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# Acclimation strategy to increase phenol tolerance of an anaerobic microbiota

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## HIGHLIGHTS

- Acclimation strategy minimized anaerobic digester performance loss caused by phenol.
- Biogas production restart threshold increased from 680 to 1480 mg/L of phenol.
- Increasing phenol pressure led to the emergence of a major archaeal OTU.
- Important elasticity allowed *Bacteria* to adapt to increasing phenol concentrations.
- *Clostridiales* and *Bacteroidales* were the dominant orders after acclimation.

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## ABSTRACT

A wide variety of inhibitory substances can induce anaerobic digester upset or failure. In this work the possibility to improve the resistance of an anaerobic microbiota to a common pollutant, the phenol, was evaluated in a lab-scale semi-continuous bioreactor. An acclimation strategy, consisting in a regular step-wise adaptation of the microbiota to stressful condition was employed. Degradation performances were monitored and molecular tools (16S sequencing and ARISA fingerprinting technique) were used to track changes in the microbial community. The acclimation strategy progressively minimized the effect of phenol on degradation performances. After 3 successive disturbance episodes, microbiota resistance was considerably developed and total inhibition threshold increased from 895 to 1942 mg/L of phenol. Microbiota adaptation was characterized by the selection of the most resistant *Archaea* OTU from *Methanobacterium* genus and an important elasticity of *Bacteria*, especially within *Clostridiales* and *Bacteroidales* orders, that probably enabled the adaptation to more and more stressful conditions.



## 1. Introduction

The production of bioenergy from waste is an essential component in the global development of sustainable energy sources (De Vrieze et al., 2013). Anaerobic digestion (AD), which is the most prominent bioprocess technology for waste recovery worldwide, uses non-specific and highly diverse microbial consortia to produce energy rich compounds, such as alcohols, volatile fatty acids and biogas from organic substrates. Methane-rich biogas provides a versatile carrier of renewable energy, which can partly replace fossil fuels for heat, power generation and as vehicle fuel after upgrading to concentrate the methane. Anaerobic bioreactors are maintained on the basis of decades of observed relationships between performance and operating parameters. However, poor operational stability still prevents AD from being commercialized as widely as it could (Chen et al., 2008). Indeed, a large variety of inhibitory substances, which can either be substantially present in wastes, or released during their degradation can induce anaerobic digester upset or failure (Chen et al., 2008). Therefore considerable research efforts have been made to understand the mechanisms and the controlling factors of inhibition (Chen et al., 2008).

AD of organic matter is a complex microbial multistep process. The efficient and stable operation of methanogenic bioreactors relies on synergistic interactions of an intricate community of microbes including hydrolytic and fermenting bacteria, specialized acidogenic and acetogenic syntrophs, and methanogenic archaea. They have diverse and parallel pathways for substrate metabolism, and specific sensitivities to environmental conditions (Carballa et al., 2015). Besides technological improvements of the processes such as reactor design, AD efficiency could be further optimized by

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adapting its microbiota with external environmental levers, such as operating parameters, toward the desired functioning. Carballa et al. recently reviewed strategies currently applied to shape the reactor microbiome (Carballa et al., 2015). In particular, to confront future disturbances, the best strategy could consist in provoking a preliminary adaptation of the microbial communities to stressful conditions (McMahon et al., 2004). Pulse-feeding promotes for example higher capacity to confront perturbations (De Vrieze et al., 2013). In this framework, the study described herein aimed at evaluating the possibility to improve the resistance of a complex anaerobic microbiota to phenol.

Cellulose was chosen as a representative substrate for AD. Indeed, an important amount of lignocellulose, which is a major component of the biosphere is available. It also represents the most voluminous waste produced by our society (Lynd et al., 2002). Phenol was selected to inhibit the microbiota, as it is often recovered in various substrates treated with anaerobic digestion. Phenols can be produced from biodegradation of naturally occurring aromatic polymers such as humic acids and tannins or from degradation of xenobiotic compounds such as pesticides (Fang et al., 2006). Different pre-treatments applied to increase biogas production efficiency from lignocellulosic materials are also known to result in the production of phenolic compounds (Monlau et al., 2014). They are regularly detected at concentrations reaching up to several grams per liter in different types of effluents from petrochemical or paper industry (Rosenkranz et al., 2013a; Veeresh et al., 2005) or in contaminated sewage sludge or municipal solid waste (Hoyos-Hernandez et al., 2014). Although phenol can be biodegraded to harmless compounds under methanogenic conditions, high concentrations are toxic to different groups of microorganisms involved in methane production (Chapleur et al., 2015a; Olguin-Lora et al., 2003; Poirier et al., 2016a; Rosenkranz et al., 2013a). Phenol is indeed a membrane-damaging microbiocide that affect membrane proteins and alter the cell wall permeability, inducing progressive leakage of intracellular constituents (McDonnell and Russell, 1999). In particular, it was observed that unexpected phenol shock loadings within unacclimated anaerobic digesters can induce major disruptions in AD bioprocess, leading to the decrease of biogas production rate and longer treatment durations (Veeresh et al., 2005).

Our work was based on the presupposition that a regular stepwise adaptation of the microbial community to stressful condition could strengthen the microbial ecosystem against the disturbance. Based on this presupposition, the impact of increasing phenol levels on the performances of a semi-continuous anaerobic bioreactor degrading cellulose was comprehensively analyzed during extended reactor operation. Degradation performances were monitored throughout the experiment. Additionally, molecular tools (automated ribosomal intergenic spacer analysis (ARISA) and 16S sequencing assays) were used to track changes in the bacterial and archaeal communities as phenol level was modified.

## 2. Material and methods

### 2.1. Experimental set-up

A continuously stirred lab-scale bioreactor (Biostat B Sartorius, total volume 6.6-L, working volume 5-L) was set-up in mesophilic conditions. Temperature was maintained at 35 °C by recirculating water in the jacket of the double walled bioreactor. At start-up, the bioreactor was inoculated with a sludge recovered from a full-scale mesophilic household waste digester (Varennes-Jarcy, France, characteristics of the sludge: total solid = 33.7 g/L, volatile solid = 13.2 g/L). 1 L of sludge was mixed with 4 liters of biochemical methane potential buffer (BMP, ISO 11734:1995) to seed the reactor). The reactor was fed twice daily with a solution of cellulose (15 g of cellulose (alpha cellulose, Sigma–Aldrich) per L in BMP buffer). Feeding was followed by mixture removal to maintain a constant volume. In total 200 mL per day were added and removed, hydraulic retention time (HRT) was about 25 days, organic loading rate (OLR) circa 0.6 kg/m$^3$. Three successive disturbance episodes of increasing importance were applied. Each episode was composed of a perturbation period (P) during which phenol concentration was increased progressively until biogas production nearly stopped, a stabilization period (S) during which phenol concentration was maintained at a high level for at least one HRT and a recovery period (R) during which phenol concentration was decreased if necessary in order to enable the biogas production to restart at a significant level before the next perturbation. To set-up these perturbation episodes, phenol was diluted in the cellulose feeding solution at different levels of concentration according to the objective (increasing, maintaining or decreasing the perturbation). Cellulose and phenol were the only sources of carbon. Mixing speed was controlled and maintained at 70 rpm throughout the experiment. Bioreactor was closed hermetically to maintain anaerobic conditions. Biogas was collected through a port at the top of the bioreactor and production rate was measured with a tipping bucket gas counter (volume of the bucket 10 mL). Biogas was ultimately collected in a bag to measure its composition. The pH and the temperature in the reactor were continuously measured. Samples were collected weekly through a sampling pipe, centrifuged at 10,000*g* for 10 min at 4 °C, frozen directly in liquid nitrogen and kept at −80 °C until used for analysis of biomass and chemical parameters. The digester was operated for 562 days. It reached a steady state after nearly 3 HRT (IS, initial stabilization) and was maintained in stable functioning conditions (SF period) for more than 1 HRT before being disturbed. Experiment physico-chemical parameters measured during this period were considered as the control reference parameters of a non-disturbed digester. The three successive disturbance episodes were called 1, 2 and 3 and their perturbation, stabilization and recovery periods were called P1, S1, R1, P2, S2, R2, P3, S3 and R3. R3 step was divided in R3a (high level of phenol) and R3b (very low level of phenol). They started respectively at days: 114, 182, 220, 247, 288, 324, 380, 448, 484 and 528. These dates were chosen as the experiment progressed on the basis of the evolution of the main physico-chemical parameters, especially biogas production (see Section 3).

### 2.2. Analytical methods

Biogas collected was analyzed every two days using a micro GC (CP4900, Varian) exactly as described in (Chapleur et al., 2014). Ratio of cellulose conversion to biogas was calculated by dividing measured biogas production ($CH_4$ and $CO_2$) by theoretical biogas production if all carbon of cellulose was converted into biogas. Dissolved organic carbon (DOC) was measured in the samples' supernatant (Bioritech Model 700) as described in (Chapleur et al., 2014). The concentrations of formic, acetic, propionic, butyric, valeric and lactic acids (volatile fatty acids, VFA) were measured by conductometric detection, using a Dionex 120 equipped with an IonPAc ICE-AS1 column (9 mm × 250 mm). Mainly acetic and propionic acids were detected. Chemical oxygen demand (COD) was measured with LCK514 kit (Hach Lange) and phenol concentration was measured with LCK346 kit (Hach Lange) according to the manufacturer's instructions.

### 2.3. Microbial structure analyses

Total DNA from samples' pellet was extracted using Power Soil DNA Isolation Kit (Mobio Laboratories Inc. Carlsbad) according to