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Biomethanation and microbial community changes in a digester treating sludge from a brackish aquaculture recirculation system



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HIGHLIGHTS

- Saline seed & stepwise increased OLR enhance specific CH₄ yield from salty sludge.
- Stable digester performance w/o severe VFA accumulation even at 4.4 kg COD/(m³ day).
- OLR adjustment & fecal substrate substantially influence the digester microbiomes.
- Most abundant methanogen is *Methanosarcina* in both the inoculum and digestates.
- Increasing similarity in microbial community of inoculum and digestates at low OLR.

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ABSTRACT

Using a high-salinity-adapted inoculum and a moderate stepwise-increased organic loading rate (OLR), a stable digester performance was achieved in treating sludge from a brackish aquaculture recirculation system. The specific methane yield was distinctly enhanced, reaching 0.203 L CH₄/g COD_{added}, compared to literature values (0.140–0.154 L CH₄/g COD_{added}) from the salty sludges. OLR adjustment and the fecal substrate substantially influenced population changes in the digester. Within the bacterial subpopulations, the relative abundance of *Bacillus* and *Bacteroides* declined, accompanied by the increase of *Clostridium* and *Trigonala* over time. The results show *Trigonala* was derived from the substrate and accumulated inside the digester. The most abundant methanogen was *Methanosarcina* in the inoculum and the digestates. The *Methanosarcina* proliferation can be ascribed to its metabolic versatility, probably a feature of crucial importance for high-salinity environments. Other frequently observed methanogens were outcompeted. The population similarity at the genus level between inoculum and digestates declined during the initial stage and afterwards increased.

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1. Introduction

Marine/brackish recirculation aquaculture systems (RAS) provide a stable production rate of seafood including fish. It is considered as an environmentally friendly and highly efficient approach to satisfy the demand of seafood, while meeting the increasingly stringent regulations. Nevertheless, the relatively small amount of organic-rich streams, for instance, backwash water from drum-filters which are commonly employed as solids separation unit in RAS, still constraints its sustainability and wide-spread application (Zhang et al., 2013). The streams contain fish faeces and a small fraction of uneaten feed and are also characterized

by high salt concentrations (Gebauer, 2004; Gebauer and Eikebrokk, 2006; Zhang et al., 2014). The high salinity of the sludge limits its land application as fertilizer and direct discharge into a sewer system. Thus, this waste stream is impending to be properly disposed of.

Anaerobic digestion (AD) is a relatively simple and efficient biological approach to simultaneously achieve sludge stabilization and recover bioenergy in the form of biogas from organic wastes. Moreover, during AD, nutrients such as ammonia and phosphorus are released from nitrogenous and/or phosphorous rich organic matter, which offers the feasibility to recover the nutrients from digestate. Therefore, AD is seemingly a technological alternative of interest to treat the organic streams from marine/brackish RAS.

There have been a few studies on AD of sludges from brackish/marine RAS (Gebauer, 2004; Gebauer and Eikebrokk, 2006;

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Mirzoyan and Gross, 2013). However, Gebauer and Eikebrokk (2006) and Gebauer (2004) reported that the performance of a completely stirred tank reactor (CSTR) fed with sludge with a salinity of 35.0 g/L was not stable at an OLR of 3.1 kg COD/(m³ day). After two times dilution of the sludge using tap water in the study (Gebauer, 2004), a stable reactor performance was reached. The cited results indicate that the unstable reactor performance may be attributed to the non-high salinity adapted inoculum, that is: a mixture of sewage sludge and cow manure (Gebauer, 2004) and a similar inoculum that was first fed with saline substrate for a period of only 4 months (Gebauer and Eikebrokk, 2006). Moreover, high salinity causes long adaptation periods and inhibition to AD microbial activity (Chen et al., 2008). The minimum SRT and adaptation periods are required to avoid a washout of the microbial population with low growth rates in digesters treating saline wastes when non-high salinity acclimatized inoculum is employed. The presence of well-adapted inoculum to saline conditions is crucial, particularly for shortening the reactor start-up procedure and for warranting functional halophilic anaerobic species that are required for efficiently treating saline waste(waters) (Lefebvre and Moletta, 2006). Moreover, the presence of a well-adapted inoculum influences the stability of the microbial profile and their dynamics in digesters (Angelidaki et al., 2006). In addition, Ganesh et al. (2013) and Wang et al. (2013) employed a strategy, i.e. stepwise increases in OLR, via which the stable digester performances were achieved. The strategy is of great importance to the stable performance and the presumably well-adapted community in the studies. In the AD of salty sludges from marine/brackish RAS, stepwise increases in OLR probably are an efficient strategy to achieve the stable performance of the digester even at OLRs exceeding 3.1 kg COD/(m³ day) without causing digester perturbation.

In the anaerobic treatment of fish excreta that also contains diverse anaerobic microorganisms, the community in digesters may not only be influenced by the inoculum, but also by the fecal feedstock. Hence, it is of great interest and significance to examine the influences of inoculum and feedstock on the predominant species in a digester (Cardinali-Rezende et al., 2011). Thus far, population dynamics at both bacterial and archaeal levels in saline digesters treating brackish/marine RAS sludges have been rarely studied. Most importantly, a better understanding of microbial dynamics may be conducive for elaborating the impact of feeding conditions on an alternation of microbes over time in the digester fed with fecal sludge.

The main aims of this work were to: (1) verify the hypothesis that proper inoculum and stepwise increased OLR enhance the methane yield from the brackish RAS feedstock by comparing experimentally derived methane yields with the ones reported in the literature; (2) preliminarily understand the influences of the inoculum, the fecal waste and also operational conditions (organic loading rate and sludge retention time) on biodiversity and abundance of the bacterial and archaeal populations under the saline condition.

2. Material and methods

2.1. Source and characteristics of substrate and inoculum

The substrate was collected from a 60 μm mesh fine sieve of a brackish RAS in the Netherlands. The brackish fish farm mainly cultures turbot with a temperature of 17.5–18.0 °C. The substrate characteristics are presented in Table 1. The substrate, also referred to as the concentrated sludge, was stored at –26 °C within 4 h after sampling. The inoculum, with a salinity of approximately 17 g/L, was taken from one of the full-scale digesters of a fish processing

Table 1
Characterization of the concentrated sludge from the brackish RAS.

Parameter & units	Value
pH	5.8–6.3
Salinity (g/L)	13.5–14.7
EC (mS/cm)	20.4–24.7
ORP (mV)	–217 to –196
TS (g/L)	82.0–136.3
VS (g/L)	57.7–110.4
TSS (g/L)	50.1–110.7
VSS (g/L)	45.5–94.3
TCOD (g O ₂ /L)	77.2–143.1
TP (mg P/L)	813–1793
TN (mg N/L)	2400–4933
NH ₄ -N (mg N/L)	172–400
Ca ²⁺ (mg/L)	353 ± 64 ^a
K ⁺ (mg/L)	422 ± 50 ^a
Mg ²⁺ (mg/L)	346 ± 11 ^a
Na ⁺ (mg/L)	4532 ± 65 ^a
Cl ⁻ (mg/L)	10,004 ± 176 ^a
SO ₄ ²⁻ (mg/L)	1197 ± 201 ^a

^a Indicates the values were the averages of 4 samples; within the 4 samples collected at different time from the brackish fish farm, the difference in the charge balance between anion and cations was less than 7%. The difference might be caused by the variations in the characteristics of the sludges with seasons, particularly NH₄⁺.

factory in the Netherlands. The VSS/VS ratio of the sludge (Table 1) is 80–85%. The main volatile content of the sludge was particulate, which requires a sufficiently long retention time for hydrolysis. Thus, anaerobic digestion of the sludge under mesophilic condition would ensure sufficient hydrolysis of particulate matter.

2.2. Operation of semi-continuous digester

A completely mixed tank reactor (Fig. 1) with a working volume of 4.0 L was operated at mesophilic condition (35 ± 1 °C). The digester was mechanically stirred at a speed of 80 rpm. The operational period was divided into 5 phases. During the first three phases with stepwise increases in OLR, the digester was fed with 250 mL of substrate every two days, while keeping the SRT at 32 days. During the last two phases with decreases in OLR, the feeding volume of the sludge was reduced to 190 mL and the resulting SRT was 42 days. The following parameters were analyzed during the operational period: total solids (TS), volatile solids (VS), total suspended solids (TSS), volatile suspended solids (VSS), total and soluble chemical oxygen demand (COD_{tot} and COD_{sol}), total ammonia nitrogen (TAN), total nitrogen (TN), reactive phosphorus (RP), total phosphorus (TP), volatile fatty acids (VFA), pH, electrical conductivity (EC) and salinity. Biogas, pH and methane production were automatically recorded by an automation system.

2.3. Analysis methods

TS, VS, TSS, and VSS were analyzed following standard methods (APHA, 2005). Alkalinity was measured by an auto-titration instrument (702 SM Titrino, Metrohm, Switzerland). COD, TAN, TN, TP, and RP were measured using the kits (Merck, Germany). The substrate samples and digestates for analyses of COD, TP, and TN, were first homogenized using a digital ultrasonicator with model 250 (Branson, USA). Afterwards, the dilution of the samples was carried out for the analyses, assuring Cl⁻ less than 2.5 g/L to eliminate the interferences for COD analyses. Samples for analyses of COD_{sol}, NH₄-N, RP, and VFA were filtered by syringe membrane filter (0.45 μm, Whatman, Germany). pH of the digestate samples was checked every two days with a multi-functional meter (InoLab® Multi 720, WTW). EC (mS/cm) and salinity (g/L) were measured using a portable conductivity meter (Cond 340i, WTW, Germany).

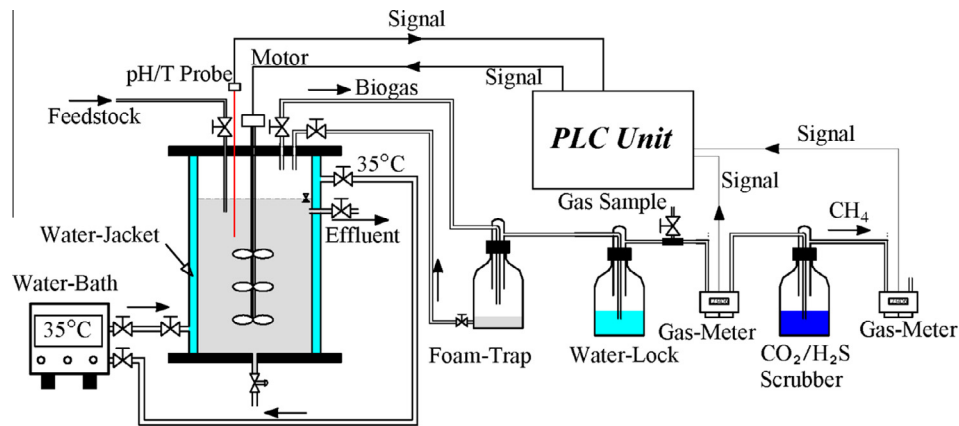


Fig. 1. Set-up of CSTR, (PLC: programmable logic controller).

VFAs were measured by a gas chromatography (GC) with a FID detector (Agilent 7890A, USA) (Helium as carrier gas with a flow rate of 1.8 mL/min; Column: Agilent 19091F-112 (240 °C, 25 m × 320 μm × 0.5 μm, Oven temperature: 80 °C). Biogas composition was analyzed using a GC (Varian CP 4900 with Thermal Conductivity Detector (TCD) and columns, i.e. Mol Sieve 5A PLOT (10 m × 0.53 mm) and PoraPlot U (10 m)). The specifications of the GC were as follows: Channel 1, Mol Sieve 5A PLOT column with a temperature of 80 °C, and argon as carrier gas (1.47 mL/min); Channel 2: PoraPlot U column with a temperature of 65 °C and helium as carrier gas (1.47 mL/min).

Acetotrophic specific methanogenic activities (SMA) (Zhang et al., 2014) were measured in duplicate using an AMPTS II (Bioprocess Control, Sweden). The slope of linear part of the accumulative methane production was calculated and divided by biomass to obtain the SMA with the unit of g COD-CH₄/(g VSS day).

2.4. Microbial community analysis

The microbial composition and diversity of the biomass was investigated using 454 pyrosequencing. The biomass samples included one inoculum sample, two substrate samples, i.e. Sample 1 that was taken in July 2012 (day 131) and Sample 2 that was taken in January 2013 (day 317) from the brackish RAS, and three digestate samples. These digestates were sampled at days 129, 229, and 358. All the biomass samples were pretreated before DNA extraction. Firstly, 5 mL fresh samples were washed with 10 mM phosphorus buffer solution, and then they were centrifuged at 10,000g for 3 min. The supernatant was removed and then the biomass pellets were stored at –26 °C until further use.

DNA extraction was performed using a MoBio UltraClean microbial DNA isolation kit (MoBio Laboratories, Inc., CA, USA) following the manufacturer's protocol. A combination of heat, detergent, and mechanical force against specialized beads was involved in this process. A minor modification out of the protocol was that twice bead-beating (5 min) and heating (65 °C, 5 min) were applied in sequence in order to enhance the lysis efficiency of microbial cells. Successful DNA isolation was confirmed by agarose gel electrophoresis and the concentration of DNA was verified by Nanodrop 1000 equipment (Thermo Scientific, Waltham, MA, USA).

The amplification of the 16S rDNA gene was performed by Research and Testing Laboratory (Lubbock, TX, USA) with universal primers U515F (GTG YCA GCM GCC GCG GTA A) and U1071R (GAR CTG RCG RCR RCC ATG CA) and archaeal primers Arch341F (CCCTAYGGGGYGCASCAG) and Arch958R (YCCGGCGTTGAMTC-CAATT), followed by pyrosequencing by using a Roche 454 GS-FLX system (454 Life Science, Branford, CT, USA) with titanium

chemistry. By testing on Ribosomal Database Project (RDP) (Maidak et al., 1997), both forward and reverse primers target over 90% bacterial and archaeal DNA. The post analysis of pyrosequencing data was performed by combining different programs from the quantitative insights into microbial ecology (QIIME, version 1.6.0) pipeline (Caporaso et al., 2010). The 97% identity threshold (i.e. the 3% dissimilarity level) was used to group operational taxonomic units.

3. Results

3.1. Batch feeding mode

The digester was fed semi-continuously with the RAS sludge as described in Section 2.2. The pH, biogas production, and methane production were monitored online and used for following the 2-day batch-feeding cycle (Fig. 2). The gas production rate increased the first 5 h of the batch feeding, after which a gradual decrease was observed, following the substrate consumption. The methane content peaked immediately after feeding, after which it gradually decreased and stabilized after 10 h during the remaining cycle. The pH values varied from 7.5 to 7.6 with a minimum at 4–5 h after starting the feed cycle, reflecting rapid acid formation following the feed dose. The almost negligible pH change agreed with the strong buffer capacity of the digestate in the digester, which was measured as the total supernatant alkalinity ranging from 11.3 to 14.1 g CaCO₃/L. The high alkalinity was a result of a high concentration of bicarbonate, kept in solution by the high total ammonia concentration of 3.0–4.7 g NH₄-N/L.

3.2. Performance of the digester

This study was conducted for a period of 398 days, including 5 experimental stages, namely, start-up (Stage I), stabilized phase (Stage II), stepwise increasing OLR (Stage III), transitional phase (Stage IV), and low OLR also as lengthened SRT (Stage V).

3.2.1. Stage I and II

Stage I (day 0 to day 55) was conducted as a start-up period at an OLR of 2.8 kg COD/(m³ day). During this stage, biogas and methane flow rates fluctuated, accompanied by VFAs fluctuations (Figs. 3 and 4). However, the gradually stabilizing methane production, the decreasing VFA concentrations, and increasing COD removal efficiency from day 33 to day 55 (Figs. 3 and 4) indicated an improved adaptation of the anaerobic consortium to the substrate. The observed VFA peaks in Stage I had no substantial effect on the pH which ranged from 7.4 to 7.8 (Fig. 4), which is likely

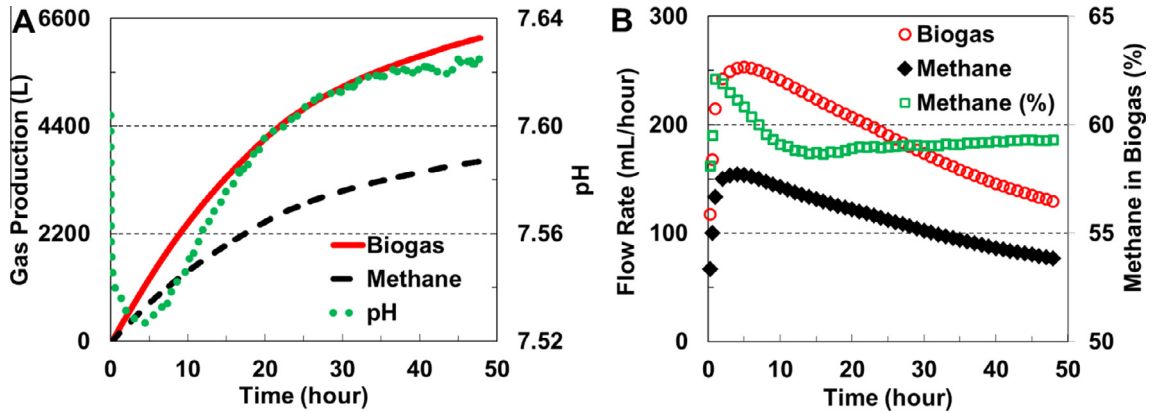


Fig. 2. Batch feeding regime: (A) cumulative biogas and methane productions and pH variations in the digester; (B) methane flow rates and CH₄ percentage in biogas.

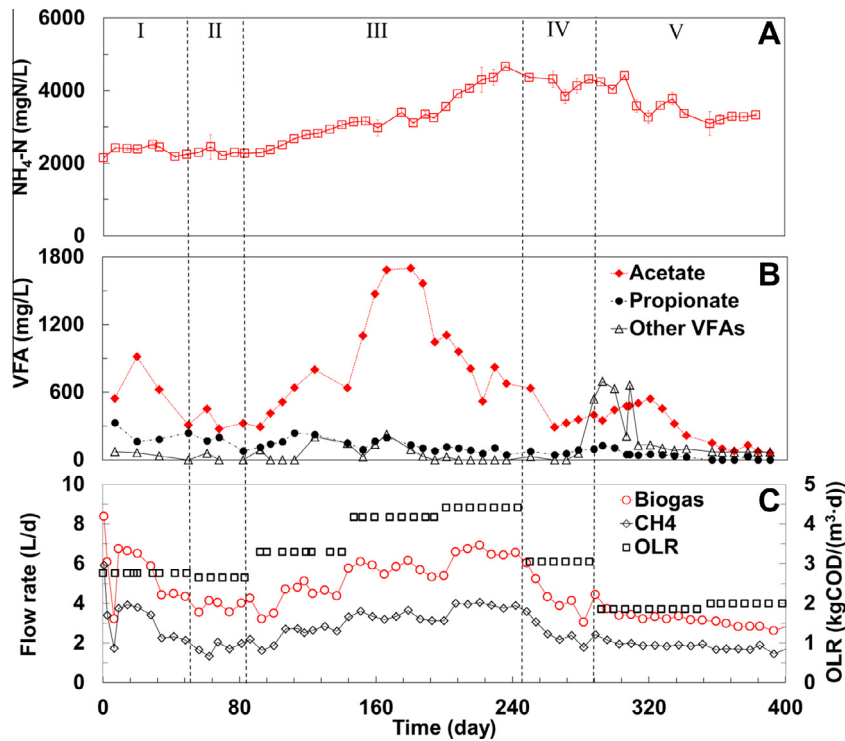


Fig. 3. (A) Digester NH₄-N concentration; (B) digester VFA concentrations; (C) applied OLR and both the biogas and CH₄ flow rate of the digester; other VFAs represent isobutyrate, butyrate, iso-valerate, valerate, iso-caproate, and caproate.

related to the strong buffer system as described in Section 3.2. From day 33, after 1 SRT had elapsed, biogas and methane productions became relatively stable, approximately 4.56 L/day and 2.52 L/day, respectively. Acetate diminished to approximately 312 mg/L, with a propionate concentration of 237 mg/L at the end of the start-up period of Stage I.

From day 56, Stage II (day 56–91) started at an OLR of 2.7 kg COD/(m³ day). In this period biogas, methane production, total COD, VS and VSS of the sludge were stable (Figs. 3 and 4). Removal efficiencies of total COD, VS and VSS were approximately 60%, 66%, and 67%, respectively. The salinity and pH remained at approximately 22 g/L and 7.8, respectively. Moreover, the total acetate concentration was relatively stable in the range of 312–450 mg/L, and the propionate acid concentration remained at a low level, i.e. less than 237 mg/L. The biomass' SMA values on day 57 and day 80 were 0.13 and 0.11 g COD-CH₄/(g VSS day), respectively, indicating a higher methanogenic activity in this

period, compared to 0.07 g COD-CH₄/(g VSS day) on day 7. This result illustrates a possible enrichment of methanogenic archaea in quantity or enhancement to their activity when the community was adapted to the prevailing conditions. The SMA increase is in accordance with the stable methane production and VFA profile in Stage II. Thus, all monitored parameters indicated that a stable and active operational condition of the digester had been reached.

3.2.2. Stage III

The adjustment of OLR in this study was based on literature review (Gebauer, 2004; Gebauer and Eikebrokk, 2006) and the performance of the digester in this present study. Stage III (day 92–249) is characterized by a moderate stepwise increasing OLR, from 2.7 to 3.3, 4.2 and then to 4.4 kg COD/(m³ day). Fig. 3C shows that with the stepwise increased OLR, biogas and methane productions were stable at each OLR and no substantial fluctuations of gas productions after stepwise changing OLR were observed. On average

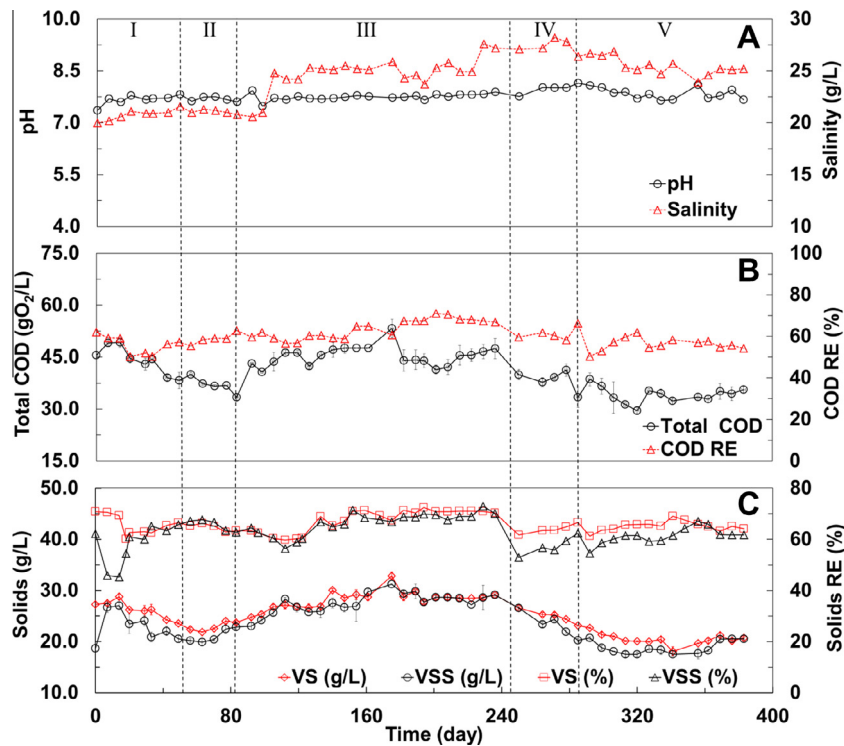


Fig. 4. pH and salinity of the digestate; (B) digestate total COD concentration and total COD removal of the digester (RE represents removal efficiency); (C) solids (VS and VSS) concentration and solids (VS and VSS) removal efficiency.

the methane productions at the three OLRs (3.3, 4.2 and 4.4 kg COD/(m³ day)) were stabilizing at approximately 2.70, 3.34 and 4.03 L/day, respectively. Notably, the total ammonia concentration (Fig. 3A) increased from 2269 to 4664 mg NH₄-N/L with the elevated OLRs. This very likely resulted from the increased total nitrogen concentration of substrate from 3150 to 4933 mg N/L, accompanied with the elevated OLR. Partially it might also be related to enhanced protein degradation. Additionally, an increase in the sludge COD_{sol} to COD_{tot} ratio with the stepwise OLR increase was observed during the period. The COD_{sol} and COD_{tot} increased, respectively, from 5940 and 45,600 mg O₂/L on day 92 to 9940 and 53,267 mg O₂/L on day 189. The ratio increased from about 13% (days 92–151) to approximately 19% (days 152–200), which was mainly related to the accumulation of acetate and propionate during this stage (Fig. 3B) as explained below. After adjusting OLR from 2.7 kg COD/(m³ day) to 3.3 kg COD/(m³ day), acetate started to accumulate gradually from 294 mg/L to 798 mg/L and then dropped to 636 mg/L during the period. In this period, propionate increased from 81 mg/L to 237 mg/L and then decreased to 148 mg/L. The other major accumulating VFAs were butyrate, isovalerate, and isocaproate. When the OLR increased further from 3.3 to 4.2 kg COD/(m³ day), total acetate quickly increased to 1686 mg/L and remained at that level for 14 days, and then decreased. Afterwards, when OLR was increased further to 4.4 kg COD/(m³ day), acetate first slightly built-up and then decreased to 522 mg/L. At this OLR, no further VFA accumulation was observed: acetate stabilized and propionate and the other VFAs remained relatively stable. The methane production was also stable in this period (Fig. 3C). It can be concluded that the performance of the digester was stable at the OLR of 4.4 kg COD/(m³ day) in terms of methane production and VFA profile in the digestate.

In addition, during Stage III, pH had stabilized between 7.5 and 7.9. Salinity increased from 21 to 28 g/L, which could be related to continuous release of ammonia, resulting from the increased OLR and the ongoing increasing hydrolytic capacity of the

methanogenic biomass. The improved sludge stabilization rate is illustrated by the modest VSS increased from 22 g/L at an OLR of 2.7 kg COD/(m³ day) to only 27 g/L at an OLR of 4.4 kg COD/(m³ day). The COD and solids removal efficiencies increased (Fig. 4B and C). Moreover, at the OLR of 4.4 kg COD/(m³ day), VS, VSS and COD removal efficiencies stabilized approximately at 71%, 70% and 69%, respectively. All the observed parameters indicated that even at the OLR of 4.4 kg COD/(m³ day), a stable performance of the digester was reached, accompanied by stable methane production and without severe inhibition from the increased NH₄-N.

3.2.3. Stage IV

In Stage IV (days 250–291), OLR was decreased to 3.1 kg COD/(m³ day). The SRT was increased from 32 days to 42 days to investigate the effect of increased SRT on waste stabilization and biogas production. During this period, biogas production decreased due to the reduced OLR till day 264. Afterwards biogas and methane productions gradually stabilized (Fig. 3C). Ammonia concentration firstly decreased to 3840 mgNH₄-N/L, and then seemed to stabilize at approximately 4300 mgNH₄-N/L, which was only slightly lower than that at the OLR of 4.4 kg COD/(m³ day) in Stage III. Possibly, the hydrolytic capacity of the sludge was only fully adapted after operating the reactor for 6 SRTs. Acetate decreased to 288 mg/L. Propionate was lower than 96 mg/L during the whole Stage IV and no substantial variation of the other VFAs was observed. pH increased from 7.8 to 8.2 during this period. Biomass concentration (VSS) decreased from 27 g/L to 20 g/L over time due to the decreased OLR. COD of the sludge decreased from 40 g/L to 33 g/L and COD removal efficiency fluctuated between 60% and 66%.

3.2.4. Stage V

The purpose of Stage V (days 292–398) with an SRT of 42 days was to evaluate the effect of lengthened SRT on sludge

stabilization, specific methane yield and the microbial community in the digester. Biogas and methane productions decreased from day 293 till day 313 from the onset of Stage V due to the lowered OLR. Then after day 313, averaged biogas and methane productions seemed to be stabilized at approximately 3.26 L/day and 1.92 L/day, respectively. Remarkably, when OLR decreased from 3.1 to 2.0 kg COD/(m³ day), NH₄-N gradually decreased and stabilized at about 3300 mg/L after day 341 (Fig. 3A), which represents a drop of 35% in OLR vs 23% in NH₄-N. In Stage V the NH₄-N concentrations were much higher than during the first operational Stages. Acetate concentrations increased from 348 to 540 mg/L on day 320. Propionate had a slight increase as well. However, there was a sudden surge of butyrate, valerate, particularly isovalerate and isocaproate indicated as other VFAs in Fig. 3B, which might be related to the degradation of material with lower degradability at the lower OLR (Ramdani et al., 2010) or toxicity of high content of ammonia to acetogens. From day 341 to day 398, acetate was lower than 216 mg/L and stabilized at 108 mg/L and propionate remained at low levels, i.e. less than 126 mg/L. However, the other VFAs were not detected in the sludge except for isocaproate maintaining at approximately 70 mg/L.

Digester pH gradually decreased from 8.2 to 7.7 between days 292 and 313. Then from day 314, pH remained at approximately 7.70. Salinity decreased from 28 g/L and remained at approximately 25 g/L in this stage. From day 327 onwards, VS, VSS, and COD of the sludge seemed to stabilize. The average removal efficiencies of VS, VSS and COD at steady state from day 327 were approximately 66%, 62%, and 57%, respectively. The performance of the digester, in terms of stable pH, salinity, VS, VSS, COD removal and gas productions, as well as VFA maintained stable since day 327 till day 398 as shown in Figs. 3 and 4.

3.3. Microbial community

3.3.1. Bacterial community composition

Among the Shannon diversity indices of bacterial populations listed in Table 2, sample 1, representing substrate sample 1 and the inoculum had the highest and lowest values, respectively. This means that the substrate sample 1 contained the most diverse bacterial communities, whereas the inoculum had the least biodiversity. Additionally, no substantial variation in the Shannon index of the bacterial community was observed over time.

The predominant phyla in the two substrate samples as shown in Fig. 5A were Bacteroidete (28.8% and 31.6%), Verrucomicrobia (22.5% and 34.0%) and Acidobacteria (4.7% and 5.3%), while Firmicutes and Bacteroidete were the most dominant phyla in the inoculum and the digestates. The most dominant phylum of the digestates shifted from Bacteroidete (51.1%→22.7%) to Firmicutes (33.7%→65.0%). The levels of Firmicutes and Bacteroidete in the digester seemed to approach to those of the inoculum. Additionally, Chloroflexi ranged from 1.4% to 6.2% in the digestates, and this

phylum was also present in the inoculum at a level of 1.3%. However, the levels of Chloroflexi in the substrate samples were lower than 0.3%.

The predominant genera in the inoculum included *Finegoldia* (22.3%) and *Clostridium* (19.3%) (Fig. 6A). In the substrate samples, the dominant genera, *Verrucomicrobium* (22.4% and 26.0%) and *Rhodobacter* (10.4% and 11.8%), were quite different from the inoculum. Intriguingly, the predominant genera of the 3 digestates were *Bacillus* (23.5%, 20.5% and 8.7%), *Clostridium* (8.2%, 13.6% and 15.5%), *Trigonala* (3.5%, 15.0% and 19.2%) and *Bacteroides* (18.2%, 3.7% and 6.5%), which were apparently deviating from either the inoculum or the substrate samples. The content of *Bacillus* in the digestate substantially decreased over time from 23.5% to 8.7%. The genus likely originated from the inoculum, became relatively dominant in the bacterial community and then diminished. In contrast, in the digester the presence of *Clostridium* likely originating from the inoculum increased from 8.2% (day 139) to 15.5% (day 358). Interestingly, compared to the dominant genera in the digestate, it seems that only *Trigonala* originating from the substrate with a low level (0.1%) accumulated from 3.5% (day 139) to 19.2% (day 358). The increase in the content of *Bacteroides* from 7.0% in the inoculum to 18.2% in the digestate on day 139 is also shown in Fig. 6. Afterwards, the content of *Bacteroides* on day 229 dropped to 3.7% and further increased on day 358. Therefore, the results of the distribution at genus level of the bacterial community also indicated the more similarity between the inoculum and the digestate of the digester. Nevertheless, some genera originally from the substrate such as *Trigonala* could also thrive over time in the digester.

3.3.2. Archaeal community composition

Fig. 5B shows the phylum level distribution of the archaeal community of the samples. In the inoculum 99.4% of phyla were Euryarchaeota. In contrast, the predominant phyla of the substrate samples and digestates mainly contained Crenarchaeota (65.8% and 88.4%) and Euryarchaeota (34.0% and 11.5%). This was also reported in the waste stabilization ponds and UASB reactors treating brackish aquaculture sludge in the study (Mirzoyan et al., 2012). In the digestates, the composition of dominant phyla was very similar to the Sample1 before day 229 and there was a twist in the decrease in the content of Crenarchaeota and the increase in Euryarchaeota. The Shannon diversity indices (Table 2) show that the diversity of the archaeal community in the digestates also seemingly increased, as the index increased from 1.056 on day 129 to 2.797 on day 358. The relative abundance of *Candidatus Nitrosopumilus* decreased from 95.0% on day 139 to 39.9% on day 358. *Methanosarcina* genus increased over time (Fig. 6B). After the digester operation for 358 days, *Methanoculleus* and *Methanobacterium* accumulated in the digester, both of which were probably originating from the inoculum. However, *Methanosaeta* that was the predominant methanogen in the substrate disappeared over time (Fig. 6B). Thus, from the analyses of both the phylum and genus levels of the archaeal community in all the samples, the similarity of the species of methanogens in the digestate with those in the inoculum also seemed to increase.

4. Discussion

With stepwise increase in the OLR, stable performance of the digester was achieved without severe VFA accumulation. The methane yield was enhanced and the solids and COD removal efficiencies also increased. Particularly at the OLR of 4.2 kg COD/(m³ day), gradual accumulation of acetate (up to 1698 mg/L) occurred but with no severe accumulation of the other VFAs. Meanwhile, ammonia concentration was also increasing. All these

Table 2
Shannon biodiversity index of bacteria and archaea obtained from pyrosequencing data.

Sample name	Bacteria (primer U515F)	Archaea (primer Arch341F)
Inoculum	5.502	1.577
Sub 1	7.084	1.408
Sub 2	5.779	1.914
Day 129	6.446	1.056
Day 229	6.239	1.186
Day 358	6.428	2.797

It was defined by the 97% identity threshold (i.e. 3% dissimilarity).

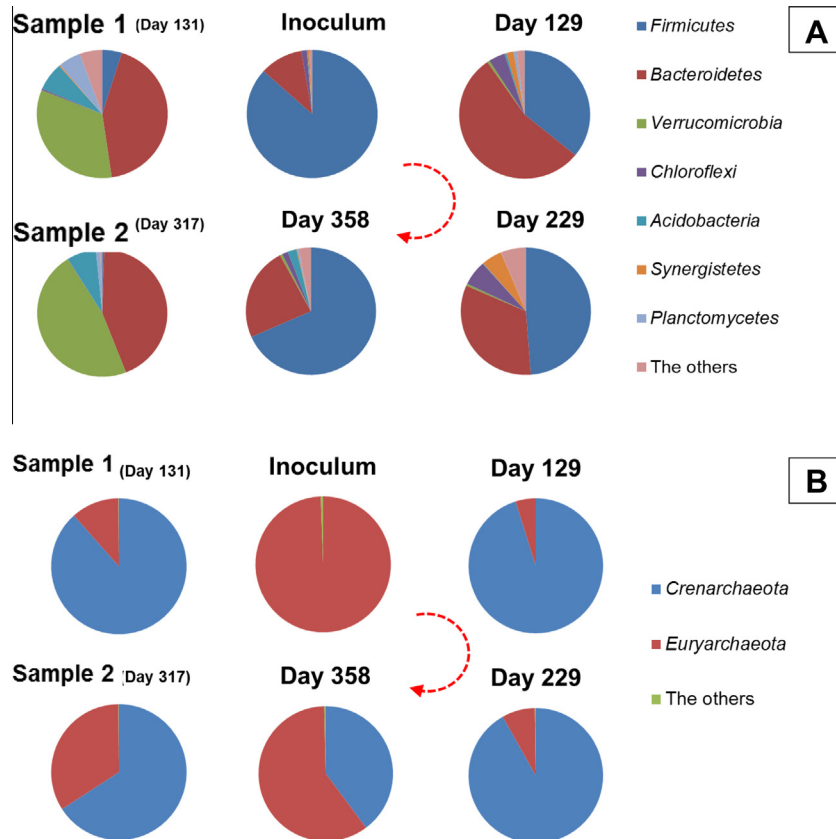


Fig. 5. Phylum level distribution of bacterial (A) and archaeal (B) community in inoculum, substrate (samples 1 and 2) and the digestate of the digester.

indicate that during this period, the activities of hydrolytic bacteria and acetogenic bacteria may not have been inhibited. However, the activity of methanogens was likely inhibited. On day 179 (OLR of 4.2 kg COD/(m³ day)), acetotrophic SMA is 0.08 g COD-CH₄/(g VSS day), which was only 2/3 of that operated under an OLR of 2.7 kg COD/(m³ day) in the stabilized stage. Nevertheless, even with the further increase in OLR, the digester seemed to stabilize in terms of reduced acetate concentrations until a relatively stable concentration of 600 mg/L was reached. Moreover, the acetate accumulation was accompanied by an increased ammonia concentration (Fig. 3). This may indicate that the increased ammonia concentration of 3140 mgNH₄-N/L might have slightly inhibited the activity of methanogens (Chen et al., 2008; Gebauer and Eikebrokk, 2006). However, afterwards methanogens were acclimatized to high ammonia concentration of up to 4400 mgNH₄-N/L (Fig. 1A). Hansen et al. (1998) also reported that methanogens could adapt to even higher ammonia concentration such as 6000 mg NH₄-N/L without causing reduction of methane production.

Specific methane yields (SMYs) reported in literature on AD of salty sludges from marine RAS were 0.154 L CH₄/g COD_{added} at 2.90 kg COD/(m³ day) with an SRT of 55 days, 0.140 L CH₄/g COD_{added} at 3.1 kg COD/(m³ day) at an SRT of 60 days and 0.136 L CH₄/g COD_{added} at 3.12 kg COD/(m³ day) at an SRT of 24 days and 0.184 L CH₄/g COD_{added} at 1.24 kg COD/(m³ day) at an SRT of 60 days (Gebauer, 2004; Gebauer and Eikebrokk, 2006). Compared to those reported values, the enhanced specific methane yields of 0.186 L CH₄/g COD_{added} at 3.30 kg COD/(m³ day) and 0.203 L CH₄/g COD_{added} at 4.42 kg COD/(m³ day) were achieved in the current study at the SRT of 32 days. Such values are also comparable to those reported by Kugelman and Van Gorder in the AD of sludge from fresh RAS (Kugelman and Van Gorder, 1991). The above literature results and current experimental data indicate

that high salinity may be responsible for the lower specific methane yields (Gebauer, 2004; Gebauer and Eikebrokk, 2006). Nonetheless, in the present study, the increased specific methane yields were obtained at a low SRT of 32 days. This probably benefited from the inoculum that was already acclimatized to high salinity and the stepwise increased OLR.

Additionally, the sudden buildup of butyrate, valerate, particularly isovalerate, and isocaproate concentrations occurred after lowering OLR from 3.30 to 1.87 kg COD/(m³ day) at an SRT of 42 days, accompanied by a gradual increase in acetate concentration. This might be related to the enhanced hydrolysis of material with low degradability or biomass decay after increasing SRT (Siegrist et al., 2002). De la Rubia et al. (2006) also reported that SRT presented a significant role in the VFA composition. Moreover, with longer SRT (42 days) in the present study, a higher specific methane yield was achieved, compared with that at an SRT (32 days), which is also reported by Gebauer (2004). A long SRT not only promotes higher conversion rates of particulate volatile solids into soluble products but also prevents methanogens from being washed out from the digester (Zhang and Noike, 1994). This is in accordance with the results of acetotrophic SMA assessment (Table 3). The results showed a higher SMA at longer SRTs. The applied SRT strongly influenced the biodiversity of communities in the digester, particularly methanogens that grow slowly, that is, have a long generation time (Zhang and Noike, 1994). An obvious example in this study is that the relative abundance of *Methanosarcina* increased by about five times under the SRT of 42 days compared to that under the SRT of 32 days.

Immigration happened across the entire experiment because the real marine/brackish RAS sludges were continuously supplied, which contained high levels of microorganisms. The dominant phyla of the substrate samples (sample 1 and sample 2) were

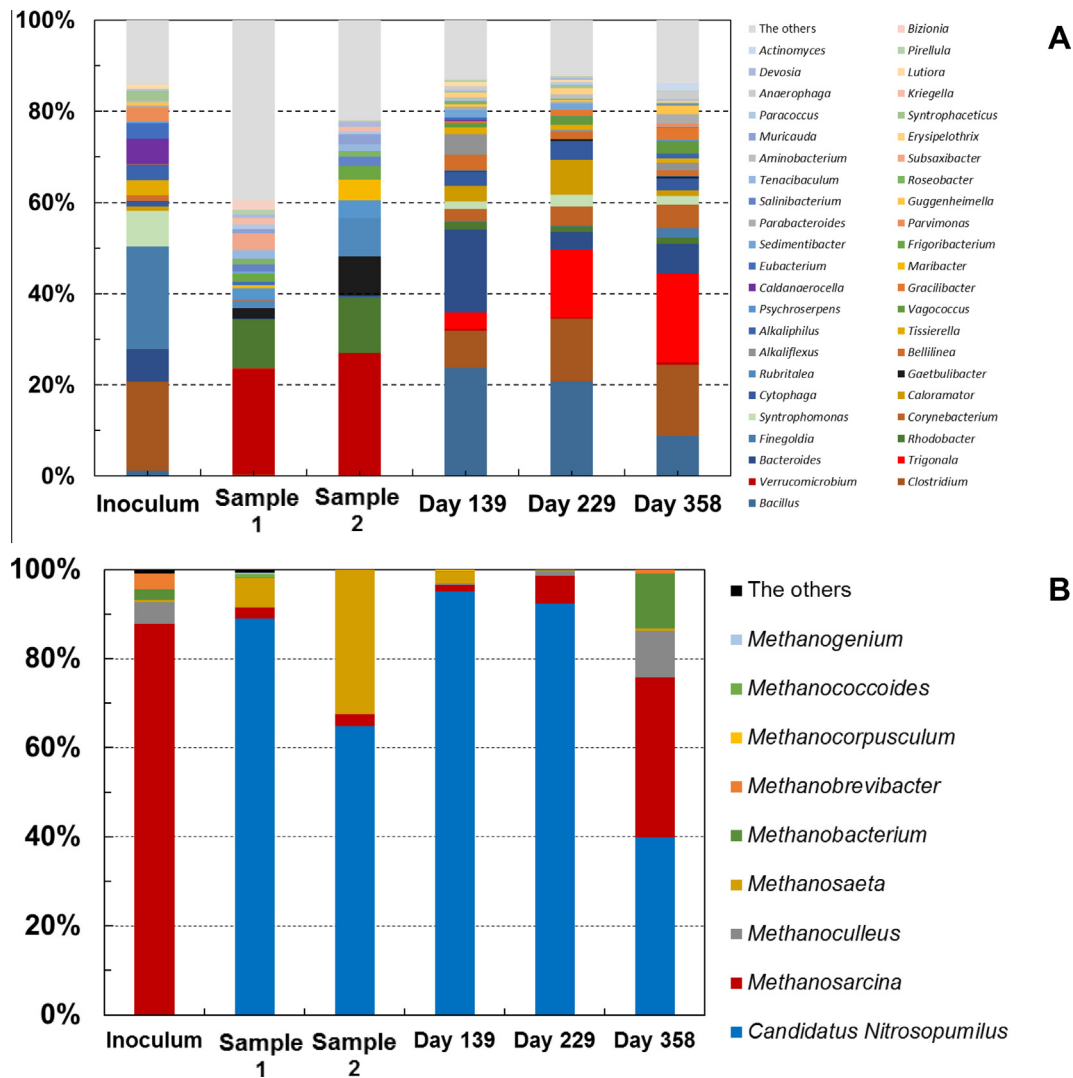


Fig. 6. Genus level of bacterial (A) and archaeal (B) community in inoculum, substrate (samples 1 and 2) and the digestates in time sequence: the species, whose sums of percentage in all the samples are less than 1%, are classified as 'the others'.

Table 3
Specific methanogenic activity (SMA) in the digester at steady state.

Stage	Number	OLR (kg COD/ m ³ /day)	SRT (day)	SMA (g COD-CH ₄ / g VSS day)	Sampling day
I	1	2.77	32	0.07 ± 0.01	7
II	2	2.66	32	0.12 ± 0.01	57
III	3	3.30	32	0.11 ± 0.01	80
	4	4.18	32	0.09 ± 0.02	149
IV	5	4.42	32	0.08 ± 0.01	179
	6	3.05	42	–	246
V	7	1.90	42	0.14 ± 0.01	325
	8	2.00	42	0.12 ± 0.01	353

Verrucomicrobia and Bacteroidetes, which have been reported often present in soils as well as oceans (Freitas et al., 2012), fecal wastes (Leitch et al., 2007) including fish faeces and biofilters of marine aquaculture systems (Schreier et al., 2010). Verrucomicrobia is ubiquitous in the ocean and may play an important role in the carbon cycle in the ocean (Freitas et al., 2012). Therefore, in this brackish RAS, Verrucomicrobia may also play a major role in the carbon conversion as it is present in a high fraction in the sludges collected from the farm, which might be critical to the carbon cycle in the marine/brackish RAS. At the phylum level of bacteria, no

substantial differences were observed between the two substrate samples. Apparently, Verrucomicrobia were not present in the digestate samples and the inoculum. Likely, Verrucomicrobia were not sustained in the digester probably due to the higher temperature (35 °C) and the obligate anaerobic conditions (Pol et al., 2007).

Bacteroidetes were present in all the samples. This phylum presented a high fraction in the substrate from the brackish fish farm. The predominant phyla in the inoculum and the digestate samples included Firmicutes and Bacteroidetes, which are known to be ubiquitous in anaerobic digesters (Nelson et al., 2011). However, that is quite different from municipal sludge digesters of sewage treatment plants where at phylum level generally Chloroflexi is the major bacterial community (Nelson et al., 2011; Riviere et al., 2009). Firmicutes outcompeted Bacteroidetes, and gradually became the dominant phylum in the digester over time. This phenomenon occurred in accordance with the OLRs increase from 2.7 to 4.4 kg COD/(m³ day). Some studies have shown that an increase in OLRs may cause the shift of dominant phyla from Bacteroidetes to Firmicutes (Wang et al., 2015), because of higher demand of the hydrolysis capacity that Firmicutes are more capable of (Ersahin et al., 2016). The disparity of the inoculum and the digester samples on day 358 at the phylum level decreased with the long-term operation of the digester.

The four major genera in the digestate samples in the present study were *Bacillus*, *Clostridium*, and *Trigonala*, which are classified into the phylum of Firmicutes, and *Bacteroides* classified within the phylum of Bacteroidetes. *Bacillus* excretes proteases that hydrolyze proteins to peptides and amino acids and further release ammonia (Kim et al., 2002). *Clostridium* also enables to produce extracellular enzymes to hydrolyze cellulosic materials, protein, and lipid, and also is involved in acetogenesis (Li et al., 2013). In addition, the members of the genus *Bacteroides* are also reported to take important roles in the hydrolysis of protein, lipid, cellulose and also some biopolymers such as polysaccharides (Li et al., 2013). Interestingly, *Trigonala elaeagnus*, a species recently discovered and classified within *Trigonala*, was apparently originating from the fecal sludge of the brackish RAS rather than from the inoculum since it was not detected in the inoculum. The genus was even enriched in the digester on the long term. The functionality of the species in the digester is not clear and needs to be further elucidated. There was a shift between the genera in the digester over time. *Bacillus* was dominant when the digester was operated at high OLR, but over time and with the lowered OLR *Bacillus* gradually lost its dominant role. Thus, the diminished fraction of *Bacillus* in the digester probably was due to the lowered OLR, particularly reduced protein loading to the digester. The abundance of *Clostridium* might explain the sudden buildup of VFA at the OLR of 1.87 kg COD/(m³ day) since *Clostridium* as acid-producing bacteria can be beneficial to the acidification process (Zheng et al., 2013) and then enhance VFA production from the particulate organic waste with low anaerobic digestibility particularly at longer SRT. Additionally, the decline in the percentage of *Bacteroides* might also be related to the reduced particulate macromolecules loading because *Bacteroides* are regarded as degraders of protein, lipid and hemicellulolytic polymer and producers of formate and lactate (Li et al., 2013).

It is very intriguing to find a high percentage of ammonia-oxidizing archaea (AOA), *Candidatus Nitrosopumilus* as the single species of *Candidatus Nitrosopumilus* detected in the sludges from the brackish RAS (Fig. 6B). *Candidatus Nitrosopumilus* has been reported to play a significant role in a biofilter of a marine shrimp RAS (Brown et al., 2013). It is suspected that *Candidatus Nitrosopumilus* plays a key role in the nitrogen metabolism in the digester as neither significant removal of ammonia nor generation of nitrite has been detected in the triplicate batch tests seeded with the digestates (data not shown). The relative abundance of *Nitrosopumilus maritimus* in the archaeal populations (Fig. 6B) decreased with time, particularly after lowering OLR.

Methanosarcina in the inoculum and the digestates were apparently abundant methanogens and should play a significant role in biomethanation of the salty organic waste in the present study. That differs from the full-scale municipal sludge digesters where the common dominant methanogenic species is *Methanosaeta* (Sundberg et al., 2013). The difference might be related to the prevailing high salinity conditions in both the full-scale digester where the inoculum was taken from and in the digester in the present study. In addition, hydrogenotrophic *Methanoculleus* and acetoclastic *Methanosaeta* were also present in the inoculum and digestates. Three pathways for biomethanation co-existed in the inoculum, which was also reported in the waste stabilization ponds (Mirzoyan et al., 2012). However, 2 pathways in the UASB of the study (Mirzoyan et al., 2012) were present. The difference might be related to the different operation conditions of the UASB (Mirzoyan et al., 2012) with the digester in the current study, and the waste stabilization ponds (Mirzoyan et al., 2012).

The changing OLRs lead to the variation of dominant methanogenic species. The methanogenic community's diversity in terms of Shannon index was lower at short SRTs (i.e. higher OLR) than at long SRTs (i.e. lower OLRs) (Table 2). *Methanosaeta* were

outcompeted by *Methanosarcina* over time at lower OLR. At high OLR on day 139, *Methanosarcina* and *Methanosaeta* were the dominant methanogens, which could be related to the availability of high acetate concentrations (Fig. 3). However, at lowered OLR, *Methanosarcina* became more abundant than the other methanogens, which is likely related to its metabolic versatility (Li et al., 2013). *Methanosarcina* are skilled in three different pathways, namely, using acetate, CO₂ and H₂ and by dismutating methyl compounds to grow and produce methane. Additionally, *Methanobacterium*, mainly *Methanobacterium petrolearium* commonly isolated from saline environments (Mori and Harayama, 2011), and *Methanoculleus* capable of using CO₂ and H₂ and formate to produce methane (Li et al., 2013), were also enriched over time. This was possibly resulting from the increased SRT. In short, the elevated percentage of methanogens in the archaeal community in the digestates is in agreement with the enhanced SMA shown in Table 3.

5. Conclusions

The use of a high-salinity-adapted inoculum and a moderate stepwise-increased-OLR strategy substantially enhanced digester performance. Specific methane yield reached 0.203 L CH₄/g COD_{added} at 4.4 kg COD/(m³ day), which is distinctly higher than literature values, 0.140–0.154 L CH₄/g COD_{added} from the salty sludges. The applied OLR and fecal substrate substantially influence the diversity and composition of digester biocommunity. *T. elaeagnus* and *Candidatus Nitrosopumilus* originated from substrate accumulated in the digester. However, their functionality in the digester needs further investigation. *Methanosarcina* outcompeted other methanogens presumably due to their metabolic versatility under saline conditions. Reducing OLR increases the community similarity between inoculum and digestate at the genus level over time.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2016.04.120>.

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