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Microbial community redundancy in anaerobic digestion drives process recovery after salinity exposure

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ABSTRACT

Anaerobic digestion of high-salinity wastewaters often results in process inhibition due to the susceptibility of the methanogenic archaea. The ability of the microbial community to deal with increased salinity levels is of high importance to ensure process perseverance or recovery after failure. The exact strategy of the microbial community to ensure process endurance is, however, often unknown. In this study, we investigated how the microbial community is able to recover process performance following a disturbance through the application of high-salinity molasses wastewater. After a stable start-up, methane production quickly decreased from 625 ± 17 to 232 ± 35 mL CH_4 L^{-1} d^{-1} with a simultaneous accumulation in volatile fatty acids up to 20.5 ± 1.4 g COD L^{-1}, indicating severe process disturbance. A shift in feedstock from molasses wastewater to waste activated sludge resulted in complete process recovery. However, the bacterial and archaeal communities did not return to their original composition as before the disturbance, despite similar process conditions. Microbial community diversity was recovered to similar levels as before disturbance, which indicates that the metabolic potential of the community was maintained. A mild increase in ammonia concentration after process recovery did not influence methane production, indicating a well-balanced microbial community. Hence, given the change in community composition following recovery after salinity disturbance, it can be assumed that microbial community redundancy was the major strategy to ensure the continuation of methane production, without loss of functionality or metabolic flexibility.

1. Introduction

Anaerobic digestion (AD) of nitrogen- and or salt-rich feedstocks, such as animal manure (Usack and Angenent, 2015), slaughterhouse waste (Franke-Whittle and Insam, 2013; Pitk et al., 2013) and aquaculture sludge (Zhang et al., 2016) leads to high total ammonia nitrogen (TAN) and salt, mainly monovalent cations (Na^+ and K^+), concentrations in the mixed liquor of the digester. This may encounter process stability issues, *e.g.* volatile fatty acid (VFA) accumulation and/or a variable biogas production and composition that have to be avoided to guarantee continuous high biogas production rates. The concentrations of total ammonium and salt that cause process disturbance in AD have been determined in numerous studies, but this led to a very broad concentration range that provokes 50% inhibition of methane production, *i.e.* 1.7–14 g N L^{-1} for TAN (Chen et al., 2008) and 4.4–17.7 g L^{-1} for Na^+ (Feijoo et al., 1995). Several factors also influence the degree of toxicity of both salt and TAN, including pH, temperature, presence of other cations, and organic loading rate (Chen et al., 2008; Fang et al., 2011; Garcia and Angenent, 2009; Moestedt et al., 2016; Rajagopal et al., 2013). Especially for TAN, which can be present as ammonium ion (NH_4^+) and the free ammonia (NH_3) form, both an increase in pH and temperature engage a shift to free ammonia. Free ammonia is, in general, more toxic than the ammonium ion, due to its ability to freely migrate through the microbial cell membrane, thus, influencing intracellular pH and proton transport across the membrane (Gallert et al., 1998).

The accumulation of VFA and instable methane production as a

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result of an increase in TAN and/or salt concentration is the consequence of the overall high susceptibility of the methanogenic (archaeal) community to high salt and total/free ammonia concentrations, compared with the bacterial community (De Vrieze et al., 2012). This is related with often observed shifts from acetoclastic to hydrogenotrophic methanogenesis with increased total/free ammonia and salt concentrations (Niu et al., 2013; Schnurer and Nordberg, 2008; Werner et al., 2014). Hence, an increased level of microbial community dynamics could be observed in response to process disturbances that are accompanied with (sudden) changes in operational parameters (Goux et al., 2015; Li et al., 2015b; Niu et al., 2015a; Regueiro et al., 2014a).

To determine to which extent microbial community dynamics influence functional stability, several aspects need to be taken into account. As the microbial community shows a constant degree of dynamics, even at a constant feeding regime and operational conditions (De Vrieze et al., 2013; Fernandez et al., 1999), it is not always straightforward to differentiate between natural fluctuations and actual shifts with respect to process disturbances. If the shift in the microbial community is confirmed as beyond natural fluctuations, its influence on process performance needs to be estimated. For example, a shift from acetoclastic methanogenesis to syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis often maintains process stability (Werner et al., 2014; Westerholm et al., 2011). This shift, however, often coincides with a decrease in acetate oxidation and even methane production (Schnurer et al., 1999). It is, therefore, important to estimate to which extent community dynamics can prevent process failure. A shift in the microbial community in response to a specific disturbance could coincide with a change in (species) diversity (Briones and Raskin, 2003; Curtis and Sloan, 2004). Microbial community diversity and functional stability often do not show a positive correlation (Baho et al., 2012; Dearman et al., 2006; McCann, 2000), which emphasizes the equal importance of functional resilience and/or redundancy to ensure process stability in AD (Niu et al., 2015a; Werner et al., 2011). The microbial community can either maintain a stable composition (resistance), temporarily change in composition (resilience) or shift to a new composition (redundancy) in response to a disturbance (Allison and Martiny, 2008). After the disturbance has disappeared, the return to a microbial community with similar functional stability as the original state has to be confirmed to ensure resistance to future perturbations.

In this research, molasses wastewater with a high salinity and nitrogen content was used as feedstock to provoke a disturbance in AD after which the feedstock was switched again to waste activated sludge with low salinity and nitrogen content. This was carried out to test the ability of the microbial community to return to its original state and performance. After this, an alternative disturbance was applied through the addition of urea to evaluate the functional resilience or redundancy of the microbial community following the previous disturbance. The archaeal and bacterial community were evaluated in terms of functional performance (methane production) and ecology with respect to the different disturbances.

2. Material and methods

2.1. Inoculum and feedstocks

The anaerobic sludge inoculum was obtained from a full-scale digester treating brewery wastewater (Van Steenberge, Ertvelde, Belgium) (Table 1). The molasses wastewater was acquired from the company AVEBE (Veendam, The Netherlands), and the waste activated sludge (WAS) was collected from the municipal wastewater treatment plant Dendermonde (Dendermonde, Belgium) (Table 2).

Table 1
Characteristics of the anaerobic sludge that was used to inoculate the digesters (n = 3).

Parameter	Value
pH	7.33 ± 0.04
Total solids, TS (g L^{-1})	59.5 ± 3.4
Volatile solids, VS (g L^{-1})	49.3 ± 3.2
Conductivity (mS cm^{-1})	5.71 ± 0.47
Total VFA	0 ± 0
Acetate	0 ± 0
Propionate	0 ± 0
Total ammonia nitrogen, TAN (mg L^{-1})	220 ± 5
Free ammonia (mg L^{-1})	0.45 ± 0.01

Both the molasses wastewater and WAS were stored at 4 °C until use.

2.2. Experimental set-up

Glass Schott bottles with a liquid volume of 800 mL and a total volume of 1 L were used as digesters. A rubber stop was used to ensure anaerobic conditions, and a plastic tube was installed as gas outlet through the rubber stop. The biogas was collected by means of water displacement in inverted plastic tubes (Fig. S1). To avoid CO_2 in the biogas from dissolving in the water, the pH was maintained below a value of 4.3. The inoculum was diluted with tap water until a volatile suspended solids concentration of 10 g L^{-1} was obtained. The digesters were operated in a continuous stirred tank reactor mode, and incubated in a temperature controlled room at 34 ± 1 °C. Manual feeding took place three times a week.

2.3. Digester operation: start-up and experimental phases

In the start-up phase (day 0–34), the nine digesters were operated in the exact same way (Table 3 and Table S1). Initially (day 0–20), only WAS sludge was used as feedstock, and the hydraulic retention time (HRT) was decreased from 40 to 20 days, while from day 21–34 molasses wastewater was slowly added at an HRT of 20 days to avoid overloading of the digester, and to guarantee a smooth transition between the two feedstocks.

The experimental phase consisted of 3 main phases (Table 3). In Phase 1 (day 35–83), molasses was used as single feed in each of the nine digesters. A differentiation between the three digesters, each of which were run in triplicate, was initiated in Phase 2 (day 84–167). To Digester A, molasses was continued to be added as single feedstock, whilst for the other two digesters (Digester B and C), the feed was switched back to the same WAS that was used during the start-up phase. Glycerol was added as a co-substrate to Digester B and C to obtain a similar organic loading rate in all digesters, because the COD content of the WAS was too low to ensure an organic loading rate of 2.0 g COD L^{-1} d^{-1}, as was the case in Digester A. Glycerol was selected as co-substrate, because crude glycerol is often co-digested with other feedstocks to increase methane production (Fountoulakis et al., 2010). In Phase 3 (day 168–245), molasses was still the only feed for Digester A. In both Digester B and C, WAS and glycerol also still served as substrate, but to Digester C, first NH_4Cl and then urea was added (Fig. S2) to slowly increase the free ammonia concentration to obtain a final additional TAN concentration of 2.0 g N L^{-1}. The organic loading rate and HRT were maintained at 2.0 g COD L^{-1} d^{-1} and 20 days, respectively, during the entire experimental phase in each of the digesters.

The volumetric biogas production and composition were measured three times per week, and reported at standard temperature (273 K) and pressure (101,325 Pa) conditions (STP). The pH

Table 2
Characteristics of the waste activated sludge and molasses wastewater (n = 3).

Parameter	Waste activated sludge	Molasses wastewater
pH (−)	6.83 ± 0.04	5.63 ± 0.04
Total solids, TS (g kg^{-1})	58.7 ± 0.2	52.6 ± 0.6
Volatile solids, VS (g kg^{-1})	20.2 ± 0.2	36.7 ± 1.7
Total COD (g kg^{-1})	29.6 ± 2.0	40.0 ± 1.4
Conductivity (mS cm^{-1})	2.70 ± 0.36	13.40 ± 0.45
Total VFA (mg COD kg^{-1})	0 ± 0	560 ± 19
Acetate (mg COD kg^{-1})	0 ± 0	560 ± 19
Propionate (mg COD kg^{-1})	0 ± 0	0 ± 0
Total ammonia nitrogen, TAN (mg N kg^{-1})	249 ± 7	132 ± 10
Kjeldahl nitrogen, TKN (mg N kg^{-1})	1650 ± 70	3290 ± 90
COD:N ratio	18.0 ± 1.4	12.2 ± 0.5
TS:VS ratio	2.90 ± 0.03	1.43 ± 0.07
COD:VS ratio	1.47 ± 0.10	1.09 ± 0.06

Table 3
Overview of the main difference between the treatments in the different phases of the experiment. The organic loading rate and hydraulic retention time were kept constant at 2.0 g COD L^{-1} d^{-1} and 20 days, respectively, in each treatment. WAS = waste activated sludge.

Phase	Period	Feed composition		
		Digester A	Digester B	Digester C
Start-up	Day 0–34	WAS + molasses	WAS + molasses	WAS + molasses
Phase 1	Day 35–83	molasses	molasses	molasses
Phase 2	Day 84–167	molasses	WAS + glycerol	WAS + glycerol
Phase 3	Day 168–245	molasses	WAS + glycerol	WAS + glycerol + urea/NH_4Cl

in the mixed liquor of each digester was also measured three times per week. Samples for total VFA, TAN and conductivity measuring were taken once per week. Conductivity can be considered an overall estimation of salinity in the mixed liquor. Samples were collected from the inoculum and the mixed liquor at the end of each phase for microbial community analysis, and stored at −20 °C until analysis.

2.4. Microbial community analysis

The DNA extraction was carried out as described by Vilchez-Vargas et al. (2013). The quality of the DNA extracts was validated by means of agarose gel electrophoresis and via PCR, using the protocol of Boon et al. (2002) with the bacterial primers P338 and P518r (Muyzer et al., 1993). The quality of the PCR product was determined with agarose gel electrophoresis to ensure that no inhibition of the PCR took place. The DNA extracts were sent to LGC Genomics GmbH (Berlin, Germany) for Illumina sequencing on the Miseq platform. The amplicon sequencing and data processing were carried out as described in SI (S4).

2.5. Statistical analysis

Separate tables containing the abundances of the OTUs (operational taxonomic units) and the taxonomic assignments were generated for the bacteria and archaea (Supplementary file 2). The R Studio version 3.2.3. software (http://www.r-project.org) (R Development Core Team, 2013) was used for statistical analysis. The packages phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen et al., 2016) were used for microbial community analysis. The community composition of the biological replicates was statistically compared by means of analysis of variance (ANOVA, *aov* function) to validate that both the bacterial and archaeal community did not significantly differ between biological replicates. Heatmaps were generated using the weighted average values of the biological replicates by means of the pheatmap package. Differences in order-based Hill's numbers (Hill, 1973) between the digesters were defined via ANOVA and the post-hoc Dunn's Test of Multiple Comparisons (dunn.test package) with Benjamini-Hochberg correction. Separate non-metric distance scaling (NMDS) plots of the bacterial and archaeal community data were created based on the (un)weighted Unifrac and Bray-Curtis distance measures. Significant differences in community composition between digesters and/or between different phases were identified by means of pair-wise Permutational ANOVA (PERMANOVA) with Bonferroni correction, using the *adonis* function (vegan). The identification of a subset of OTUs that best describe the overall community pattern was carried out *via* a stepwise exploration according to description of the *forward selection/backward elimination* algorithm (Clarke and Warwick, 1998). This was done in two steps. First, the algorithm was run for 50 times, randomly selecting 60% of the total OTUs for inclusion in the stepwise exploration. In the second step, samples similarities were identified using a much reduced number of subset of OTUs by maximising correlation (pearson correlation) of the Bray-Curtis distances measure between samples considering the subset of OTUs and between the samples considering all OTUs.

2.6. Chemical analyses

Total solids (TS), VS, total suspended solids (TSS), volatile suspended solids (VSS), Kjeldahl nitrogen (TKN) and TAN were determined by means of standard methods (Greenberg et al., 1992). The free ammonia concentration was calculated based on the TAN concentration, pH and temperature (Anthonisen et al., 1976). A C532 pH and C833 conductivity meter (Consort, Turnhout, Belgium) were used to measure pH and conductivity, respectively.

The biogas composition was measured with a compact gas chromatograph (Global Analyser Solutions, Breda, The Netherlands), and concentrations of different VFA were also analysed by means of gas chromatography (GC-2014, Shimadzu®, The Netherlands), as described in SI (S5).

2.7. Data deposition

Data deposition: the sequences reported in this paper have been deposited in the European Nucleotide Archive (ENA) database (Accession numbers LT624945-LT625937 for bacteria and LT625938-LT626064 for archaea).

3. Results

3.1. Impact of feed composition on digester performance

The shift in feedstock from WAS to molasses wastewater during the start-up resulted in an increase in methane production to 416 ± 15 mL CH L^{-1} d^{-1} on day 35 (Fig. 1). The pH reached a value of 7.37 ± 0.04 (Fig. S3), and VFA remained below the detection limit (Fig. S4), indicating a stable start-up. During Phase 1, feeding of molasses wastewater initially resulted in an increase in methane production with a maximum value of 625 ± 17 mL CH_4 L^{-1} d^{-1} on day 37 (Fig. 1), and this corresponded with a COD conversion efficiency of 89.3 ± 2.4%. After this, methane production decreased to a value of 232 ± 35 mL CH_4 L^{-1} d^{-1} (33.2 ± 5.0% conversion of the COD in the molasses wastewater to CH_4) on day 84. A concomitant increase in total VFA concentration to 20.5 ± 1.4 g COD L^{-1} was observed, which corresponded with 51.2 ± 4.0% of the COD in the molasses wastewater converted to VFA (Fig. S4). This points to a strong inhibition of the AD process. This decrease in methane production and increase in VFA concentration coincided with an increase in conductivity to 26.8 ± 0.7 mS cm-1 on day 84 (Fig. 2). Similarly, an increase in the TAN concentration (Fig. S5) could be observed, while free ammonia concentration (Fig. S6) initially increased to a maximum value of 81 ± 12 mg N L^{-1} after which it decreased to 34 ± 3 mg N L^{-1} at the end of Phase 1.

The switch to WAS and glycerol as main feedstock in Phase 2 in Digester B resulted in an increase in methane production to 248 ± 17 mL CH_4 L^{-1} d^{-1} on day 168, while VFA decreased to values below detection limit towards the end of Phase 2, indicating process recovery. In contrast, methane production in Digester A in which molasses wastewater was still used as feedstock decreased further to a value of 62 ± 15 mL L^{-1} d^{-1} (8.9 ± 2.1% conversion of the COD in the molasses wastewater to CH_4) and VFA further accumulated to 30.3 ± 1.8 g COD L^{-1}, which corresponded with 75.7 ± 5.2% of the COD in the molasses wastewater converted to VFA. The conductivity reached a value of 29.6 ± 0.6 mS cm^{-1} in Digester A on day 168, while it was a factor 6 lower in Digester B,

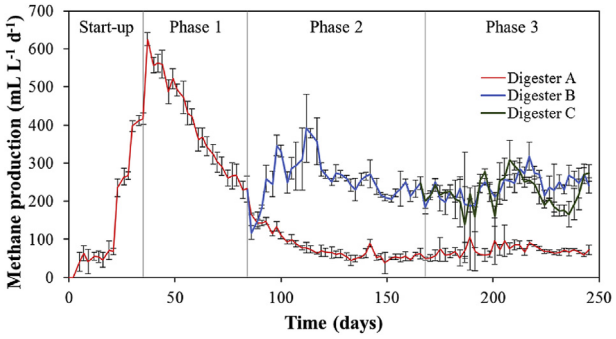

Fig. 1. Methane production in the three different digesters. Error bars represent standard deviations of the biological replicates. In the Start-up phase and Phase 1, the average value (n = 9) was calculated over Digester A, B and C, as these were still biological replicates. In Phase 2, the average value (n = 6) was calculated over Digester B and C, as these were still biological replicates, while Digester A (n = 3) was considered separately, due to a different treatment. In Phase 3, Digester A, B and C (each n = 3) were considered separately, due to a different treatment.

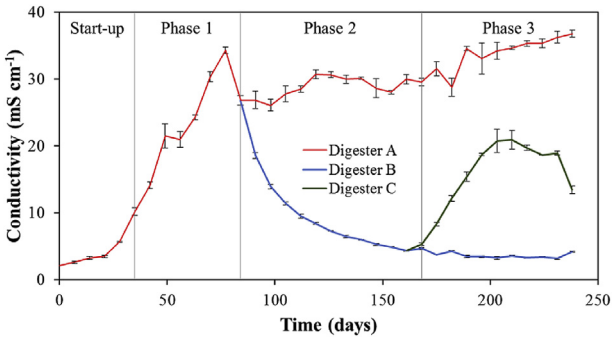

Fig. 2. Conductivity in the mixed liquor of the three different digesters. Error bars represent standard deviations of the biological replicates. In the Start-up phase and Phase 1, the average value (n = 9) was calculated over Digester A, B and C, as these were still biological replicates. In Phase 2, the average value (n = 6) was calculated over Digester B and C, as these were still biological replicates, while Digester A (n = 3) was considered separately, due to a different treatment. In Phase 3, Digester A, B and C (each n = 3) were considered separately, due to a different treatment.

with only 4.68 ± 0.12 mS cm^{-1}. A similar observation was made for TAN, with values of 1.5 ± 0 and 0.7 ± 0 g N L^{-1} in Digester A and B, respectively.

In the final phase (Phase 3), Digester A showed similar values to Phase 2 in terms of methane production and VFA concentration. Similarly, Digester B maintained stable methane production and VFA remained below the detection limit, which indicates that process recovery was maintained. The addition of NH_4Cl and urea to Digester C resulted in an increase in TAN and free ammonia concentration, yet, this did not result in a decrease in methane production, and VFA remained below 0.5 g COD L^{-1} up to day 196. Towards the end of Phase 3, however, a minor increase in VFA concentration up to 1.2 ± 0.8 g COD L^{-1} or 3.0± 2.0% of the COD in the molasses wastewater was observed, while this was not the case for Digester B.

3.2. Microbial community changes in response to feed composition and operational conditions

Microbial community analysis resulted in an average of 11,762 ± 4650 reads and 994 OTUs for the bacterial community, while 37,018 ± 19,424 reads and 127 OTUs were obtained for the archaeal community. No significant differences ($P < 0.01$) were observed in bacterial and archaeal community composition between biological replicates of the same digester.

3.2.1. Microbial community composition

The bacterial community was mainly dominated by the Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes phyla (Fig. 3a). The Inoculum did not show a distinct dominance of any of the bacterial phyla, with the exception of a slightly higher relative abundance (23.1%) of the Proteobacteria phylum. In contrast, Actinobacteria mainly dominated Digester B and C, representing on average 38.4 ± 6.0% of total bacterial reads, and this was mainly related with the increased relative abundance of Actinomycetales order (Fig. S7). Firmicutes dominated Digester A, with an average of 65.5 ± 6.6% of total bacterial reads, and the Clostridiales and Lactobacillales were the two main orders. A slight increase in Firmicutes relative abundance was, however, observed in Digester C, compared with Digester B, which was related with a relative increase in Lactobacillales. The difference in dominance between the Actinobacteria and Firmicutes phylum was clearly reflected in the composition of the feedstocks, *i.e.* WAS (47.4% of total reads for Actinobacteria) and molasses wastewater (93.8% of total reads for

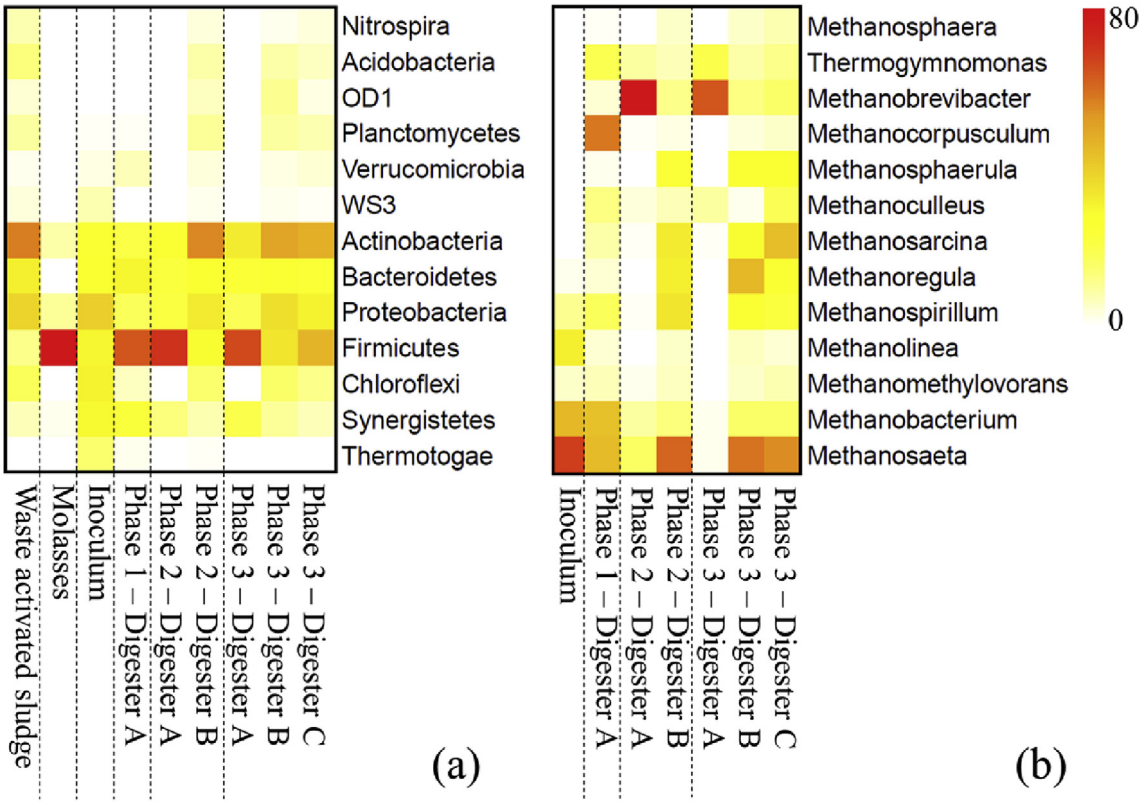

Fig. 3. Heatmap showing the square root transformed relative abundance of (a) the bacterial community at the phylum level, and (b) the archaeal community at the genus level. Weighted average values of the biological replicates are presented. The colour scale ranges from 0 (white) to 80% (red) relative abundance. In Phase 1, the average value (n = 9) was calculated over Digester A, B and C, as these were still biological replicates. In Phase 2, the average value (n = 6) was calculated over Digester B and C, as these were still biological replicates, while Digester A (n = 3) was considered separately, due to a different treatment. In Phase 3, Digester A, B and C (each n = 3) were considered separately, due to a different treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Firmicutes), respectively.

The archaeal community contained both acetoclastic and hydrogenotrophic methanogens, yet, their relative abundance strongly differed between the different digesters (Fig. 3b). Digester B and C were mainly dominated by OTUs belonging to the acetoclastic *Methanosaeta* genus (35.9 ± 4.2% of total archaeal reads), which was also the case for the inoculum sample (49.3%), while Digester A was dominated by OTUs belonging to the hydrogenotrophic *Methanocorpusculum* (Phase 1, 35.4%) and *Methanobrevibacter* (Phase 2 & 3, 54.7 ± 17.5%) genera. Digester B also showed an increased relative abundance of the *Methanosarcina* genus (20.0%). No methanogens were detected in the WAS and molasses wastewater feedstocks.

3.2.2. Microbial community dynamics

The evolution in community composition over time, and with respect to the different digesters was analysed via NMDS of the weighted Unifrac distance measure (Fig. 4). Both for the bacterial and archaeal community, four distinct clusters could be identified, containing the Inoculum sample (Cluster 1), Digester A in Phase 1 (Cluster 2), Digester A in Phase 2 & 3 (Cluster 3) and Digester B and C in Phase 2 & 3 (Cluster 4). A significant difference in community composition was observed between Digester B and C on one hand and Digester A on the other hand for bacteria ($P = 0.0006$) and archaea ($P = 0.009$). A significant difference was also observed between the Inoculum and Digester B and C for bacteria ($P = 0.0012$) and archaea ($P = 0.0006$). This indicates a strong deviation from the original community, both for archaea and bacteria, despite similar operational conditions for the Inoculum and Digester B in Phase 2 & 3. Similar observations were made based on the unweighted Unifrac and Bray-Curtis dissimilarity measures (Fig. S8).

For the bacterial community, only OTU851 (Unclassified Bacteria) showed a significant correlation ($P = 0.001$) with the Bray-Curtis dissimilarity matrix. In contrast, in the archaeal community four OTUs, *i.e.* OTU5 (*Methanobrevibacter*, $P = 0.003$), OTU28 (*Methanobacterium*, $P = 0.001$), OTU267 (*Methanobacterium*, $P = 0.022$), and OTU372 (*Methanoregula*, $P = 0.001$) were identified with a significant correlation with the Bray-Curtis dissimilarity matrix.

3.2.3. Microbial community redundancy and functionality

Microbial community redundancy, *i.e.* the change in microbial community organization in response to a disturbance (Werner et al., 2011) was evaluated *via* alpha diversity analysis by means of the Hill diversity order numbers (Hill, 1973).

A clear difference could be observed between the three Hill diversity orders for the bacterial community. A significantly lower richness (H_0) was observed between the Inoculum sample and Digester B in Phase 2 ($P = 0.0152$) and Phase 3 ($P = 0.0203$) (Fig. 5a), while this was not the case for H_1 and H_2 (Fig. 5b and c). The diversity in Digester B Phase 2 ($P = 0.0050$ for H_0 and 0.0106 for H_1), Digester B Phase 3 ($P = 0.0052$ for H_0 and 0.0104 for H_1), and Digester C Phase 3 ($P = 0.0157$ for H_0 and 0.0408 for H_1) was significantly higher, compared with Digester A. The H_2 diversity in the Inoculum was significantly higher than for Digester A in Phase 1 ($P = 0.0184$) and Phase 2 ($P = 0.0192$).

The archaeal species richness (H_0) was significantly lower in the

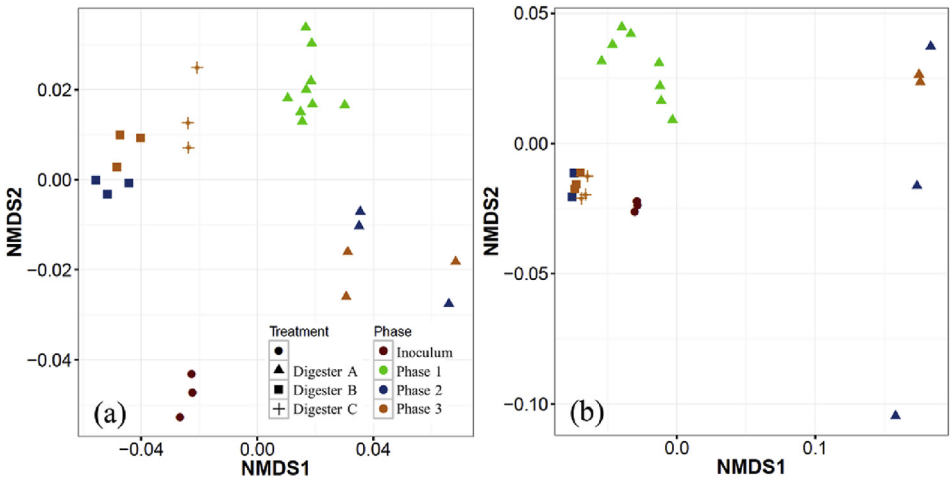

Fig. 4. Non-metric distance scaling (NMDS) analysis of the weighted Unifrac distance indices of the (a) bacterial, and (b) archaeal community at OTU level. Different symbols and colours are used for different digesters and phases, respectively.

Inoculum compared with Digester B in Phase 2 ($P = 0.0287$), and Digester B ($P = 0.0319$) and Digester C ($P = 0.0313$) in Phase 3 (Fig. 5a), while this was not the case for H_1 and H_2 (Fig. 5b and c). Each of the three order numbers of the archaeal community was significantly lower in Digester A than Digester B and C (Phase 2 & 3). No significant differences were detected between Digester B and C, both for bacteria and archaea ($P > 0.2$).

4. Discussion

A clear shift in community composition was detected with respect to the shifts between WAS and molasses wastewater as main feedstock, both for the bacterial and archaeal community. Despite a retrieval of similar operational parameters in Phase 3, the microbial community did not return to its initial state following the disturbance phase, despite similar conditions. A decrease in alpha diversity was observed during the disturbance period (Phase 1), yet, it increased again following stabilization of the AD process in the final phase (Phase 2 & 3). The application of a mild disturbance in Phase 3 did not entail a strong effect on process or community stability.

4.1. Salt stress drives bacterial and archaeal community composition

Stable methane production was initially observed during the start-up phase, as VFA remained below the detection limit. Stable methane production was, however, not maintained, because during Phase 1 methane production and pH decreased, and VFA accumulated, indicating process failure. Both the increase in ammonia concentration and/or salinity (as measured by conductivity) could be considered responsible for this. As the TAN concentration did not reach values higher than 1.9 g N L^{-1} in Phase 1 & 2, and also free ammonia remained below 90 mg N L^{-1}, ammonia toxicity was most likely not the main cause of process failure (Angelidaki and Ahring, 1993; Rajagopal et al., 2013; Sung and Liu, 2003). In contrast, salinity reached values up to 35 mS cm^{-1} in Phase 1, while it has been postulated that overall conductivity in AD should be maintained below 30 mS cm^{-1} to avoid salt stress (Chen et al., 2008; De Vrieze et al., 2012). The problem of salt inhibition during AD of molasses or vinasses wastewaters has been reported in numerous studies (De Vrieze et al., 2014; De Vrieze et al., 2015b; Fang et al., 2011; Syutsubo et al., 2013), thus, supporting our findings.

The switch in feedstock from WAS to molasses also resulted in a shift in the microbial community. The increase in Firmicutes, more specifically the Clostridiales and Lactobacillales orders, in Digester A and the increase in Actinobacteria in Digester B and C appears to be a direct consequence of the feedstock microbial composition itself, *i.e.* the molasses wastewater and WAS, respectively. This is in line with other studies in which the importance of the feedstock for shaping the community in AD has been demonstrated (Li et al., 2015a; Lu et al., 2013; Zhang et al., 2014; Ziganshin et al., 2013). In line with the results in our study, the increased relative abundance of the Actinobacteria often has been observed in sludge digesters (Chouari et al., 2005; De Vrieze et al., 2015c; Sundberg et al., 2013). The microbial composition of the feedstock is, however, not the only factor that determined the microbial community in the digesters. A dominance of the Clostridiales order often has been observed at suboptimal conditions for methanogenesis (*e.g.* increased ammonia and salt concentrations), irrespective of feedstock composition (Alsouleman et al., 2016; De Vrieze et al., 2015c; Muller et al., 2016). The shift in the methanogenic community from the acetoclastic *Methanosaeta* to the hydrogenotrophic *Methanobrevibacter* and *Methanocorpusculum* is also related with the increased salinity and process deterioration in general (Goberna et al., 2015; Walter et al., 2016). This potentially reflects the shift from acetoclastic methanogenesis to syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis, as reported earlier for Clostridiales and *Methanobrevibacter* or other hydrogenotrophic methanogens (Muller et al., 2016; Werner et al., 2014).

4.2. The microbial community does not return to its original composition following the disturbance phase

The significant shift in microbial community composition between the Inoculum and Digester A in Phase 1 was as anticipated, given the strong change in operational conditions. In Digester B in Phase 2 & 3, by adjusting the feed, operational conditions were readjusted to the initial situation, yet, neither the bacterial, nor the archaeal community returned to its original composition. This is related with the high diversity of the bacterial community. Multiple bacterial species are able to occupy the same niche in AD, for example the fermentation of carbohydrates into VFA (Xia et al., 2015), thus, a different community can be used to obtain a similar process performance, as observed earlier (Goux et al., 2015). This was confirmed by the operational parameters in Digester B in Phase

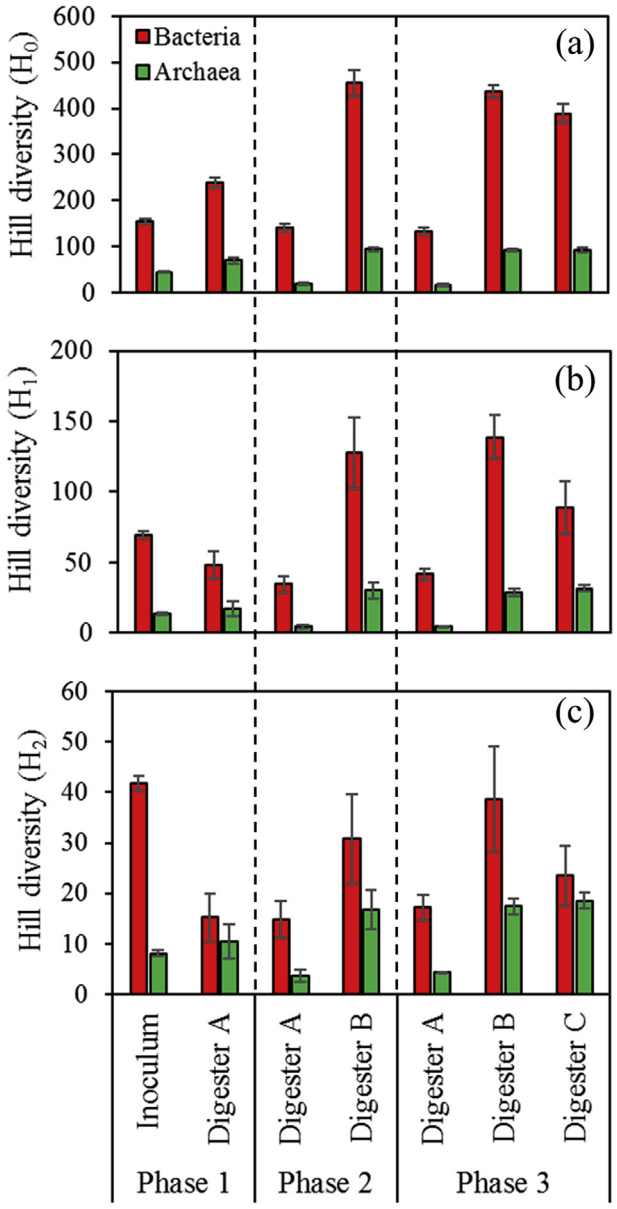

Fig. 5. Alpha diversity of the samples for each digester in the different phases. The three Hill order diversity numbers (a) H_0 (richness, number of OTUs), (b) H_1 (exponential value of the Shannon index) and (c) H_2 (inverse Simpson index) were calculated both for bacteria and archaea. Error bars represent standard deviations of the biological replicates. In Phase 1, the average value (n = 9) was calculated over Digester A, B and C, as these were still biological replicates. In Phase 2, the average value (n = 6) was calculated over Digester B and C, as these were still biological replicates, while Digester A (n = 3) was considered separately, due to a different treatment. In Phase 3, Digester A, B and C (each n = 3) were considered separately, due to a different treatment.

2 &3. It also relates with the often observed (high) bacterial community dynamics over time, despite constant or similar operation (De Vrieze et al., 2016; Klang et al., 2015; Pycke et al., 2011). An alternative explanation might be the change in feedstock, *e.g.* the addition of glycerol to the feed, which has been shown to potentially influence microbial community structure (Zhang et al., 2014). However, it was shown by De Vrieze et al. (2015c) that digesters with similar operational conditions contained closely related microbial communities.

The significant change in the overall archaeal community between the Inoculum and Digester B in Phase 2 & 3 was not anticipated, given the much lower archaeal diversity compared with bacteria, as observed in this and other studies (Nelson et al., 2011; Sundberg et al., 2013). The archaeal community dynamics as a function of time at stable conditions are in most cases also lower than for bacteria (De Vrieze et al., 2013; Regueiro et al., 2014b; Town et al., 2014), as long as no (severe) process disturbance, as was the case in Phase 1, leads to (partial) inhibition of the methanogens (Poirier et al., 2016; Williams et al., 2013). *Methanosaeta* can be considered a key methanogen in the AD processes, because it is the, thus far, only known acetoclastic methanogen with a sufficiently low K_s value to efficiently convert acetate directly to methane at low acetate concentrations (Conklin et al., 2006; De Vrieze et al., 2012). This allows *Methanosaeta* to become dominant at low acetate concentrations, which is in contrast with *Methanosarcina* that has a higher Ks value and, thus, lower affinity for acetate (Conklin et al., 2006; De Vrieze et al., 2012). Despite the fact that a clear revival of the *Methanosaeta* genus can be observed in Digester B, following the disturbance in Phase 1, its relative abundance is clearly lower compared with the Inoculum. This 'hiatus' in the methanogenic community was taken in by *Methanoregula*. The *Methanoregula* OTU372 significantly correlated with the Bray-Curtis dissimilarity matrix, which confirms its clear contribution to the methanogenic community shift. As *Methanoregula* is a hydrogenotrophic methanogen that can also use formate as substrate (Yashiro et al., 2011), it cannot take over the acetoclastic methanogenesis. Hence, this points to a partial shift in methanogenesis pathway caused by the disturbance, even though operational conditions, such as pH, TAN and VFA concentration, were similar in digester B in Phase 3 compared with the Inoculum. The inability of *Methanosaeta* to regain its previously uncontested position in the methanogenesis process was most likely hampered by its low growth rate (Conklin et al., 2006; De Vrieze et al., 2012), and the absence of *Methanosaeta* sp. in the WAS sludge, making feedstock inoculation not possible.

4.3. The microbial community retains its redundancy and metabolic potential following recovery after process disturbance

Rather than maintaining a constant composition, the resilience, resistance or redundancy of the microbial community is important to sustain process performance (Allison and Martiny, 2008; Niu et al., 2015b; Werner et al., 2011). In this research, during process disturbance, the microbial community was unable to sustain the methane production process, most likely due to the high salinity, caused by the molasses wastewater. In Phase 2 & 3, Digester B regained process stability, reflected in the increase in methane production and removal of residual VFA. Both on the bacterial and archaeal level, the community did not rebound to its original composition. This demonstrates microbial community redundancy, rather than resilience or resistance as the major strategy behind AD process recovery (Allison and Martiny, 2008; Langer et al., 2015).

The metabolic potential of the microbial community was estimated by basic alpha diversity analysis through the Hill diversity order numbers. The different diversity order numbers, both for bacteria and archaea, indicated a similar, and sometimes even significantly higher diversity in Digester B and C (Phase 2 & 3) in comparison with the Inoculum. Hence, this proves that the metabolic potential of the microbial community is retained after process disturbance.

To verify to which extent the microbial community would be able to deal with other mild disturbances after process recovery, a mild (free) ammonia disturbance was provoked in Digester C. Both methane production and pH values were maintained at similar levels as Digester B, and only a minor accumulation of VFA was observed towards the end of Phase 3. The overall bacterial and

archaeal community did not show a significant change in composition compared with Digester B. However, an increase in relative abundance of the Firmicutes phylum and *Methanosarcina* genus, as observed earlier for (free) ammonia disturbance in AD (De Vrieze et al., 2015a; Niu et al., 2015a), again demonstrates the redundant character of the microbial community.

5. Conclusions

The application of a disturbance, in this case molasses wastewater, resulted in a significant shift in bacterial and archaeal community composition, following recovery after the disturbance. Despite the fact that the microbial community did not return to its original position, microbial diversity was recovered. Hence, microbial redundancy was shown to be the major strategy behind process recovery, continued operational stability and microbial flexibility in AD in response to a common disturbance, *i.e.* most likely a high salinity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.12.042.

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