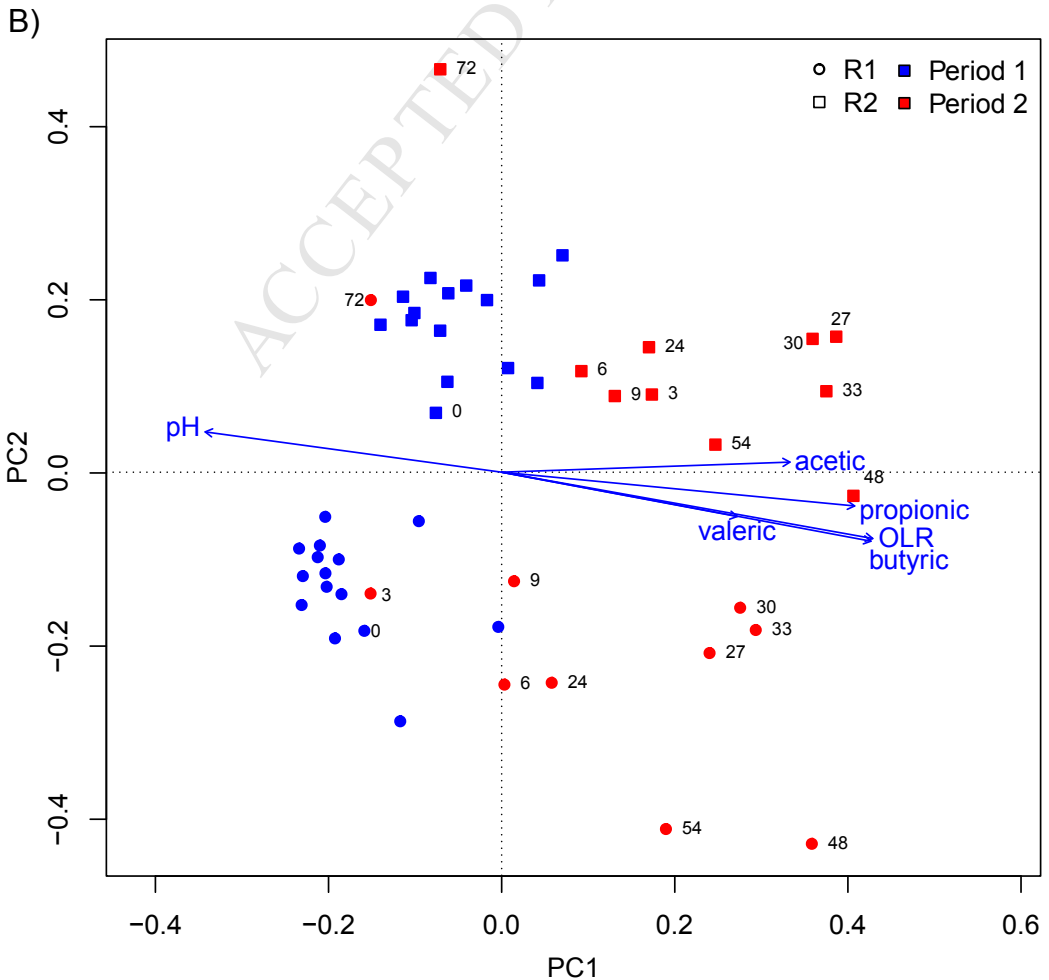

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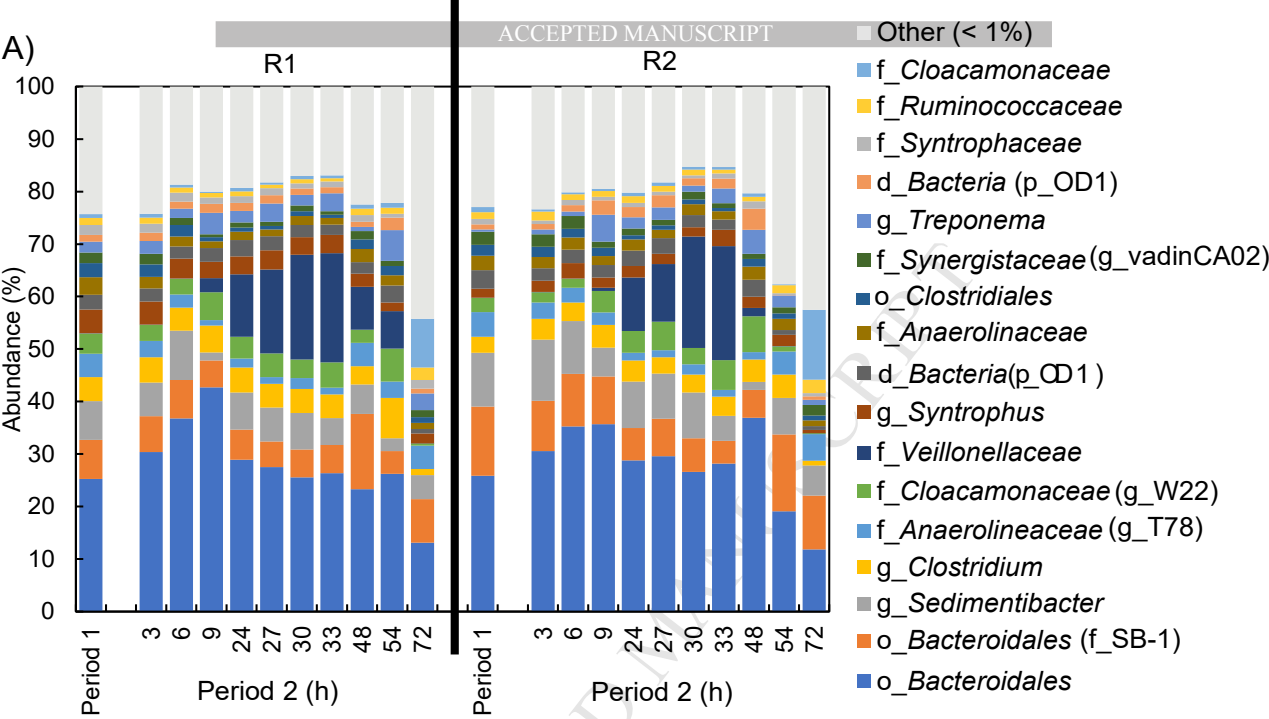

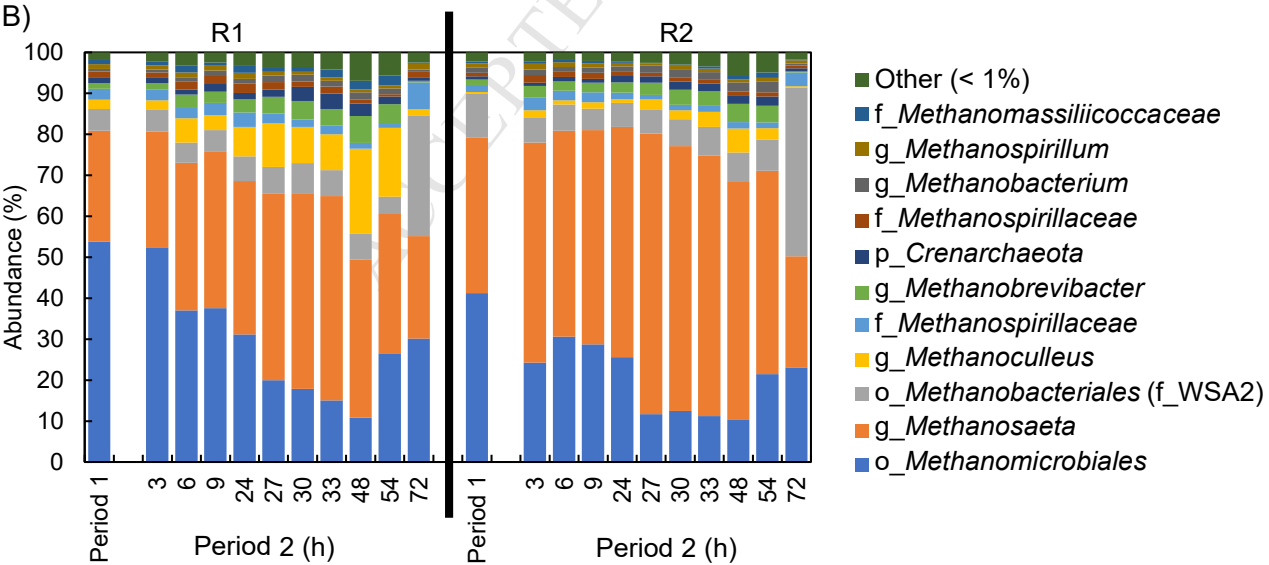

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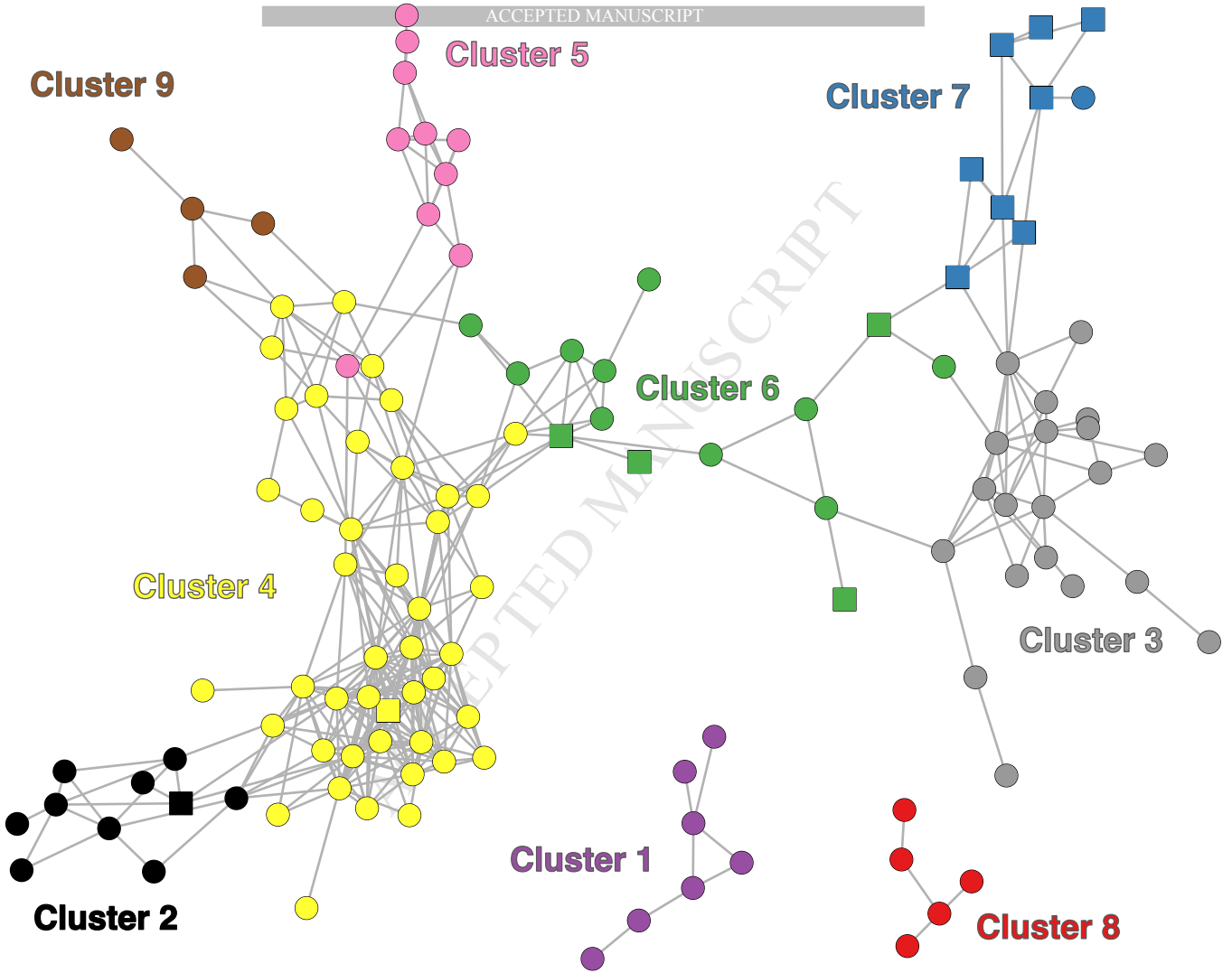

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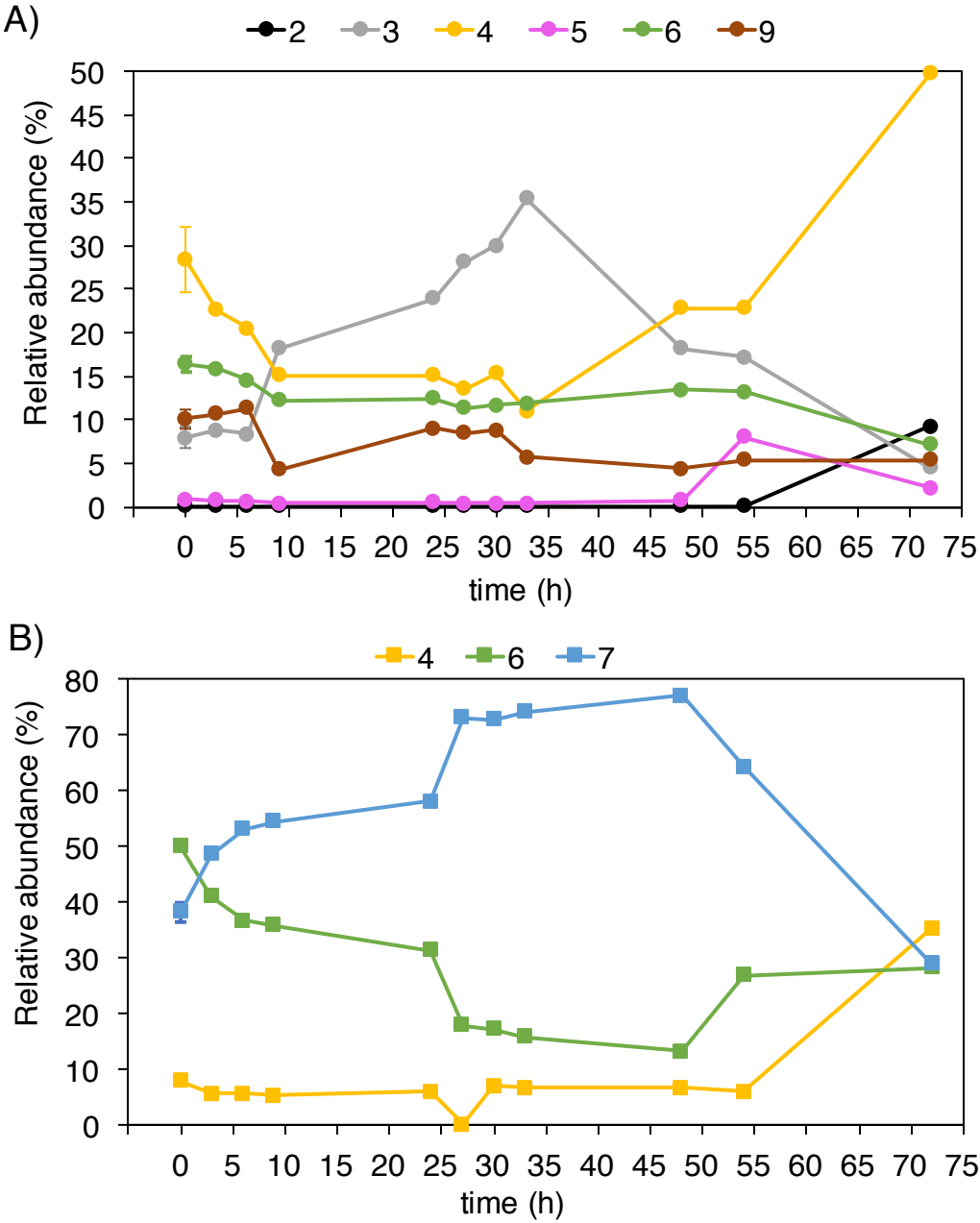
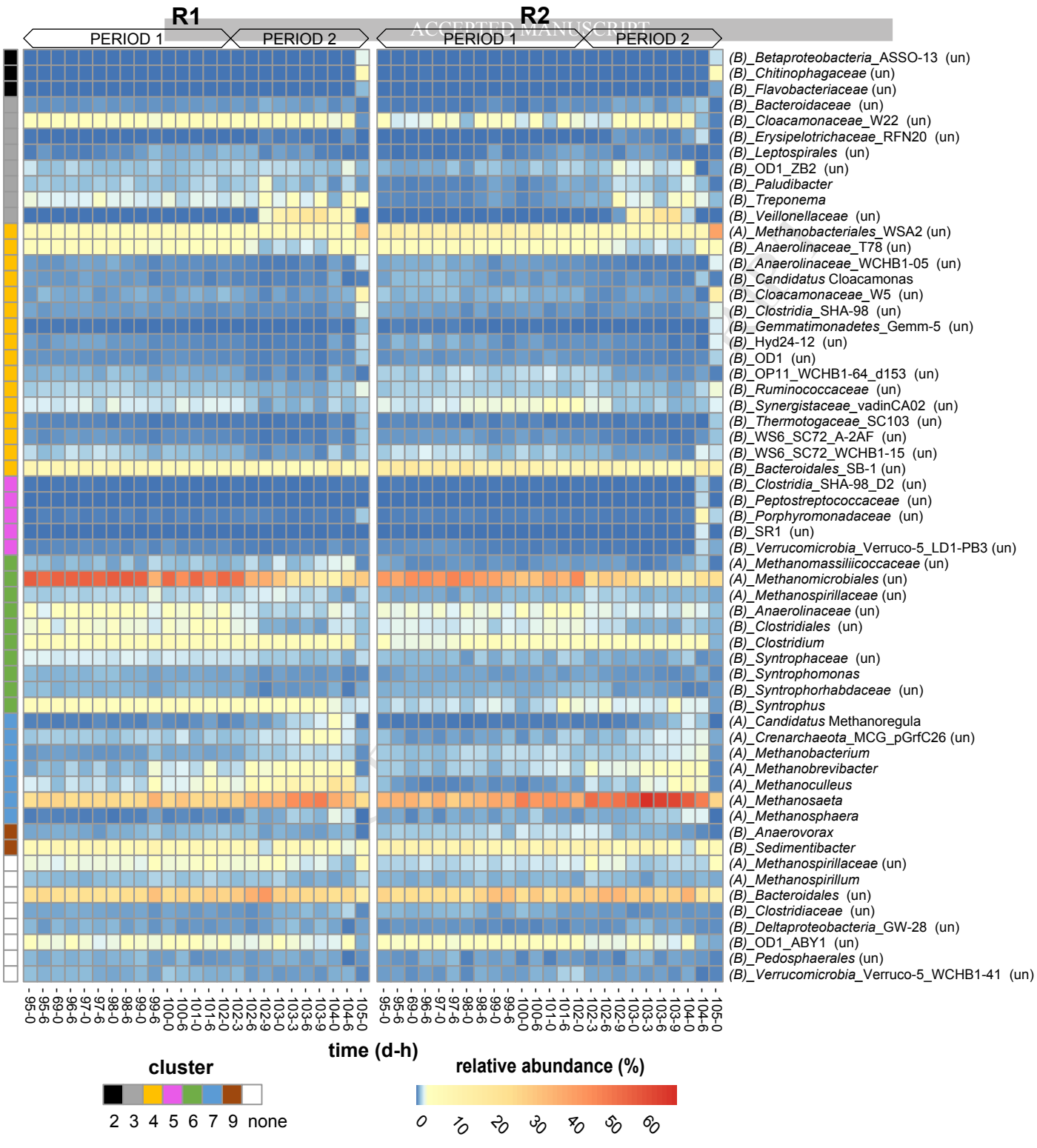

A)
2
3
4
5
6
9
Relative abundance (%)
time (h)
B)
4
6
7
Relative abundance (%)
time (h)

ACCEPTED MANUSCRIPT



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Highlights

- Operational parameters similarly varied after few hours of organic overloading in replicated reactors.
- *Methanosaeta* sp. incremented with increasing VFA concentrations.
- The organic overloading induced a redistribution in the equitability of microorganisms and a loss of *Archaea* species.
- Community structure was not resilient changing rapidly after the OLR increased.
- Fast temporal replacement of functionally redundant microorganisms was observed.

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