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Key microbial communities steering the functioning of anaerobic digesters during hydraulic and organic overloading shocks

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HIGHLIGHTS

- Feedstock changes in anaerobic co-digestion processes provokes overload shocks.
- Hydraulic and organic overloads cause an increase in *Bacteroidetes* and *Actinobacteria*.
- Both overloads cause drop in *Syntrophomonadaceae* and *Pseudomonadaceae*.
- *Tissierellaceae* family only increases during the organic shock.

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ABSTRACT

Overloading is one of the most typical process disturbance in anaerobic digesters, resulting in volatile fatty acids (VFAs) accumulation. This work aimed to study the microbial community dynamics during hydraulic (decreasing the hydraulic retention time (HRT)) and organic (increasing the organic loading rate maintaining the HRT constant) overload shocks in anaerobic reactors treating agro-industrial wastes, as well as during the recovery period. In both cases, the organic loading rate increased from 2 to 10 g COD L^{-1} d^{-1}, resulting in VFAs accumulation up to 9 g L^{-1}. Both overloads were correlated to an increase in *Bacteroidetes* and *Actinobacteria* phyla and with a drop in *Syntrophomonadaceae* and *Pseudomonadaceae* families. In contrast, *Tissierellaceae* family only increased during the organic shock. Active Archaea decreased in both overloads, going from *Methanosaeta* dominance to *Methanosarcina* prevalence. During the recovery period, *Porphyromonadaceae* family increased its presence and *Clostridium* genus recovered values prior to perturbation.

1. Introduction

The success of anaerobic co-digestion (ACo-D) processes is based on the significant improvement in biogas production when organic-rich co-substrates are treated with base substrates (Mata-Álvarez et al., 2000). However, this technology frequently suffers from fluctuations in the organic loading rate (OLR). When the OLR increases rapidly (i.e. overloading), the process performance is deteriorated. This is mainly caused by the dynamic nature of the wastes used as main substrate (manure for example suffered seasonal changes (Regueiro et al., 2014a,b)) or as co-substrates (they could change regularly depending on availability). As a consequence, the composition of the reactoŕs feeding changes, making very difficult to keep a constant OLR in co-digestion systems (Steinberg and Regan, 2011). But also the ambition to produce more biogas (more energy) provokes that practitioners press the reactor, and the thin line that separates stable and instable conditions can be broken, leading to system disturbance. Overload shock can be produced by an increase in the feeding flow rate (i.e., increasing the amount treated every day: hydraulic overload) or by an increase in the organic matter content of the feeding (i.e., maintaining the same flow rate but treating a steam with higher organic matter content: organic overload). Not only does the type of overload affect the reactor performance differently, but also the change rate. If the change takes place smoothly enough so as the digester is able to manage the fluctuation, it is possible to avoid the system collapse, but if it happens rapidly and the reactor is not able to absorb the drastic change, it induces a system failure (Leitao et al., 2006a).

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The result of overloading events is a process imbalance, causing volatile fatty acids (VFAs) accumulation and lowering the biogas production (Leitao et al., 2006b). But, of course, it also affects microbial community structure and composition.

A recent review (Carballa et al., 2015) indicated several benchmark microbial communities related to stable performance for anaerobic digestion reactors depending on substrate type or reactor configuration. The author's highlighted different microbial communities in steady-state systems related with the substrate, i.e. *Clostridia* class for degrading both protein and cellulose, *Bacilli* class related to fat and carbohydrate anaerobic digestion and *Syntrophomonas* genus as propionate and butyrate degrader. They also indicated a positive relationship between the presence of the phylum *Firmicutes* working with solid-based anaerobic digesters. On the contrary, little information is available on the impact of reactor overloading on Bacteria domain, which is essential to understand overloading events in anaerobic co-digestion systems. Lerm et al. (2012) did not detect major shifts in *Clostridia* class and *Bacteroidetes* phylum due to organic shocks, whilst Kampmann et al. (2012) showed an increase in *Pelotomaculum* and *Petrimonas* sp. Kundu et al. (2013) did not observe clear differences between the organic shock and the hydraulic shock in the microbial community.

Regarding the archaeal domain, Methanosaeta is the dominant genera in stable anaerobic reactors (Carballa et al., 2015), whilst *Methanosarcina* appeared as crucial in many overloaded systems (Hori et al., 2006; Kundu et al., 2013; McMahon et al., 2004), probably explained by the fact that this genus is able to use both methanogenic routes, the hydrogenotrophic and the acetoclastic. Other authors, such as Lerm and colleagues (2012), also showed a dominance of other hydrogenotrophic methanogens, like *Methanospirillum hungatei* and *Methanoculleus receptaculi*, after an organic shock.

After OLR stress, system recovery becomes essential, and a drastic decrease in the OLR is the most common way to solve the problem (Rétfalvi et al., 2011), trying to remove those VFAs accumulated during the disturbance period. But this strategy implies extended recovery periods (with low methane production) or even a new start-up if the OLR decrease does not suffice to solve the problem. To detect an impending acidification of the biogas reactors early enough to counteract the acidification process, it would be helpful to find indicator microorganisms showing the futurity acidification before chemical parameter like pH-value changes. Therefore, the detection of these early-warning microbial indicators, or at least microbial indicators to help the practitioners to shorten the recovery periods is of great importance. Ali Shah et al. (2014) recommended the addition of an inoculum rich in *Methanosarcina* sp. to establish proper conditions to regenerate methanogenic activity after process disturbances. Tale et al. (2011) indicated that bioaugmented systems with propionate-utilizing Bacteria recovered stable performance relatively sooner than the non-bioaugmented ones. Williams et al. (2013) showed that supplementation of trace elements resulted to be a successful remediation strategy to overcome a propionic acid accumulation event. However they applied the strategy once the disturbance had occurred. The knowledge of the microbial dynamics during both the disturbance time and the recovery period can help us to design "Ad-hoc" strategies, in order to apply one microbial-based control handle before the process deterioration.

The aim of this study was to investigate the effects of controlled overloading events on the microbial community structure in co-digestion systems, applying denaturing gradient gel electrophoresis (DGGE)+sequencing and 16S rRNA gene sequencing with Illumina MiSeq to follow the bacterial domain and fluorescence in situ hybridization (FISH) to follow the archaeal domain. Particularly, the type of overloading shock (hydraulic and organic) and the recovery period were assessed.

2. Methods

2.1. Experimental set-up

One continuously stirred tank reactor (160 rpm, Heidolph RZR 2041), with a working volume of approximately 10 L, was operated in two consecutive experimental runs: Experiment 1 (hydraulic overload) and Experiment 2 (organic overload). A mixture (50:50, v/v) of two anaerobic sludges, one from a sewage sludge anaerobic digester and the second from a brewery wastewater anaerobic reactor, was used as inoculum. These two sludges were selected to obtain a greater initial microbial diversity. They had been previously characterized (Regueiro et al., 2012) and they present several microbial populations with different and complementary microbial activities. The initial in-reactor inoculum concentration was 15 g of volatile suspended solids (VSS) per liter.

The substrates used were: pig manure (PM), fish waste (FW), beet molasses residues (MR) and biodiesel waste (BW). They were chosen according to their representativeness of typical Galician agro-industries and also due to their different physico-chemical characteristics (Table S1), thus ensuring in order to assure the development of different microbial communities inside the reactor. All residues were physico-chemically characterized (Table S1). Reactor was fed once a day with a mixture of PM, FW and MR (60–20–20, COD basis,) in both experiments. The mixing ratio is in COD basis, expressed in percentage, which means that if the total OLR was 1 g COD L^{-1} d^{-1} (with 60–20–20 of pig manure, fish waste and molasses residues, respectively) the 60% of the total OLR (0.6 g COD L^{-1} d^{-1}) was due to the pig manure organic matter and the other substrates meant each 0.2 g COD L^{-1} d^{-1} of the total OLR. Substrate mixture was prepared every week, diluted with tap water according to the applied OLR, and stored at 4 °C. The organic overload at 10 g COD L^{-1} d^{-1} was provoked by incorporating to the feeding mixture biodiesel waste. This substrate was selected in order to provoke a clear shock in the organic load with a minimum change in the microbial community due to the new substrate addition. The amount of biodiesel waste that it is necessary to achieve 5 g COD L^{-1} d^{-1} is really low (30 mL), since this substrate has higher organic matter content (Table S1). Moreover, no microorganisms were detected in this co-substrate matrix in previous studies (Regueiro et al., 2014b). Therefore, the impact of the entrance of this new substrate on microbial community was expected to be minimal. Temperature, pH, stirring speed, biogas production (Ritter milligascounters, Dr. Ing. Ritter Apparatebau GmbH, Bochum, Germany) and biogas composition (AwiFLEX, AWITE Bioenergie GmbH) were monitored on-line. Samples of reactor mixed liquor were taken three times per week for VFAs, total COD, total suspended solids (TSS), VSS, alkalinity and ammonium determinations. Biomass samples were taken once a week, except during the perturbation and the recovery periods where a more frequent sampling (every 2–3 days) was performed.

2.2. Operational strategy

The two experiments were carried out consecutively in time and they were divided in four periods: start-up (0–30 days), increase of OLR until 2 g COD L^{-1} d^{-1} (31–97 days) and steady state performance (98–150 days), the perturbation period (151–189 in experiment 1 and 151–175 in experiment 2) and the recovery period (in experiment 1 it lasted only 15 days (190–204) to observe the recovery during approximately one HRT, whilst in experiment 2 it lasted from day 176 to day 237, to observe the complete disappearance of the cumulated VFAs in the system). Table S2 shows the conditions of each operational period for each experiment (1 and