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# Anaerobic digestion of biowaste under extreme ammonia concentration: Identification of key microbial phylotypes

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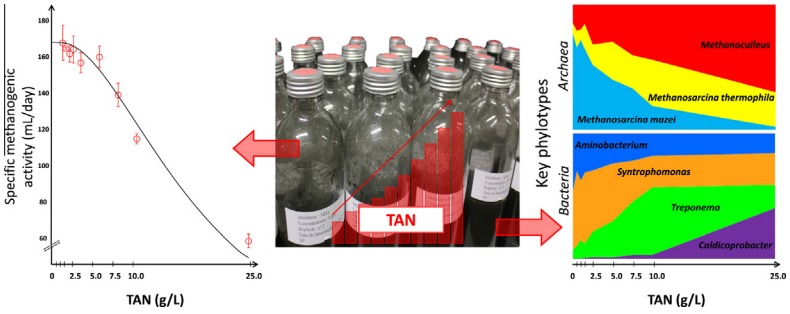
## HIGHLIGHTS

- Microbial community shifts were investigated from 0.0 up to 50.0 g/L of TAN.
- Ultimate $CH_4$ yield was not impaired by TAN up to 25.0 g/L.
- *Methanosarcina* populations were present up to 25.0 g/L of TAN.
- *Treponema* relative abundance increased with TAN concentration up to 10.0 g/L.
- Abundant *Caldicoprobacter* populations detected from 5.0 up to 25.0 g/L of TAN.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Ammonia inhibition represents a major operational issue for anaerobic digestion (AD). In order to get more insights into AD microbiota resistance, anaerobic batch reactors performances were investigated under a wide range of Total Ammonia Nitrogen (TAN) concentrations up to 50.0 g/L at 35 °C. The half maximal inhibitory concentration ($IC_{50}$) value was determined to be 19.0 g/L. Microbial community dynamics revealed that above a TAN concentration of 10.0 g/L, remarkable modifications within archaeal and bacterial communities occurred. 16S rRNA gene sequencing analysis showed a gradual methanogenic shift between two OTUs from genus *Methanosarcina* when TAN concentration increased up to 25.0 g/L. Proportion of potential syntrophic microorganisms such as *Methanoculleus* and *Treponema* progressively raised with increasing TAN up to 10.0 and 25.0 g/L respectively, while *Syntrophomonas* and *Ruminococcus* groups declined. In 25.0 g/L assays, *Caldicoprobacter* were dominant. This study highlights the emergence of AD key phylotypes at extreme ammonia concentrations.



## 1. Introduction

Anaerobic digestion (AD) is a bioprocess widely implemented to stabilize organic waste and simultaneously produce methane-rich biogas which can be used to generate energy and heat. However, anaerobic microorganisms responsible for biological conversion of organic material into methane ($CH_4$) are vulnerable to a wide variety of inhibitory substances or factors which result in decreased production yields and potentially in process failure (Chen et al., 2008). For example, high concentration of ammonia is regularly mentioned as the primary cause of digester failure (Yenigün and Demirel, 2013), even though nitrogen is an essential nutrient for the growth of microorganisms at low level. Many traditional AD substrates such as livestock manure, slaughterhouse byproducts and food industrial residues contain a high nitrogen concentration due to the presence of organic nitrogen such as urea and proteins which readily release ammonia upon digesters during their anaerobic degradation (Rajagopal et al., 2013).

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Total Ammonia Nitrogen, abbreviated as TAN, is a combination of unionized free ammonia (FAN or $NH_3$), and ammonium ion ($NH_4^+$). The chemical equilibrium between FAN and TAN concentrations mainly depends on pH and temperature (Anthonisen et al., 1976). Additionally, ionic strength is also supposed to have a significant influence on FAN concentration particularly in concentrated solutions (Nielsen et al., 2008). FAN is considered more toxic to anaerobes than TAN. The most widely accepted mechanism explaining FAN inhibition is based on change of intracellular pH due to its passive diffusion through cell walls, increasing the maintenance energy requirement and inhibiting specific enzyme reactions (Wittmann et al., 1995).

TAN has been reported to induce 50% reduction in $CH_4$ production at a wide range concentrations varying from 1.7 g/L to 14.0 g/L (Chen et al., 2008). According to Sung and Liu (2003), total inhibition occurs in the range of 8.0–13.0 g/L of TAN depending on pH and inoculum. Similarly, toxic effect of FAN was observed from 150 mg/L to 1200 mg/L (Rajagopal et al., 2013; Yenigün and Demirel, 2013). The wide range of inhibiting FAN or TAN concentrations can be ascribed to the differences in nature of substrates, environmental conditions (pH, temperature, hydraulic retention time) and acclimation of inoculum (Van Velsen, 1979).

The inhibitory effect of ammonia is known to mainly influence the phase of methanogenesis in bioreactors (Calli et al., 2005). Under ammonia stress, acetotrophic methanogens are usually considered to be more vulnerable than the hydrogenotrophic ones (Angelidaki and Ahring, 1993; Koster and Lettinga, 1984). Thanks to stable isotope-based analytical techniques, a metabolic shift in methanogenesis from the acetoclastic mechanism to the syntrophic pathway in which hydrogenotrophic methanogens are involved, was pointed out when ammonia concentration increases (Schnürer and Nordberg, 2008). Other studies revealed that increasing concentrations of ammonia induced a growth of Syntrophic Acetate Oxidation Bacteria (SAOB) and hydrogenotrophic methanogens (Karakashev et al., 2006; Westerholm et al., 2012). Nevertheless, in these latter studies, acetoclastic methanogens were still detected. Conflicting results tended to demonstrate that acetoclastic methanogenesis was predominant under ammonia stress (Calli et al., 2005; Fotidis et al., 2014). Furthermore, Hao et al. (2015) indicated that besides the SAO pathway, acetoclastic methanogenesis catalyzed by *Methanosarcina* spp. was still activated at high ammonia levels.

The aim of the present study is to assess the amplitude of the inhibition effect of ammonia on AD over a wide range of concentrations. AD performances degradation patterns were investigated until reaching a total inhibition of biogas production at extremely high ammonia levels up to 50.0 g/L of TAN. Further objectives were to describe the impact on microbial communities' dynamics and to identify the key phylotypes involved in ammonia resistance within AD microbiota.

## 2. Methods

### 2.1. Experimental set-up

30 anaerobic batch bioreactors were set up using serum bottles (1000 mL, working volume of 500 mL). All digesters were initially seeded with 20 g of centrifuged methanogenic sludge as inoculum. 50 g of mashed biowaste were added as substrate corresponding to an initial organic loading of 10 g COD/g COD (12 g COD as substrate vs 1.2 g COD as inoculum). Biochemical $CH_4$ potential buffer (International Standard ISO 11734 (1995)) was added to reach a total working volume of 500 mL. Mashed biowaste was provided by an industrial food waste deconditioning unit (Chemaudin, France). Inoculum was sampled from a 50 L laboratory anaerobic bioreactor incubated at 35 °C. This reactor had been previously inoculated with 20 L of methanogenic sludge collected from a full-scale mesophilic digester treating wastewater primary and excess biological sludge in a municipal wastewater treatment plant (Achères, France) and fed with mashed biowaste in order to acclimatize the microbial consortium to this substrate.

$NH_4Cl$ (99.998%, Sigma Aldrich) was added in order to reach 10 different TAN concentrations (0.0, 0.5, 1.0, 1.5, 2.5, 5.0, 7.5, 10.0, 25.0 and 50.0 g/L). Consequently, pH was measured for each sample in order to determine FAN concentration. Resultant TAN and FAN concentrations at the beginning of each digestion test were slightly different from the planed ones. This was mainly attributed to the background TAN concentrations contained in the substrate and inoculum. Initial equivalent FAN concentrations ranged from 47 mg/L up to 494 mg/L. All incubations were performed in triplicates.

The 30 bioreactors thus set up were sealed with a screw cap and a rubber septum and headspaces were flushed with $N_2$ (purity > 99.99%, Linde gas SA) in order to assure anaerobic conditions. Time zero ($T_0$) samples were taken and all reactors were incubated without agitation, in the dark, at 35 °C. Liquid samples (8 mL) were periodically taken through the septum and centrifuged at 10,000 × *g* for 10 min. The pellets and supernatant thus obtained were stored separately at −20 °C for analysis of biomass and chemical indicators respectively. Digestion tests were run for 160 days until all daily biogas productions decreased below 10 mL.

### 2.2. Analytical methods

Biogas accumulation in the headspace was measured using a differential manometer (Digitron 2082P). Headspace gas analysis was performed using a micro GC (CP4900, Varian) as described in Chapleur et al. (2014). These data were used to calculate gas production and composition, at standard temperature and pressure. Grofit package of R CRAN software (version 3.1.2) was employed to fit the cumulative $CH_4$ production data to a modified Gompertz three-parameter model (Eq. (1)) where $M(t)$ is the cumulative $CH_4$ production (mL) at time $t$ ($d$); $P$ is the ultimate $CH_4$ yield (mL); $R_{max}$ is the maximum $CH_4$ production rate (mL/d); $\lambda$ is the lag phase ($d$); $e$ is the exponential:

$$M(t) = P \times \exp\left\{-\exp\left[\frac{R_{max} \times e}{P} \times (\lambda - t) + 1\right]\right\} \quad (1)$$

The impact of ammonia on AD performances was evaluated by calculating the half maximal inhibitory concentration ($IC_{50}$) value. $R_{max}$ values were plotted for the different incubation as a function of initial TAN concentration. As described in Chapleur et al. (2015a), this curve was fitted to the Hill model by non-linear regression using the algorithm of Marquard and a non-linear regression program proposed by Duggleby.

Volatile Fatty Acids (VFA) concentrations were measured by ionic chromatography coupled to conductometric detection, using a Dionex 120 equipped with IonPAc ICE-AS1 column (9 mm × 250 mm). The mobile phases were heptafluorobutyric acid (0.4 mmol/L) and tetrabutylammonium hydroxide (5 mmol/L). Acetate, propionate, butyrate, lactate, formate and valerate were quantified.

Ammonium concentration was measured according to the Nessler's reagent colorimetric method following the French standard (NF T 90-015). FAN concentration was calculated from the equilibrium of Eq. (2) (Anthonisen et al., 1976):

$$\mathrm{FAN} = \frac{10^{\mathrm{pH}}}{\exp\left(\frac{6334}{T}\right) + 10^{\mathrm{pH}}} \times \mathrm{TAN} \quad (2)$$

where $T$ represents the temperature (K).