The active microbial community more accurately reflects the anaerobic digestion process: 16S rRNA (gene) sequencing as a predictive tool

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Abstract
Amplicon sequencing methods targeting the 16S rRNA gene have been used extensively to investigate microbial community composition and dynamics in anaerobic digestion. These methods successfully characterise amplicons, but do not distinguish micro-organisms that are actually responsible for the process. In this research, the archaeal and bacterial community of 48 full-scale anaerobic digestion plants was evaluated on DNA (total community) and RNA (active community) level via 16S rRNA (gene) amplicon sequencing. A significantly higher diversity on DNA compared with the RNA level was observed for archaea, but not for bacteria. Beta diversity analysis showed a significant difference in community composition between the DNA and RNA of both bacteria and archaea. This related with 25.5 and 42.3% of total OTUs for bacteria and archaea, respectively, that showed a significant difference in their DNA and RNA profiles. Similar operational parameters affected the bacterial and archaeal community, yet, the differentiating effect between DNA and RNA was much stronger for archaea. In conclusion, a clear difference in active (RNA) and total (DNA) community profiles was observed, implying the need for a combined approach to estimate microbial community stability in anaerobic digestion.

Keywords
Biogas, Illumina sequencing, methane, methanogenesis, microbiome

INTRODUCTION

Anaerobic digestion (AD) relies on complex microbial communities for the conversion of organic waste streams into biogas. Physico-chemical parameters reflect the current state of the process, and do not always accurately reflect microbial community composition, dynamics or activity [1,2]. To implement more direct microbial process control of the AD process, we need to extend our knowledge of the interaction between the temporal trajectories of microbial community structure and operational parameters.

The advent of high-throughput sequencing techniques in AD research resulted in a significant increase in our understanding of the (active) microbial community [3]. The DNA based techniques have delivered significant insights, but they do exhibit important shortcomings in their ability to reveal the active microbial community in AD. Hence, an alternative approach is needed to bridge the knowledge gap on active microbial communities, (potential) collaboration and complete functionality prediction.

In this research, the microbial community in full-scale AD plants was evaluated through amplicon sequencing of the 16S rRNA gene and the 16S rRNA transcripts to directly compare the total and active microbial community. This is in contrast to most other approaches that make use of different techniques to make an estimation of the difference between the active and total microbial community. The bacterial and archaeal (methanogenic) differential abundance and activity patterns were identified, and related to the sensitivity of the methanogenic community to variations in operational

parameters in AD.

MATERIALS AND METHODS

Sample and data collection

Digestate samples were collected from 48 full-scale AD plants in Belgium in 1 L air-tight containers, and immediately transported to the laboratory. Upon arrival in the laboratory, samples were homogenized, and three replicate 1.5 mL subsamples were taken, and stored at -80°C until DNA and RNA extraction. Another 10 mL subsample was stored at -20°C for VFA analysis. A 50 mL sample was stored at 4°C for total ammonia nitrogen, conductivity, volatile solids (VS), total solids (TS) and cation analysis. Sample pH was measured directly upon arrival in the laboratory. Information concerning the sludge retention time (SRT) and temperature was obtained directly from the operator.

Amplicon sequencing and data analysis

Total DNA and RNA were co-extracted from the same sample to avoid biases related to variable cell lysing efficiency. The RNA PowerSoil® Total RNA Isolation Kit in combination with the RNA PowerSoil® DNA Elution Accessory Kit (Mobio Laboratories Inc., Carlsbad, CA, USA) was used for simultaneous RNA and DNA extraction. The RNA extracts were subjected to DNase treatment using the DNase I Kit for Purified RNA in Solution (Mobio Laboratories Inc.) for removal of residual DNA. The RNA was subsequently converted to cDNA using the qScriberTM cDNA Synthesis Kit (Mobio Laboratories Inc.). The final quality of the cDNA and DNA was validated by 1% agarose gel electrophoresis and PCR analysis.

The cDNA and DNA extracts were sent to LGC Genomics GmbH (Berlin, Germany) for sequencing on the Illumina Miseq platform. Sequencing was performed by targeting the V3-V4 hypervariable region of the 16S rRNA (gene) using bacterial primers 341F and 785R. A nested approach was used for the archaea, with the archaea specific primers 340F and 1000R for the first PCR run, followed by universal primers 341F and 806R for the second PCR run. Statistical analyses were performed in R Studio, version 3.2.3. using the packages vegan and phyloseq for community analysis.

Amplicon sequencing and data analysis

Total solids (TS), volatile solids (VS) and TAN were determined according to standard methods. The pH and conductivity were measured with a C532 pH and C833 conductivity meter (Consort, Turnhout, Belgium), respectively. The concentrations of the different VFA were analysed by means of gas chromatography.

RESULTS AND DISCUSSION

Microbial diversity in the total (DNA) and active (RNA) community: bacteria vs. archaea

Basic alpha diversity analysis showed a significantly higher richness ($P < 0.0001$) and overall diversity ($P < 0.0001$), based on the Shannon, Simpson and Fisher's alpha, on DNA level compared with the RNA level for archaea, while Pielou's evenness was similar (Figure 1). In contrast, none of the diversity indices showed a significant difference ($P > 0.05$) between DNA and RNA for bacteria. Beta diversity analysis revealed a highly significant ($P = 0.0001$) community differentiation pattern between DNA and RNA for archaea using the unweighted Unifrac distance measure (Figure 2).

This contrast between the DNA and RNA level indicates a high functional specialization, despite the high metabolic potential through a high archaeal diversity. Lin, et al. [4] observed a centralization of functionality for methanogenesis, based on functional pathway prediction, despite a high alpha diversity. This relates with the fact that only two major pathways are responsible for methane production in AD, *i.e.* hydrogenotrophic and acetoclastic methanogenesis, which do not require a diverse archaeal community. Most digesters in our study were dominated by hydrogenotrophic methanogens, both on DNA and RNA level, and this points to an even higher degree of functional

specialization. The high archaeal diversity at the DNA level can be considered a pool of "reserve players" that are not active, but can take over when digester conditions change, related to the susceptibility and narrow optimal operational parameter range of most methanogens [5,6]. Overall, the clear differentiation between the DNA and RNA profile, based on alpha and beta diversity measures, but related with operational data, reflects a well-organized methanogenic community.

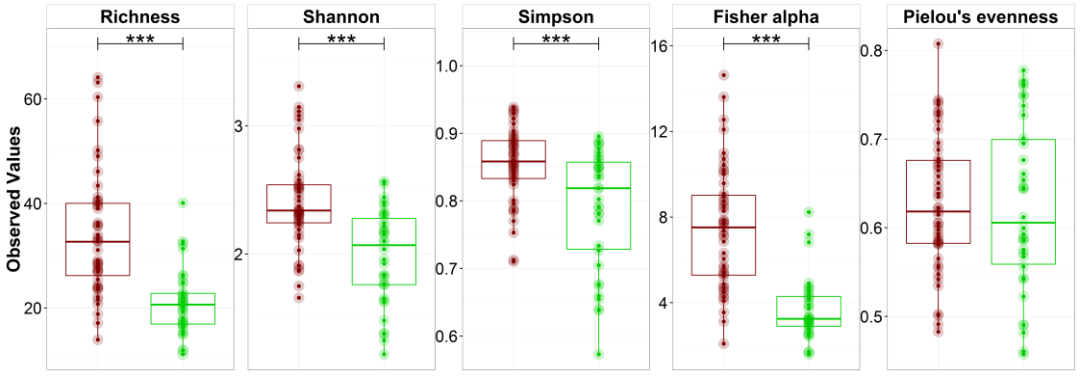

Figure 1. Boxplots of the alpha diversity indices of the archaeal community on DNA (red) and RNA (green) level. Significant differences between DNA and RNA are indicated (***).

The differentiation between the DNA and RNA profile in terms of alpha diversity that was observed for the archaeal community was not observed for the bacterial community. This indicates a similar structural organization of the total and active bacterial community. Beta diversity analysis of the bacterial community, however, revealed a significant differentiation between the total and active community, although this was not as strong as for the bacterial community, which indicates a similar bacterial community structure on DNA and RNA level, but a difference in composition (Figure 2).

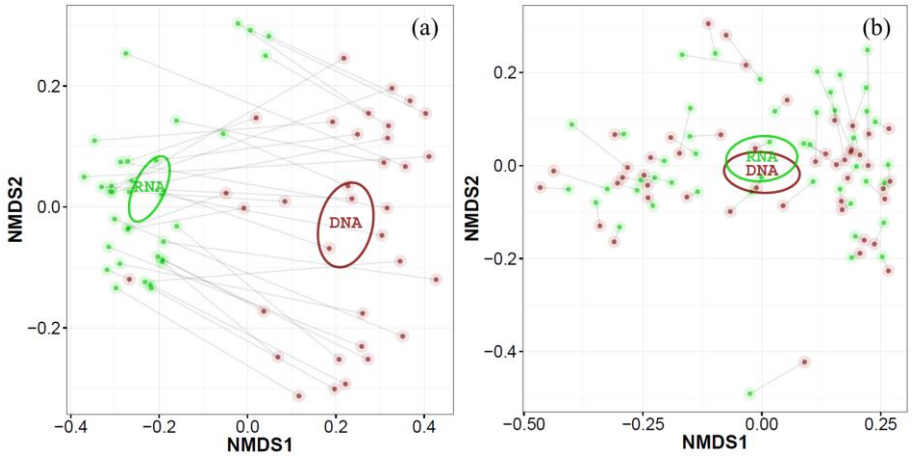

Figure 2. Non-metric distance scaling (NMDS) analysis of the unweighted Unifrac distance indices of the (a) archaeal, and (b) bacterial community at OTU level. The DNA (red) and RNA (green) based community profiles of the same samples were connected by means of a grey line. The circles represent the 95% value of the standard error of the average value of the DNA (red) and RNA (green) indices.

The high degree of variance between DNA and RNA based on the unweighted Unifrac measure confirms that the presence/absence of different OTUs and not their relative abundance is responsible for the difference between the bacterial DNA and RNA profile [7], yet, this strongly depends on sequencing depth, which was in this case similar for the RNA and DNA data. The similarity of the structural organization of the bacterial community on DNA and RNA level is the consequence of the inherent different involvement and properties of the bacterial and archaeal community in the AD process. While archaea only have to perform two methanogenic pathways in AD, the bacterial community carries out numerous pathways, which requires a higher active community diversity.

Associations between operational conditions on the total and active microbial community

The overall archaeal community was primarily shaped by temperature, pH, TAN, free ammonia, conductivity, VS, TS (P = 0.001). The Na^+ (P = 0.006), K^+ (P = 0.002), propionate (P = 0.003) and total VFA (P = 0.002) also had a strong impact on the archaeal community (Figure 3). A similar observation was made for the bacterial community. The significant (P < 0.001) difference between DNA and RNA profiles, observed *via* beta diversity analysis, was confirmed.

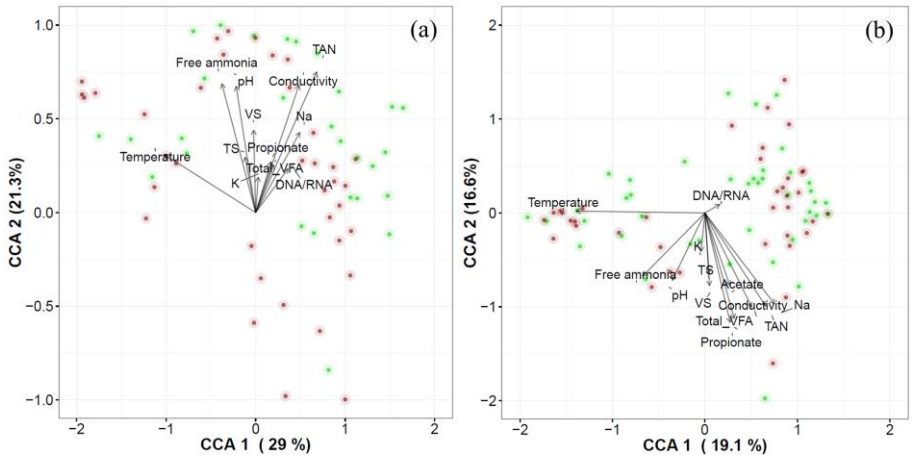

Figure 3. Canonical correspondence analysis of the (a) archaeal, and (b) bacterial community, including the DNA (red) and RNA (green) profile of each sample at OTU level. PERMANOVA was carried out to evaluate the effect of operational parameters on community composition, and significant (P < 0.01) correlations are presented by the arrows.

CONCLUSIONS

An increased level of specialization was observed in the active archaeal community. In contrast, the total and active bacterial community showed a similar community structure, but, community composition more strongly differed between the total and active community. Similar factors shaped the archaeal and bacterial community. The clear difference between RNA and DNA based community screening confirms the importance of this combined approach to obtain a general overview, not only on the total and active community, but also in terms of potential collaboration and competition. These results then serve as a basis for integrated process engineering of the anaerobic digestion process.

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