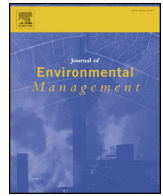




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## Research article

# <sup>1</sup>H NMR metabolomics of microbial metabolites in the four MW agricultural biogas plant reactors: A case study of inhibition mirroring the acute rumen acidosis symptoms

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## ABSTRACT

In this study, nuclear magnetic resonance (<sup>1</sup>H NMR) spectroscopic profiling was used to provide a more comprehensive view of microbial metabolites associated with poor reactor performance in a full-scale 4 MW mesophilic agricultural biogas plant under fully operational and also under inhibited conditions. Multivariate analyses were used to assess the significance of differences between reactors whereas artificial neural networks (ANN) were used to identify the key metabolites responsible for inhibition and their network of interaction. Based on the results of nm-MDS ordination the subsamples of each reactor were similar, but not identical, despite homogenization of the full-scale reactors before sampling. Hence, a certain extent of variability due to the size of the system under analysis was transferred into metabolome analysis. Multivariate analysis showed that fully active reactors were clustered separately from those containing inhibited reactor metabolites and were significantly different. Furthermore, the three distinct inhibited states were significantly different from each other. The inhibited metabolomes were enriched in acetate, caprylate, trimethylamine, thymine, pyruvate, alanine, xanthine and succinate. The differences in the metabolic fingerprint between inactive and fully active reactors observed in this study resembled closely the metabolites differentiating the (sub) acute rumen acidosis inflicted and healthy rumen metabolomes, creating thus favorable conditions for the growth and activity of pathogenic bacteria. The consistency of our data with those reported before for rumen ecosystems shows that <sup>1</sup>H NMR based metabolomics is a reliable approach for the evaluation of metabolic events at full-scale biogas reactors.

## 1. Introduction

Design and operation of full-scale reactors for waste management are an exercise in ecosystem management. Reactor communities contain thousands of interacting microbial populations, even when a single substrate is provided (Fernandez et al., 2000). Parallel processing of substrates through complex interconnected metabolic pathways has been proven to be a more efficient and robust approach in many systems. Parallel processing of substrate was correlated with greater functional stability of methanogenic reactor communities, despite nutritional perturbations (Hashsham et al., 2000), as mixed microbial systems are functionally equivalent to a network of metabolic activities of many interdependent populations working together as a community

to convert organic substrates to methane via the well-recognized anaerobic food chain. However, the inhibition of microbial activity by perturbation takes place well before these perturbations in metabolic flow are reflected at the level of the residing complex microbial communities and their community structure (Kolbl et al., 2017).

Today's intensive management systems of industrial biogas plants encourage the inclusion of large amounts of less conventional substrates to support high methane yields and enhance cost efficiency (Kolbl et al., 2014, 2016; Murovec et al., 2015) (substrates with high content of nitrogen, sulphur, salts and/or lipids: molasses, vinasses, paper pulp sludges, organic fractions of municipal solid wastes, discarded oils and fats). However, as the use of such substrates cannot be comprehensively tested by comparative batch or semi-continuous lab-scale and pilot-

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scale experimental tests within the prolonged time frames (e.g. years) and in combinations with many different co-substrates (Kolbl et al., 2014), these practices ultimately lead to development of suboptimal process conditions and decreased biogas yields next to accumulation of various products such as intermediate degradation products and metabolites. In turn, the suboptimal process conditions further affect metabolite turnover, the extent of hydrolysis, acidogenesis, acetogenesis next to hydrogenotrophic or acetoclastic methanogenesis conducted within microbial functional guilds, and can result in partial and complete inhibition of those processes (Westerholm et al., 2016; Jiang et al., 2018).

The intensive biogas plant management systems encourage the inclusion of large amounts of various plant, animal and industry based substrates next to those originating from wastewater treatment plant or organic fraction of municipal solid waste, that fluctuate on annual and seasonal basis. The high-energy substrates that contain high availability of fermentable carbon next to the repetitive daily loading practices with high organic loading rates in terms of volatile solids ( $> 6$  g VS/L) often lead to accumulation of large amounts of short-chain fatty acids (SCFA) and to the acidification of the reactor contents, decrease of alkalinity (dissolved inorganic carbon) and the inhibition of the underlying anaerobic processes (i.e. cessation of biogas production). The characteristics of this pathological condition (pH  $< 6.5$ , SCFA buildup) largely correspond to acute or sub-acute rumen acidosis symptoms (ARA or (S)ARA, respectively) observed in high-intensity animal production (Ametaj et al., 2010; Nocek, 1997). The enormous energy requirements of high producing cattle require rations of substrates with increasing dry matter intakes and levels of concentrate feeds, i.e. higher energy feedstuffs. These make it increasingly difficult for the animal to provide a ruminal environment that stays within all of the narrow constraints relevant for microbial physiology, and hence make rumen increasingly susceptible to (S)ARA (Hernández et al., 2014; McCann et al., 2016; Ishaq et al., 2017). (S)ARA is defined as fermentation disorder in the rumen, characterized by a lower than normal ruminal pH (rather a continuum of degrees of ruminal acidity) reflecting an imbalance between microbial production, microbial utilization, and ruminal absorption of short-chain fatty acids that have a significant impact on microbial activity, rumen function, and animal productivity and health (Hernández et al., 2014; McCann et al., 2016; Ishaq et al., 2017).

Accumulation of SCFAs (acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate, formate, lactate, etc.) has long been associated with a decrease in microbial activity in biogas reactors and process failure. In theory, the surplus of easily available organic carbon for hydrolysis and acidogenesis leads to increased SCFA content, decrease in pH that both affect underlying microbial activities of the complex anaerobic microbial communities (Bacteria, Archaea, Protozoa, Fungi, Viruses) (McCann et al., 2016; Ishaq et al., 2017). The same parameters encountered in anaerobic digestion process (devoid of the host uptake of SCFA, buffering, systemic detoxification in (S)ARA) result in reduced rates of methanogenesis and process inhibition. Therefore, the observed reduction in process functionality (biogas production or meat/milk production, respectively) are tightly linked to the buildup of SCFA and systemic acidification.

So far, most conventional approaches targeted a limited number of biogas reactor compound classes, i.e. SCFA, alkalinity, SCFA/alkalinity ratio, pH, ammonia, total soluble organic carbon concentration (TSOC) and have not attempted a comprehensive metabolomic characterization of the full-scale biogas digesters (Kolbl et al., 2014, 2017; Sasaki et al., 2014).

Metabolomics is an emerging field of "omics" science that uses high-throughput approaches, such as  $^1\text{H}$  NMR spectroscopy (Ametaj et al., 2010; Ravanbakhsh et al., 2015; Beckonert et al., 2007). High-resolution NMR spectroscopy is a quantitative nondestructive, noninvasive, nonequilibrium perturbing technique that provides detailed information on solution-state molecular structures, based on atom-centered

nuclear interactions and properties (Beckonert et al., 2007). It is a robust and reliable technique for metabolomic applications with high reproducibility as it allows the detection of a wide range of structurally diverse metabolites simultaneously down to the low micromole per liter range in 4–5 min acquisition time using current-generation high-field spectrometers (Ravanbakhsh et al., 2015; Beckonert et al., 2007). The peak integrals relate directly to the number of protons giving rise to the peak, and hence to the relative concentrations of the substances in the sample. Absolute concentrations can be obtained if the sample contains an added internal standard of known concentration (Beckonert et al., 2007; Sket et al., 2018). This approach is generally coupled to multivariate analyses to extract comprehensive metabolic information within the system enabling exploration of complex interactions between metabolites on metabolic pathways producing methane in response to exposition to environmental parameters and elucidation of the associated parameters explaining the observed differences in metabolites. In addition, two dimensional (2D) NMR spectroscopy can be useful for increasing signal dispersion and for elucidating the connectivity between signals, thereby helping to identify biochemical substances (Beckonert et al., 2007).

Although metabolomics is becoming a well-established high-throughput technique in analyses of metabolic imbalances in human intestinal tract and health in general, to our knowledge, only a few studies have used modern metabolomics approaches to explore the metabolic imbalances in ruminants (Ametaj et al., 2010; Saleem et al., 2012; Lee et al., 2012), and to the best of our knowledge, no such study has been published so far dealing with analyses of the full-scale biogas reactors.

In this study, we hypothesized that  $^1\text{H}$  NMR spectroscopic profiling would provide a more comprehensive view on microbial metabolites associated with reduction in biogas production in order to give insight into the function-metabolite association in the full-scale biogas digesters. For this we used  $^1\text{H}$  NMR spectroscopic profiling along with multivariate statistical analysis to compare metabolomes of the six biogas reactors in a full-scale 4 MW agricultural biogas plant under fully operational and also under inhibited conditions. Multivariate analyses such as permutational multivariate analysis of variance (perMANOVA) and similarity percentages (SIMPER) were used to assess the significance and extent of differences between biogas reactors whereas artificial neural networks (ANN) were used to identify the key metabolites responsible for inhibition and their network of interaction.

## 2. Materials and methods

### 2.1. Full-scale biogas reactor sample collection

The mesophilic agricultural biogas plant Vučja vas (Slovenia) was monitored during the start-up phase of its six anaerobic biogas reactors until its full 4 MW output and over the course of 2.5 years (Kolbl Repinc et al., 2018). This plant contains six biogas reactors ( $6 \times 3931 \text{ m}^3 = 23,586 \text{ m}^3$ ) and two post-reactors ( $2 \times 5350 \text{ m}^3 = 10,700 \text{ m}^3$ ) in total volume of  $34,286 \text{ m}^3$ .

The whole biogas plant is divided into two lines connecting biogas reactors F1-3 and biogas reactors F4-6 through overflow (Fig. 1). In addition to these main lines, substrate mixtures prepared in mixing shafts can be independently directed to any of the biogas reactors as all have their own sideway ports that enable boost injections (Fig. 1). The basic characteristics of biogas reactors F1-F6 (temperature, TSOC, pH, ratio slopes between 275 nm - 295 nm slope and 350 nm - 400 nm slope (Sr) as measure of molecular complexity of TSOC) were determined as described before (Kolbl et al., 2014, 2016, 2017) and are presented as Fig. S1.

Complete biogas plant is controlled and regulated via supervisory control and data acquisition (SCADA) system, which is located at the area of electro-technical chambers of biogas plant. Electricity is produced by four gas motors (MWM, Germany), each with 1 MW<sub>el</sub> capacity

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