Title: Isotope fractionation in biogas allows direct microbial community stability monitoring in anaerobic digestion

Running title: Isotope fractionation in anaerobic digestion

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Abstract

Process monitoring of anaerobic digestion is typically based on operational parameters, such as pH and volatile fatty acid concentration, that are lagging on actual microbial community performance. In this study, ^{13}C isotope fractionation in CH_4 and CO_2 in the biogas was used to monitor process stability of anaerobic digestion in response to salt stress. A gradual and pulsed increase in salt concentration resulted in a decrease in methane production. No clear shift in $\delta^{13}\text{CH}_4$ was observed in response to the gradual increase in salt concentration, and $\delta^{13}\text{CO}_2$ of the biogas showed only a clear shift after process failure, compared with the control. In contrast, both $\delta^{13}\text{CH}_4$ and $\delta^{13}\text{CO}_2$ in the biogas changed in response to the pulsed increase in salt concentration. This change preceded the decrease in methane production. A significantly different bacterial and archaeal community profile was observed between the DNA and RNA level, which was also reflected in a different relation with the $\delta^{13}\text{CH}_4$ and $\delta^{13}\text{CO}_2$ values. This shows that isotope fractionation in the biogas can predict process stability in anaerobic digestion, as it directly reflects shifts in the total and active microbial community, yet, due to its temporal character, further validation is needed.

1. Introduction

Monitoring of the anaerobic digestion (AD) process is needed to ensure continuous and high-rate methane production, irrespective of the feedstock composition. Commonly used indicators for process stability include the residual volatile fatty acid (VFA) concentration, pH, alkalinity, biogas production, and biogas composition ^{1, 2}. These indicators can be measured by regular sampling or via on-line sensors. Several on-line monitoring strategies for the abovementioned parameters have been developed, and were successfully applied in pilot- or even full-scale systems. For example, near infrared monitoring of the VFA concentration ³, an electronic nose for biogas composition analysis ⁴ have been used for direct or indirect AD process stability monitoring ⁵. The application of bioelectrochemical techniques resulted in the development of bio-sensors, mainly for on-line monitoring of the VFA concentration ⁶⁻⁹.

As these commonly used indicators directly relate to AD process deterioration, their potential to predict process failure within a reasonable timeframe, *i.e.* a timeframe that still allows process remediation or prevention of failure, is limited. Combinations and transformations of conventional parameters, which results in the definition of new monitoring parameters, can be used to gain a better insight on process failure in AD ¹⁰. The application of early-warning indicators, such as the VFA to calcium ratio ¹¹, has resulted in strategies to prevent process failure, for example by means of CaO addition to the digester ¹². Despite this more integrated approach, these indicators do not directly reflect microbial metabolic activity, and cannot serve to monitor changes in community activity and composition that may cause process disturbance. The organic matter present in the feedstocks contains both ¹²C and ¹³C as stable carbon isotopes ¹³. Stepwise degradation of these feedstocks by the micro-organisms involved in the different stages of the AD process results in a fractionation of these isotopes in the biogas, in both CH₄ and CO₂ ¹⁴. Stable isotopes can be used *via* two different ways to gain an increased insight in the AD process. First, ¹³C-enriched substrates (such as acetate or CO₂) can be used in tracer

experiments to determine the dominant methanogenic pathway and/or methanogenesis kinetics¹⁵⁻¹⁷. Second, natural abundance ratios of $^{13}\text{C}/^{12}\text{C}$ in biogas, whether or not in comparison with the original feedstock, also can be used to detect changes in metabolic pathways, because the pathway length relates with the degree of fractionation, and, thus, isotope ratio in the product¹⁸⁻²¹.

A shift in the stable isotope ratio directly relates with a change in at least one metabolic pathway, therefore, this concept may be used for direct microbial activity monitoring and process instability prediction in AD. The predictive potential of stable isotopes in biogas has already been demonstrated for on-line monitoring of process stability²² and ammonia inhibition prediction in AD²³. However, it is unclear whether isotope composition shifts in CH_4 and CO_2 reflect a measurable change in the present and/or active microbial community in AD.

In this research, NaCl was used as a model to provoke process failure in AD through salt inhibition of methanogenesis. The difference between a gradual and sudden increase in NaCl concentration on the isotope composition in CH_4 ($\delta^{13}\text{CH}_4$) and CO_2 ($\delta^{13}\text{CO}_2$) in the biogas was investigated. The total and active microbial community, analysed via the 16S rRNA gene and 16S rRNA, respectively, were hypothesized to show a clear relation with ^{13}C fractionation and $\delta^{13}\text{CH}_4$ and $\delta^{13}\text{CO}_2$ differentiation. Archaeal and bacterial community analysis was carried out separately to distinguish between the methanogenic archaea, which are more susceptible to salt stress, and the more tolerant bacteria.

2. Material and methods

2.1. Inoculum and feedstock

The inoculum for the anaerobic digesters (Table S1) was obtained from a full-scale digester treating brewery wastewater (Van Steenberge, Ertvelde, Belgium). A single batch of waste activated sludge that served as feedstock for the digesters (Table S2) was obtained from a municipal wastewater treatment plant (Dendermonde, Belgium). The waste activated sludge was stored at 4 °C until use.

2.2. Experimental design

A total of nine glass Schott bottles with a total volume of 1 L and a working volume of 800 mL were sealed with an airtight rubber stopper, and used as digesters (Figure S1). The gas outlet of each digester was connected to a water displacement set-up to determine biogas production. Biogas was sampled from the columns using a vacuum pump connected to a glass sampling tube from which biogas samples were taken for biogas composition analysis. The pH of the liquid in the tubes was maintained < 4.3 to ensure that no CO₂ from the biogas dissolved in the water. The initial biomass concentration was set at 10 g VS L⁻¹ (volatile solids) by diluting the inoculum with tap water. The digesters were operated as continuous stirred tank reactors, thus, the sludge and hydraulic retention time were the same. A constant temperature of 34 ± 1 °C was maintained *via* incubation in a temperature controlled room. Feeding was carried out manually three times per week.

2.3. Experimental operation

During the 28-days start-up period, the organic loading rate was increased to 2.5 g COD L⁻¹ d⁻¹ (chemical oxygen demand) and the sludge retention time was decreased to 20 days (Table S3) in each of the nine digesters. The organic loading rate and hydraulic retention time were

maintained constant throughout the entire experimental period (day 29 to 126) at 2.5 g COD L⁻¹ d⁻¹ and 20 days, respectively. Crude glycerol (AppliChem, Darmstadt, Germany) was used as co-substrate to the waste activated sludge to increase the organic loading rate, because the COD content of the waste activated sludge was too low to obtain a sufficiently high loading rate. Glycerol was chosen as co-substrate, because of its high biodegradability and frequent usage as co-substrate, to enhance methane production²⁴. On day 49, a differentiation was made between three digester types, each of which was operated in triplicate. In the Control digester, the same conditions as between day 28 and 48 were maintained (no NaCl addition) until the end of the experiment (day 126). In the Gradual digesters, the NaCl concentration was gradually increased to a final additional concentration of 10 g Na⁺ L⁻¹ on day 105, and this concentration was kept until the end of the experiment (Figure S2). The NaCl in the Pulse digesters was increased only twice, to 5 g Na⁺ L⁻¹ on day 63 and to a final additional concentration of 10 g Na⁺ L⁻¹ on day 91.

Biogas production and composition, as well as digester pH were monitored three times per week. Biogas production was reported at standard temperature (273 K) and pressure (101,325 Pa) conditions (STP). The conductivity, which is considered a measure of overall salinity, and the VFA and cation concentrations were measured once per week. Samples for biogas isotope analysis (CO₂ and CH₄) were also taken once per week, starting from day 49. Samples for microbial community analysis (DNA and RNA) were taken from the mixed liquor on day 0 (inoculum), 49, 77, 91 and 126, and from the waste activated sludge feedstock, and stored at -80 °C.

2.4. Microbial community analysis and data processing

Simultaneous total DNA and RNA extraction, as well as DNase treatment and conversion to cDNA of the RNA, and the quality check were carried out as described in SI (S5). The cDNA

134 and DNA samples were sent to LGC Genomics GmbH (Berlin, Germany) for Illumina
135 sequencing of the bacterial and archaeal community using the Miseq platform. Amplicon
136 sequencing and community data processing was carried out as described by De Vrieze, et al. ²⁵.
137 A separate table that contains the abundances of the operational taxonomic units (OTUs), and
138 their taxonomic assignments was generated for the bacteria and archaea (Supplementary file 2a
139 & b). All samples were rescaled by taking the proportions of each OTU, multiplying it with the
140 minimum sample size, and rounding to the nearest integer, the so-called “common-scale”
141 approach ²⁶. Sampling depth was evaluated through rarefaction curves ^{27, 28}. Statistical analyses
142 were carried out by means of R studio version 3.3.1 (<http://www.r-project.org>) ²⁹. Community
143 analysis was carried out using the phyloseq ³⁰ and vegan ³¹ packages.
144 The community composition of the triplicate digesters was statistically compared *via* repeated
145 measures analysis of variance (ANOVA, *aov* function) and a Mantel test (*mantel.rtest* function,
146 ade4 package) to validate that both the archaeal and bacterial community showed no significant
147 ($P < 0.05$) difference between biological replicates. The weighted average values of the
148 biological replicates were used to create heatmaps (pheatmap package). Non-metric
149 multidimensional scaling (NMDS) plots were generated for the bacteria and archaea separately,
150 based on the (un)weighted Unifrac and Bray-Curtis distance measures. Significant ($P < 0.05$)
151 differences in community composition between digesters and/or different time points were
152 determined *via* pair-wise Permutational ANOVA (PERMANOVA) (9999 permutations) with
153 Bonferroni correction (*adonis* function, vegan package). The PERMANOVA test is largely
154 unaffected by heterogeneity of variances or differences in sample size between groups ³². Prior
155 to PERMANOVA analysis, the multivariate homogeneity of variances was evaluated via
156 PERMDISP2 (*betadisper* function, vegan package). The PERMANOVA, complemented with
157 canonical correspondence (CCA) model analysis *via* the *envfit* function (vegan), was also used
158 to evaluate the significance of the relation of the Na⁺ concentration and the $\delta^{13}\text{CH}_4$ and $\delta^{13}\text{CO}_2$

values with both the DNA and RNA profiles using the *adonis* function (vegan), and their effect was visualized *via* CCA plotting.

2.5. Chemical analyses

Total solids (TS), VS, total ammonia nitrogen (TAN), and total Kjeldahl nitrogen (TKN) were determined with standard methods³³. The pH and conductivity were measured with a Consort C532 pH and C833 conductivity meter (Consort, Turnhout, Belgium). The NH_4^+ and Na^+ concentrations were measured by means of ion chromatography (IC, Metrohm IC 761, Herisau, Switzerland), equipped with a Metrosep C6 e 250/4 column and Metrosep C4 Guard/4.0 guard column. The eluent contained 1.7 mM HNO_3 and 1.7 mM dipicolinic acid. Samples were prepared by centrifugation at 3,000g for 5 min, followed by filtration over a 0.45 μm filter (type PA-45/25, Macherey-Nagel, Germany). Samples were diluted with milli-Q water to the desired concentration range of 10-100 mg L^{-1} . The biogas composition (CH_4 and CO_2) was measured with a compact gas chromatograph (Global Analyser Solutions, Breda, The Netherlands), and the VFA concentrations were measured via gas chromatography (GC-2014, Shimadzu®, The Netherlands), as described in SI (S6).

The $\delta^{13}\text{CH}_4$ and $\delta^{13}\text{CO}_2$ values in the biogas were determined as earlier described³⁴. Gas samples were stored in 12 mL Soda Glass Vials – Flat Bottomed (Labco limited, Lampeter, UK), prior to analysis. The vials were flushed twice with helium, and vacuumed to a residual pressure of 10 Pa.

2.6. Data deposition

The raw fastq files that were used to create the OTU table, which served as a basis for the microbial community analysis, have been deposited in the National Center for Biotechnology Information (NCBI) database (Accession number SRP129760 for bacteria & SRP129760 for

184 archaea).

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3. Results

3.1. Digester performance

During the start-up phase (day 0-27), a clear increase in CH₄ production was observed to similar values of 483 ± 118 mL CH₄ L⁻¹ d⁻¹ for Control, 469 ± 69 mL CH₄ L⁻¹ d⁻¹ for Gradual and 513 ± 52 mL CH₄ L⁻¹ d⁻¹ for Pulse before the addition of NaCl was initiated on day 49 (Figure 1a). This steady increase was also reflected in an optimal pH of 7.01 ± 0.02 for Control, 7.02 ± 0.04 for Gradual and 7.02 ± 0.03 for Pulse (Figure S3a) and total VFA remaining below 0.2 g COD L⁻¹ (Figure S3b), indicating stable operation.

The addition of NaCl did not immediately result in a decrease in CH₄ and CO₂ production, but from day 93, a clear difference was observed in CH₄ production between Control (481 ± 61 mL CH₄ L⁻¹ d⁻¹) and Gradual (367 ± 21 mL CH₄ L⁻¹ d⁻¹) and Pulse (314 ± 55 mL CH₄ L⁻¹ d⁻¹). This corresponded with a Na⁺ concentration of 7.4 ± 0.1 g L⁻¹ in gradual and 5.2 ± 0.1 g L⁻¹ in Pulse (Figure S4). This decrease was similar between Gradual and Pulse, until almost complete inhibition of methane production (less than 50 mL CH₄ L⁻¹ d⁻¹ from day 110 onwards) occurred in Gradual and Pulse, compared with 386 ± 50 mL CH₄ L⁻¹ d⁻¹ on day 126 in Control (Figure 1a). The CO₂ production showed a similar pattern as the CH₄ production, with a clear decrease in Gradual and Pulse, compared with Control towards the end of the experiment (Figure 1b).

The decrease in methane production was also reflected in a decrease in pH to 5.03 ± 0.15 in Gradual and 5.05 ± 0.06 in Pulse on day 126 (Figure S3a), presumably due to an increase in total VFA to 9.5 ± 2.2 g COD L⁻¹ in Gradual and 11.7 ± 0.5 g COD L⁻¹ in Pulse (Figure S3b). The anticipated increase in the Na⁺ concentration through the addition of NaCl was clearly reflected in the measured Na⁺ concentrations (Figure S4) and conductivity (Figure S3c). The total ammonia nitrogen concentration remained below a value of 800 mg N L⁻¹ in all digesters (Figure S3d).

3.2. ^{13}C fractionation in CH_4 and CO_2

Analysis of the natural ^{13}C abundance in CH_4 ($\delta^{13}\text{CH}_4$) and CO_2 ($\delta^{13}\text{CO}_2$) in the biogas, starting from day 49, resulted in a different pattern between the different digesters (Figure 2). The overall $\delta^{13}\text{CO}_2$ was much higher compared to $\delta^{13}\text{CH}_4$ for each of the digesters. The $\delta^{13}\text{CH}_4$ showed a similar profile in Control and Gradual, irrespective of the increase in the Na^+ concentration, yet, both with a clear increasing trend towards the end of the experiment (Figure 2a). A similar observation could be made for Pulse and Gradual, but the $\delta^{13}\text{CH}_4$ value showed a clear increase after the first Na^+ pulse to $-47.7 \pm 0.3 \text{ ‰}$ in Pulse compared with $-51.9 \pm 0.4 \text{ ‰}$ in Control (Figure 2c). After this increase, $\delta^{13}\text{CH}_4$ returned to its original value in the subsequent time points, yet, also the second Na^+ pulse resulted in a clear increase in $\delta^{13}\text{CH}_4$, especially on day 119 and 126. This, however, also coincided with an increase in $\delta^{13}\text{CH}_4$ in Control, which was only slightly lower than in Pulse.

The $\delta^{13}\text{CO}_2$ remained similar in Control and Gradual, until a clear divergence on day 105 at a Na^+ concentration of $9.6 \pm 0.9 \text{ g Na}^+ \text{ L}^{-1}$, which propagated in the following time points (Figure 2b). In contrast to Gradual, the first Na^+ pulse resulted in a clear decrease in $\delta^{13}\text{CO}_2$ (Figure 2d), which corresponded with the increase in $\delta^{13}\text{CH}_4$ (Figure 2b). In contrast to $\delta^{13}\text{CH}_4$, $\delta^{13}\text{CO}_2$ did not return to its original value, and consequent lower $\delta^{13}\text{CO}_2$ values were measured in the following time points. The second Na^+ pulse resulted in a further decrease in $\delta^{13}\text{CO}_2$ in the last two time points, which also related with the increase in $\delta^{13}\text{CH}_4$. The increase in $\delta^{13}\text{CO}_2$ in Control in the last time points was not observed in Gradual or Pulse.

3.3. Microbial community composition and dynamics

An average of $18,401 \pm 7,994$ reads representing in total 2859 operational taxonomic units (OTUs) were obtained per sample for the bacteria, while an average of $35,046 \pm 28,162$ reads

and in total 252 OTUs were obtained per sample for the archaea. Rarefaction curves were generated to evaluate coverage of the bacterial and archaeal community (Figure S5). Samples with a total reads count < 5000 were excluded from further analysis. No significant differences ($P < 0.05$) in bacterial and archaeal community composition could be detected between the biological replicates.

3.3.1. Active and total microbial community composition

The bacterial community on DNA level was mainly dominated by the Actinobacteria (22.6 ± 10.8 %), Firmicutes (15.4 ± 9.5 %), Bacteroidetes (11.8 ± 2.3 %) and Proteobacteria (11.7 ± 7.2 %) phyla (Figure 3a). A higher relative abundance of the Actinobacteria was observed for the feedstock and Control samples, while the Firmicutes showed an increased relative abundance in Gradual and Pulse, compared with Control. On order level, there was an increased relative abundance of the Clostridiales (16.0 ± 9.2 %) and Synergistales (8.3 ± 5.3 %) in Gradual and Pulse, while the Actinomycetales (29.7 ± 8.7 %) were mainly dominating Control (Figure S6).

In contrast to the DNA level, the bacterial community on RNA level consisted mainly of Proteobacteria (44.9 ± 6.5 %), and, to lesser extent, Firmicutes (13.9 ± 6.8 %) and Bacteroidetes (12.6 ± 4.6 %) (Figure 3a). The overall dominance of the Proteobacteria, Firmicutes and Bacteroidetes phyla was consistent throughout the different digesters, irrespective of the addition of NaCl. A slight increase in the Campylobacterales order could be observed in Gradual and Pulse (5.7 ± 2.0 %) in comparison with Control (0.7 ± 0.3 %) (Figure S6).

The archaeal community contained both acetoclastic and hydrogenotrophic methanogenic genera, both on DNA and RNA level (Figure 3b), but there was a clear dominance of the strict hydrogenotrophic (74.7 ± 12.4 %) over the acetoclastic (16.4 ± 10.7 %) methanogens, averaged over all samples, excluding the inoculum and feedstock. The addition of NaCl resulted in a

strong increase in the relative abundance of the hydrogenotrophic *Methanocorpusculum*, both on DNA (62.5 ± 15.8 %) and RNA (62.0 ± 18.1 %) level in Gradual and Pulse compared with Control. The acetoclastic *Methanosaeta* remained more dominant in Control, especially on DNA level (34.2 ± 8.0 %) but also, to lesser extent, on RNA level (12.8 ± 4.3 %). *Methanoregula* and *Methanospirillum* also showed a slightly higher relative abundance in Control compared with Pulse and Gradual on both the DNA and RNA level.

3.3.2. Total and active microbial community association with ^{13}C fractionation and salt concentration

A significant difference in community composition was detected between the DNA and RNA level, both for the bacteria ($P = 0.0001$) and archaea ($P = 0.0002$), based on the Bray-Curtis distance measure (Figure 4, Table S4). This was also the case for the weighted and unweighted Unifrac distance measures, both for the bacteria and archaea ($P = 0.0001$) (Figure S7).

A separate analysis of the DNA and RNA profile showed that the bacterial community composition was significantly different between Control and Gradual ($P = 0.0003$ for DNA and $P = 0.0096$ for RNA) and between Control and Pulse ($P = 0.0003$ for DNA and $P = 0.0009$ for RNA) (Figure 5a & c, S8 a & c). No significant differences in the bacterial community were detected between Gradual and Pulse on DNA ($P = 0.692$) and RNA ($P = 0.086$) level. A similar observation was made for the archaeal community. A significant difference between Control and Gradual ($P = 0.009$ for DNA and $P = 0.0021$ for RNA) and Control and Pulse ($P = 0.003$ for DNA and $P = 0.0009$ for RNA) was observed (Figure 5b & d, S8b & d).

The $\delta^{13}\text{CH}_4$ and $\delta^{13}\text{CO}_2$ showed a significant relation with the bacterial and archaeal community structure, both on DNA and RNA level, as did the Na^+ concentration (Figure 5, Table S5 & S6). On DNA level, $\delta^{13}\text{CH}_4$ and $\delta^{13}\text{CO}_2$ showed an opposite direction with respect to both the archaeal and bacterial composition shaping (Figure 5a & b), while on RNA level, they showed

286 the same direction towards the bacterial and archaeal community shaping (Figure 5c & d). The
287 $\delta^{13}\text{CO}_2$ and Na^+ concentration had an inverse relation with the bacterial and archaeal
288 community shaping, both on DNA and RNA level.

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4. Discussion

The gradual or pulsed increase in Na^+ concentration resulted in both cases in a decrease in CH_4 production and increase in VFA concentration, indicating process failure. This decrease in methane production was reflected in a shift in $\delta^{13}\text{CH}_4$ and $\delta^{13}\text{CO}_2$, but mainly $\delta^{13}\text{CO}_2$ showed a strong relation with anaerobic digestion process stability. The increase in Na^+ concentration also caused a shift in the bacterial and archaeal community, with a clear difference between the DNA and RNA level. The $\delta^{13}\text{CH}_4$ and $\delta^{13}\text{CO}_2$ showed a significant relation with the bacterial and archaeal community structure and composition, both on DNA and RNA level.

4.1. Salt stress causes a shift in the ^{13}C isotope composition of the biogas

The anticipated decrease in methane production due to the increase in Na^+ concentration was similar for the gradual and pulsed increase, and coincided with a Na^+ concentration of $10 \text{ g Na}^+ \text{ L}^{-1}$. This value fitted well within the wide concentration range of 4.4 to $26.9 \text{ g Na}^+ \text{ L}^{-1}$ causing 50% process inhibition^{35, 36}.

The $\delta^{13}\text{CO}_2$ was always higher than $\delta^{13}\text{CH}_4$, irrespective of the Na^+ concentration or other operational conditions, as also reported in other studies¹⁹⁻²¹. Heavier isotopes travel at a lower velocity, forming more stable, stronger bonds than lighter isotopes, hence, an isotope composition difference between substrate and product is commonly observed in AD³⁷. There is a clear increase in $\delta^{13}\text{CO}_2$ compared with $\delta^{13}\text{CH}_4$, because CO_2 is produced in each of the four stages of the AD process, while CH_4 is only generated in the final methanogenesis stage. This explains the inherent difference in $\delta^{13}\text{CH}_4$ and $\delta^{13}\text{CO}_2$ in AD.

The sudden increase in $\delta^{13}\text{CH}_4$ and decrease in $\delta^{13}\text{CO}_2$ in response to the sudden increase in salinity, observed on day 70 in Pulse, implies a shift in the main metabolic pathways of the AD process. The $\delta^{13}\text{CH}_4$ can be used as an estimation of the methanogenic pathway, with more depleted $\delta^{13}\text{CH}_4$ values corresponding with an increased contribution of the hydrogenotrophic

pathway^{15, 22, 38}. The increase in $\delta^{13}\text{CH}_4$ on day 70 could be considered a temporal increased contribution of the acetoclastic pathway, but this was not reflected in the DNA or RNA profile of the archaeal community. The $\delta^{13}\text{CH}_4$ returned to the same value as before the Na^+ pulse, but the $\delta^{13}\text{CO}_2$ values remained lower. This indicates a resilient methanogenic community, irrespective of the dominance of the hydrogenotrophic or acetoclastic pathway. The shift in $\delta^{13}\text{CO}_2$, in contrast to $\delta^{13}\text{CH}_4$, points to a change in the overall pathways in Pulse, and, to a lesser extent, Gradual, and this apparent pathway shift was maintained throughout the experiment. This indicates bacterial redundancy as the main mechanism behind AD process stability at Na^+ concentrations between 5 and 10 g $\text{Na}^+ \text{L}^{-1}$ ^{39, 40}. This also related to the clear shift in the bacterial community profile on DNA and RNA level on day 77 in comparison with Control. The extended increase in $\delta^{13}\text{CH}_4$ and decrease in $\delta^{13}\text{CO}_2$ towards the end of the experiment, both in Gradual and Pulse, did no longer relate to a stable metabolic pathway shift, as almost complete process failure took place.

4.2. The total (DNA) and active (RNA) microbial community differentially relate with biogas ^{13}C fractionation.

The significant difference in community composition between the total (DNA) and active (RNA) community, irrespective of the Na^+ concentration, agrees with the often-observed discrepancy between community presence and (potential) activity in AD^{41, 42}. Even though bacterial community composition significantly differed between the DNA and RNA level, a similar level of organization could be observed, as determined through NMDS analysis, which indicates functional redundancy of the bacterial community in these digesters⁴³. In contrast, the apparent difference between the total and active archaeal community structure in response to salt stress confirms the overall susceptibility of the methanogenic archaea^{41, 44}.

An opposite direction of the Na^+ concentration with $\delta^{13}\text{CO}_2$, both on DNA and RNA level in

the bacterial and archaeal community, was observed with canonical correspondence analysis. This clear inverse relation reflects a possible shift in the microbial community pathways due to the increased Na^+ concentration. Based on the higher degree of fractionation (more depleted $\delta^{13}\text{CO}_2$), the average pathway length from substrate to CO_2 increased³⁷, indicating that Na^+ stress leads to more dissipation of the available energy between a higher number of (active) micro-organisms. Although such a “meandering metabolism” in AD has been suggested to increase stability^{45,46}, this appeared to be the case only in Pulse at a Na^+ concentration between 5 and 10 g $\text{Na}^+ \text{L}^{-1}$. A further increase in salinity most likely led to a too high dissipation of the available energy, which is in contrast with the need for function centralization to ensure process stability⁴⁷.

The opposite direction of $\delta^{13}\text{CH}_4$ and $\delta^{13}\text{CO}_2$ on DNA level, but similar direction on RNA level, both for bacteria and archaea, indicated a different reflection of the total and active community with respect to $\delta^{13}\text{CH}_4$. The susceptibility of acetoclastic methanogens to salt stress⁴⁴ would lead to the assumption that the main methanogenic pathway shifted to syntrophic acetate oxidation, coupled with hydrogenotrophic methanogenesis⁴⁸, and this coincides with a more depleted $\delta^{13}\text{CH}_4$ ^{17, 49}. This corresponded with the opposite relation between the Na^+ concentration and $\delta^{13}\text{CH}_4$ with respect to both the bacterial and archaeal community, but only on RNA level. The similar direction of $\delta^{13}\text{CH}_4$ and the Na^+ concentration on DNA level indicates that the shift in the methanogenic pathway was not reflected on DNA level. A clear differential change from acetoclastic to hydrogenotrophic methanogenic genera between the DNA and RNA level could not be observed. This indicates that the shift in the active methanogenic community was related directly to specific OTUs, although this was only reflected in few low abundant OTUs, such as OTU42 (*Methanospirillum*). The discrepancy between the DNA and RNA level in terms of $\delta^{13}\text{CH}_4$ seems to be a consequence of this shift. For the methanogenic community, this discrepancy is most likely related to the low growth rate

of methanogens compared with bacteria^{50,51}. For the bacterial community, this difference, most likely, is a consequence of the narrow thermodynamic boundaries⁵², related to syntrophic interactions in the AD process^{53, 54}, which implies that a minor shift in the methanogenic pathways also impacts the bacterial pathways in AD.

4.3. Biogas natural ¹³C fractionation has the potential to be used for direct community stability monitoring in anaerobic digestion

The ability to predict process stability and, even more importantly, process instability in AD remains an important issue, due to the potential economic losses related with process failure, *i.e.* digestate disposal, decrease in electricity production and restarting/reinoculating the digester⁵⁵⁻⁵⁷. The potential of ¹³C fractionation in biogas as an early-warning system for AD process failure has been demonstrated before^{22, 23}, but in this work, a link with the microbial community is established. Prior to the decrease in pH and methane production and increase in VFA, a sudden shift in the $\delta^{13}\text{CH}_4$ and $\delta^{13}\text{CO}_2$ values in Pulse pointed towards a shift in the bacterial and archaeal pathways. However, this pathway shift was not detected in the archaeal community composition on RNA level, which indicates that there is a discrepancy between the 16S rRNA profile and actual activity, related to the only indirect relation between microbial activity and functional performance. For example, *Methanosarcina* sp. can use both the acetoclastic and hydrogenotrophic pathway of which the shift cannot be detected through 16S rRNA (gene) amplicon sequencing. Despite its low relative abundance, together with other archaeal OTUs, it could have a strong contribution to methanogenesis, related to the potential discrepancy between activity and functional performance⁴². Given its similar direction to the active and total community, $\delta^{13}\text{CO}_2$ can be used to detect overall changes in pathways. In contrast, $\delta^{13}\text{CH}_4$ can be used to assess the dominant methanogenic pathway, though this does not necessarily relate to changes in methanogenic community composition. For example, the

first increase in the Na^+ concentration in Pulse reflected a permanent change in the overall pathway length (based on $\delta^{13}\text{CO}_2$), and a temporal change in the methanogenic pathway (based on $\delta^{13}\text{CH}_4$), although this temporal change was measured in only one time point.

The results of this research demonstrated the potential to use ^{13}C fractionation in CH_4 , but mainly CO_2 , to estimate and predict process stability. However, given the temporal character of the stressor in Pulse, further research would be needed to confirm its actual potential applicability. The actual predictive potential—seems to be limited to sudden and temporal disturbances, rather than a gradual deviation from the optimal process conditions. A clear shift in $\delta^{13}\text{CH}_4$ and $\delta^{13}\text{CO}_2$ in Gradual and Pulse could be observed only in relation to Control. This will require either a supplementation with conventional parameters, such as pH and VFA, or a case-specific determination of the $\delta^{13}\text{CH}_4$ and $\delta^{13}\text{CO}_2$ boundary levels that agree with stable process operation to make a validated usage of $\delta^{13}\text{CH}_4$ and $\delta^{13}\text{CO}_2$ to detect process anomalies. A projected shift in the overall metabolic pathways of the process following a Na^+ pulse could be estimated, mainly based on $\delta^{13}\text{CO}_2$, prior to conventional operational parameters, such as CH_4 production, pH and VFA concentration. Both $\delta^{13}\text{CH}_4$ and $\delta^{13}\text{CO}_2$ showed a clear relation with the microbial community, which indicates that their variation in function of time goes beyond a mere observational interpretation of the AD process. Further research will reveal what the resolution and predictive power is of ^{13}C fractionation with respect to other AD process disturbances, and what are the required measuring frequency and reference values to obtain a clear view on microbial process stability.

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581 **Figures:**

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583 **Figure 1** (a) CH₄ and (b) CO₂ production in Control, Gradual and Pulse. Average values of the
584 biological replicates (n=3) are presented, and error bars represent standard deviations. OLR =
585 organic loading rate.

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Figure 2 $\delta^{13}\text{C}$ of CH_4 for (a) Gradual and (c) Pulse, and CO_2 for (b) Gradual and (d) Pulse in comparison with Control. The Na^+ values are the measured concentrations in the digesters (Figure S4). Average values of the biological replicates ($n=3$) are presented, and error bars represent standard deviations.

Figure 3 Heatmap showing the square root transformed relative abundance of (a) the bacterial community at the phylum level and (b) the archaeal community at the genus level, both on the DNA and RNA level. Only phyla and genera with an average relative abundance > 1 % are included. Weighted average values of the biological replicates are presented. The colour scale ranges from 0 (white) to 80 % (red) relative abundance for the bacterial phyla and from 0 (white) to 100 % (red) for the archaeal genera.

Figure 4 Non-metric multidimensional scaling (NMDS) analysis of the Bray-Curtis distance indices of the (a) bacterial, and (b) archaeal community at the OTU level. The DNA (red), representing the total community, and RNA (green), representing the active community, 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.

Figure 5 Canonical correspondence analysis of the (a) bacterial community at DNA level, (b) archaeal community at DNA level, (c) bacterial community at RNA level, and (d) archaeal community at RNA level. PERMANOVA was carried out to evaluate the relation of the ^{13}C fraction in CH_4 and CO_2 , and the measured Na^+ concentration with community composition. The length of the arrow reflects the strength of the interaction.