Exploring the anaerobic digestion microbiome during ammonia incidents via genome-centric metagenomics

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Anaerobic digestion (AD) is a complex biological process in which organic residues are degraded by different groups of microorganisms producing biogas. Typically, biogas plants are co-digesting multiple substrates with variable chemical composition, thus, fluctuations in the concentration of specific compounds might influence the overall process performance. Such a case is related with high ammonia content, which is one of the most common inhibitors of the AD process. Even though ammonia is an essential nutrient for bacterial growth (Angelidaki & Ahring, 1994) and in optimal concentrations assure the buffer capacity of the medium (Rajacopal et al., 2013), high ammonia levels might lead to severe process imbalances. Among others, ammonia rich substrates used in biogas plants include swine manure, poultry manure, food processing waste streams and high proteinaceous sludge. Furthermore, ammonia is produced as a final product of protein, urea and nucleic acids degradation. Up to now, it is generally accepted that among the microorganisms involved in the AD process, methanogenic Archaea show the greatest sensitivity in the presence of ammonia (Tian et al., 2019). However, in order to optimize the AD process, it is mandatory to decipher the metabolic capabilities of all community members, to understand the fundamental mechanisms of interspecies interactions and conclusively to shed the light upon this microbial "black box" (Zhu et al., 2019).

The purpose of this research was to elucidate the microbial dynamicity and illuminate the structure and function of the AD microbiome during ammonia toxicity incidents in biogas reactors.

Initially, batch experiments were conducted to determine the levels above which ammonia leads to severe deterioration of the process and to deeply characterize the microbial structure and progressive adaptation via genome-centric metagenomic analyses. Batch assays were performed in glass serum bottles with working volume 40 mL and total volume of 118 mL. In all assays batch bottles were inoculated with mesophilic inoculum (acquired from full-scale biogas plant), Basal Medium, glucose as organic matter and specific concentration of ammonia. An initial set of batch vials without ammonia was installed. During the exponential phase of methane productivity (indicating highest microbial activity), liquid samples were obtained and served as inoculum for the next assay. In each assay, the concentration of ammonia was stepwise increased as follows: +2 NH_4Cl/L (Batch 1), +4 NH_4Cl/L (Batch 2) and +6 NH_4Cl/L (Batch 3). During the re-inoculation phase, samples were obtained for molecular and biochemical analyses. The experiment was conducted at mesophilic (37^{o}C) conditions and all assays were performed in triplicates.

In another experimental set, we monitored the process imbalances caused by sudden ammonia incidents (shock loads) in continuously fed reactors. Triplicate Continuously Stirred Tank Reactors (CSRT) with working volume of 1.5 L operating under mesophilic conditions with Hydraulic Retention Time 25 days were fed exclusively with cattle manure. Once steady state conditions were achieved the reactors were subjected to a single inhibitory shock load by adding 6 g NH_4Cl/L. During the whole experiment all the biochemical process parameters of the reactors were recorded, and genomic DNA and RNA were extracted at two points: i) at steady state conditions before the shock and ii) 14 hours after the shock.

The results from the batch assays showed that increasing concentrations of ammonia induced inhibition of ammonia, especially after the addition of 4 g NH_4Cl/L. Specifically, the methane yield of control treatment (i.e. without ammonia) was 340 ± 14 mL CH_4/g VS. The addition of 2 g NH_4Cl/L decreased the methane yield by 5%, while 4 g NH_4Cl/L led to approximately 38% reduction and 6 g NH_4Cl/L to the significant lowering of 74%. Apart from the influence in the methane productivity, higher dosages of NH_4Cl/L prolonged the overall methanation

period. Specifically, the duration of each batch assay lasted 12, 20 and 45 days for the treatments supplemented with 2, 4 and 6 NH_4Cl/L respectively. Finally, the results from the VFA showed that with increasing concentration of ammonia there was an accumulation of propionic acid that could not be further converted into acetate and be consumed by methanogens. Metagenomic analysis showed that in total 203 Metagenome Assembled Genomes (MAGs) were extracted by the assembly and binning, whence 172 MAGs of them were of high quality. Bacteria were the dominants of the microbial community and only 8 archaeal high-quality MAGs were extracted and assigned to *Methanoculleus* sp. (3 MAGs), *Methanosarcina* sp. (2 MAGs) and *Methanomassillicoccus* sp. (1 MAG).

In replicate CSTR systems it was demonstrated that the required time for the process to reach steady state conditions was equal to 3-4 HRT. At the steady state period the produced biogas contained approximately 65% CH_4 and 35% CO_2 and the average methane production was 200 mLCH_4/g VS. When 6 g of NH_4Cl/L were injected in the reactors, the methane production rate fluctuated and other biochemical parameters, such as the pH, presented a slight drop. Nonetheless, the inhibitory shock load, in contrast with the results from the batch assays, did not show a deterioration of the process that was attributed to the formation of a stable and robust microbial community in the continuous reactors. Therefore, the methanization process was recovered shortly after the induced perturbation and at the final period of the experiment the methane production was in similar levels as in the primary steady state phase.

Investigating the inhibitory effect of ammonia on the anaerobic digestion, it was concluded that 4 g NH_4Cl/L was the threshold for the ammonia toxicity in batch assays. Continuous reactor operation was more tolerant to ammonia perturbations due to the establishment of a more resistant microbial community. Results from genome centric metagenomics and metatranscriptomic analysis of CSTRs are currently analyzed and their correlation with the biochemical parameters will be available upon the time of the conference.

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