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Effect of low temperature and municipal wastewater organic loading on anaerobic granule reactor performance

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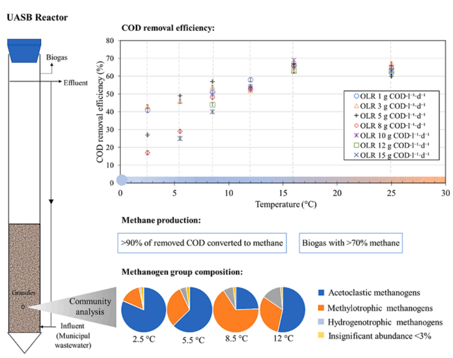
HIGHLIGHTS

- COD conversion to biogas at low psychrophilic conditions.
- Long-term UASB operation at low-temperatures is demonstrated.
- Microbial community structure indicating temperature adaptation to be evolved.
- Sustainable wastewater treatment by granulated anaerobic bioreactors.

GRAPHICAL ABSTRACT



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ABSTRACT

Biogas production and municipal wastewater COD removal at low temperatures by granulated anaerobic biomass were investigated. Two anaerobic granule reactors were operated continuously for 1025 days by stepwise increase of organic loading from 1.3 to 15.2 g $COD_{dissolved}\cdot l^{-1}\cdot d^{-1}$ at 25, 16, 12, 8.5, 5.5, and 2.5 °C. The sustained reactor performance was evaluated by COD removal efficiency, methane production, and microbial community analysis. Stable COD removal of 50–70% were achieved at 25–8.5 °C and up to 15 g $COD_{dissolved}\cdot l^{-1}\cdot d^{-1}$, and no significant temperature effect was observed on specific methane production rate and yield. Below 8.5 °C, COD removal and methane yields reduced, but still significant methane formation was observed even at 2.5 °C. More than 90% of COD removed was converted to methane. Methanogenic archaea communities showed that temperature changes affected the major methane formation pathways, which explains temperature adaptability of the granules.

1. Introduction

Anaerobic treatment has been recognized as an attractive and more sustainable alternative to conventional aerobic municipal wastewater treatment, especially for high strength wastewater, which also contributes to mitigate climate change (Lettinga et al., 2001a, Lettinga et al., 2001b). The organic load of wastewater, as a carbon rich source, could be converted to methane by anaerobic digestion, transforming low-

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value organics into a substantial renewable form of energy, and by that contributing to the circular bio-economy (Wainaina et al., 2020). Among several implemented anaerobic treatment technologies, up-flow anaerobic sludge blanket (UASB) reactors selecting for granulated biomass offers several advantages for dilute wastewater treatment (Seghezzo et al., 1998). Applications of anaerobic granulated biomass was introduced about 40 years ago (Lettinga et al., 1980) and is now regarded as an adequate methodology and a robust system for municipal wastewater treatment and energy recovery (Rosa et al., 2018).

The relatively slow growth rate and putative sensitivity of methanogens to environmental conditions have often been claimed to limit anaerobic wastewater treatment, particularly under psychrophilic condition (Lettinga et al., 2001a, Lettinga et al., 2001b). Anaerobic treatment is also vulnerable to organic overloading, which could disturb the process stability and affected the microbial community (Cardinali-Rezende et al., 2013). Previous studies have shown a significant adverse effect on the metabolic activity of mesophilic methanogens at decreasing temperatures (Kettunen & Rintala, 1997; Koster & Lettinga, 1985; Rebac et al., 1995). The degree of influence is, however, not consensually agreed upon, and relatively high methanogenic activities have been reported for the 10−15 °C range (Collins et al., 2006; Kettunen & Rintala, 1997). Furthermore, anaerobic communities of granulated sludge systems adapt to low temperatures even down to 4–10 °C (Bowen et al., 2014; McKeown et al., 2009a, McKeown et al., 2009b; Petropoulos et al., 2017). This is not surprising as abundant methanogens have been isolated from extreme cold natural environments, such as lake sediment, high arctic peat, and permafrost (Høj et al., 2008; Varsadiya et al., 2021).

Bowen et al. (2014) reported anaerobic treatment of low strength domestic wastewater in a batch system at low temperatures. Methanogenesis was inhibited due to inhibition of activity rather than the absence of methanogen population, while the acidogenic reactions still occur at all temperatures studied; 4, 8 and 15℃ (Bowen et al., 2014). In a long term study (1243 days) by McKeown et al. (2009a,b), the authors observed methanogenesis at low temperatures (4–15 ℃) using an expanded granular sludge bed (EGSB) reactor. They suggested that mesophilic inoculum could evolutionary adapt to psychrophilic operational temperatures (McKeown et al., 2009a, McKeown et al., 2009b). More recently, Petropoulos et al. (2017) studied communities from environments that have been exposed to low temperatures (4, 8 and 15 °C) for 400 days in a batch system receiving domestic wastewater. Their results implied that inoculating reactors with cold-adapted communities was a promising way to select for biomass capable of treating anaerobic wastewater treatment at low temperatures, indicating that low temperature anaerobic wastewater treatment is possible using adapted cultures (Petropoulos et al., 2017).

Even though real wastewater treatment in various continuous anaerobic reactors (e.g., UASB, anaerobic filter and hybrid system, anaerobic membrane reactor) at low temperatures, down to 3 °C, over long terms (140–540 days) is well documented, long term UASB reactor investigations of real municipal wastewater treatment at temperatures below 20 °C under variable organic loading rates (OLR) is lacking. For anaerobic wastewater treatment to become a viable and preferred treatment strategy for municipal wastewater in the northern temperate and sub-artic populated regions, stable operation and acceptable treatment performance must be demonstrated, and operational stability needs to be documented at typical variable loading. Further, if psychrophilic wastewater treatment is possible, an important design and operational question is whether such performance is a result of microbial community adaptations or phenotypic adaptations of a mesophilic generic sludge.

In this work, the long-term (1025 days) temperature effects (25, 16, 12, 8.5, 5.5 and 2.5 °C) on UASB reactor performance treating municipal wastewater over typical operational OLRs were investigated. Performance of this anaerobic granular sludge system was studied by monitoring its COD removal efficiency, determination of specific methane production rate and methane yield. Nutrient (N and P), VFA and alkalinity dynamics were closely monitored for inference on UASB operational stability. The effect of temperature and loading was investigated in an operational regime allowing for seasonal adaptations of microbial communities. Hence, the microbial community characterization of the low temperature and loading gradients was also included.

2. Materials and methods

2.1. Granule inoculum source

Granulated inoculum was kindly provided by the late Professor Rune Bakke, University of South-Eastern Norway (USN). Granules were originated from a mesophilic active pilot-scale bioreactor, operated on wastewater from different sources: (a) pulp and paper company treating cellulose and lignin-containing wastewater (Moss, Norway); (b) agriculture pilot plant treating swine and cow manure supernatant (Skien, Norway); and (c) hydrocarbon oil-containing wastewater at Bamble Industrial Park (Telemark, Norway).

2.2. Experimental set-up and operation of continuous reactors

Approximately 300 ml of settled granules inoculum were transferred to 1000 ml UASB reactors giving a 30 % (v/v) filling level. Granular sludge volumes were estimated throughout reactor operation by measuring the height of sludge blanket in UASB reactors daily. Two parallel in-house designed laboratory-scale UASB reactors (reactor A and B) were identical and operated continuously, receiving primary treated (after dissolved air flotation) municipal wastewater from the Grødaland wastewater treatment plant (WWTP), municipality of Hå, southwest Norway. The wastewater received at Grødaland WWTP is characterized by significant volumetric contributions from agricultural and food industries: (a) 167 $m^3 \cdot d^{-1}$ from an animal residual recovery plant (Biosirk Protein), (b) 3000 $m^3 \cdot d^{-1}$ from municipal wastewater of approximately 3000 houses of the community Varhaug, (c) 1910 $m^3 \cdot d^{-1}$ from a food processing plant (Fjordland), (d) 3020 $m^3 \cdot d^{-1}$ from a dairy and chicken slaughterhouse (Kviamarka), and (e) 345 $m^3 \cdot d^{-1}$ of reject water from thickening and dewatering of digested sludge at the Grødaland biogas plant. The dissolved and total COD in the inlet wastewater during UASB reactor operation fluctuated over the range 439–1473 mg $COD_{dissolved} \cdot l^{-1}$ and 502–1669 mg $COD_{total} \cdot l^{-1}$ with the mean dissolved and total COD concentration of 741 ± 7 mg $COD_{dissolved} \cdot l^{-1}$ and 819 ± 13 mg $COD_{total} \cdot l^{-1}$ (±standard error), respectively. Therefore, approximately 90% of the total COD concentration in UASB feed was dissolved COD, and the rest was suspended COD with an average concentration of 78 ± 9 mg $COD_{ss} \cdot l^{-1}$. Wastewater samples were collected weekly and stored in the dark at 2–4 °C before use (average storage time 5 days).

The UASB reactors were operated continuously by applying a stepwise increase of OLR from 1.3 ± 0.1 by intermittent increases to 15.2 ± 0.2 g $COD_{dissolved} \cdot l^{-1} \cdot d^{-1}$ at each temperature (25, 16, 12, 8.5, 5.5 and 2.5 °C, respectively). Steady-state was achieved in the reactors after the adaptation time (transient response time) when the performance parameters, i.e. the COD removal efficiencies and the daily gas production, remained relatively constant (±3%) for 4–7 days at the same temperature and OLR. OLR was controlled by adjusting the inlet wastewater flow rate (regulating the HRT) based on the inlet dissolved COD concentration. Operational characteristics during continuous UASB system operation for both reactors are shown in Fig. 1. The gradual increment of OLR was used to ensure that granules would not wash out of the system while the microorganisms were acclimating to the higher loading. Severe deterioration of granules occurred in reactor A at 5.5 °C on day 738 and reactor A operation was stopped when applying OLR of 15 g $COD \cdot l^{-1} \cdot d^{-1}$ on day 769.

The reactors used were airtight glass-type reactors (custom made by glass blower Mellum AS, Aurskog Norway) with the total volume of

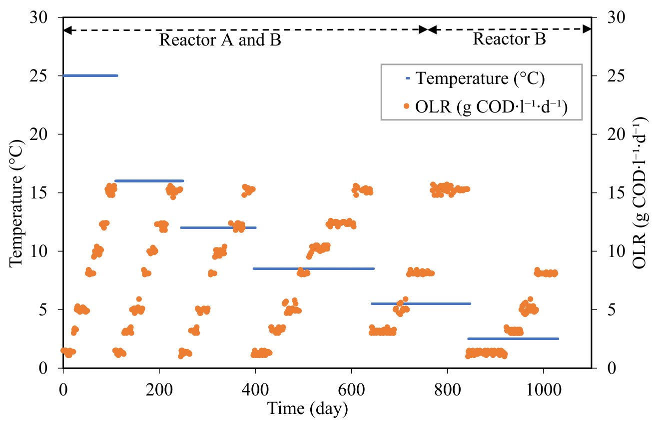

Fig. 1. The UASB reactors were operated continuously over 1025 days by the stepwise increase of OLR at decreasing temperatures. Initially, UASB reactors were started-up at 25 °C with low OLR around 1.0 g $COD_{dissolved} \cdot l^{-1} \cdot d^{-1}$ and increased gradually up to approximately 15 g $COD_{dissolved} \cdot l^{-1} \cdot d^{-1}$ before next test at reduced temperature. Reactor A operation was stopped on day 769.

1000 ml. The reactors were temperature controlled using a Lauda Alpha RA8 refrigerated water circulation unit (Lauda, Germany) with a high rate of greater than 200 $ml \cdot min^{-1}$. Glycol was added to the circulating unit to avoid freezing when applying 2.5 °C in operation on day 843. An external foam insulator was mounted to maintain the desired temperatures of 8.5 °C operations (day 520) and below. An external digital thermometer was installed inside the circulating unit as an additional temperature confirmation, and temperatures inside the reactors were measured manually on a regular basis. Cooled wastewater (2–4 °C) was fed by a peristaltic pump (Ismatec, Germany) with adjustable flow rates. Continuous recirculation to sustain mixing and a constant up-flow was achieved by pumping effluent from the top to the bottom of the reactors at 1.8 ± 0.1 $m \cdot h^{-1}$ (±standard error).

The total biogas produced (methane and CO_2) were measured using the first Milligas counter (Dr.-Ing. RITTER Apparatebau GmbH & Co., Bochum, Germany) connected to the UASB gas-outlet. Then, the CO_2 produced was absorbed using a bubble-through CO_2-absorber, containing NaOH 3 M and 0.4 % Thymolphthalein pH-indicator. Therefore, only methane went through and was measured by the second gas counter (see supplementary materials).

2.3. Analytical methods

Dissolved and total COD were determined daily using standard COD Spectroquant® test kits with a detection range of 100–1500 $mg \bullet l^{-1}$ (Merck, Germany) along with nutrients (total phosphorous, orthophosphate, total nitrogen, ammonium, and nitrate), all measured using standard Spectroquant® test kits (Merck, Germany). Samples were filtered prior to determination of dissolved components (dissolved COD, orthophosphate, ammonium, and nitrate) using a 1.2 µm glassfiber filter (GF/C, Whatman, UK). Total COD, total nitrogen, and total phosphorous analysis were performed from UASB reactor inlet and outlet without filtration. Suspended COD was calculated by subtracting total COD with dissolved COD.

Alkalinity and VFA concentrations were determined using a Titro-Line® 5000 titrator (SI Analytics, Germany) following the pH based five-point titration method. Total VFA and alkalinity were calculated concomitantly using the TITRA5 software (Moosbrugger et al., 1993). Samples were drawn and analyzed independently in duplicates for each measurement.

The total biogas and methane produced (before and after CO_2-absorber, respectively) were primarily determined using gas counters and intermittently confirmed by Agilent 7890B gas chromatography (Agilent, USA). Each gas sample was collected in duplicates in two different gas sampling bags which had a volume of 1–3 l (Tedlar®, Sigma Aldrich, Germany), and 100–200 µl of gas was withdrawn using a gas-tight syringe (SGE-Europe) from each bag and injected onto GC equipped with a thermal conductivity detector (TCD) (Agilent column, 0.32 mm diameter, 30 m length and 0.25 µm film). In the effluent, dissolved methane was estimated using the temperature adjusted Henry's coefficient for determination of the total methane produced from the UASB system (Wilhelm et al., 1977). COD mass balance was evaluated and calculated based on Henze et al. (2008). COD fractions of methane in gas phase, dissolved methane, liquid effluent, and solid in the effluent were measured on a daily basis.

2.4. Microbial community analysis

Approximately 0.25 g (wet weight) of granule samples were obtained from the middle of the sludge blanket reactors during 12, 8.5, 5.5 and 2.5 °C reactor operation carried out at different OLRs at each temperature. DNA was extracted using a DNeasy PowerWater Kit (Qiagen, Germany) as described by the manufacturer. Samples were homogenized in PowerBead tubes using the FastPrep-24™ bead beater (MPBio, USA) for 60 s prior to extraction. After extraction, DNA was checked by agarose gel electrophoresis. The DNA concentration was determined using the NanoVue™ Plus Spectrophotometer (GE Healthcare, USA) at absorbance 260 nm before sending samples for external sequencing (Macrogen Europe B.V., Netherlands). The averaged DNA concentration was approximately 100 $ng \cdot \mu l^{-1}$. The isolated DNA was stored at −20 °C until further processing.

For polymerase chain reaction (PCR) amplification, the DNA was amplified using primers for the v3–4 region of the 16S rRNA gene; B-341F (5′-CCTACGGGNGGCWGCAG) and B-805R (5′-GACTACNVGGG-TATCTAAKCC) amplifying 465 base pairs (bp) for bacterial DNA, A-340F (CCCTAYGGGGYGCASCAG) and A-760R (GGACTACCSGGG-TATCTAATCC) for archaeal DNA (Nordgård et al., 2017). Pair end sequencing was done by Macrogen Europe B.V, Maastricht (Netherlands), using the MiSeq™ platform. FLASH (fast length adjustment of short reads) software was used to assembly reading data by merging paired-end reads from next-generation sequencing experiments (Magoč and Salzberg, 2011). CD-HIT-OTU was utilized to preprocess and cluster the data with a three-step clustering to identify operational taxonomic units (OTU) and rDnaTools (Li et al., 2012).

2.5. Statistical analysis

Statistical analyses, standard errors and student t-tests at 95% confidence were calculated and applied using Excel and SigmaPlot V14.0 for Windows (SyStat Inc., USA).

3. Results and discussion

In this study, two UASB reactors (reactor A and B) were operated continuously in parallel under the same operational conditions. Based on statistical analysis (student *t*-test at 95% confidence level), the two reactors demonstrated no significant difference in terms of transient response times, COD removal efficiency, methane fraction in biogas, methane production, COD balance, and nutrient variability. However, biomass retention behavior in both reactors were different. A significant level of granule disintegration and effluent/bulk phase suspended particles was observed several times in both reactors during the acclimatization periods, especially at 5.5 °C and high OLR greater than 8.0 g $COD_{dissolved} \cdot l^{-1} \cdot d^{-1}$. However, compared to reactor B, severe granule washout occurred mainly in reactor A as the sludge bed floated, presumably due to gas entrapment at high OLR and subsequent high biogas production. This resulted in diminishing gas production and loss of COD removal capacity, and reactor A operation was stopped on day 769. Sludge bed expansion also occurred at higher temperatures due to inter granule entrapment of gas bubbles. This was mitigated by varying recirculation flow rate and direction and by mechanical wall tapping. Different granule sizes could explain the difference in the two reactors sludge behavior. Granule size was regularly measured during the experiment by observation with the use of a ruler, and larger granules were initially transferred to reactor B (2–3 mm of diameter compared to 1–2 mm of diameter in reactor A) likely resulting from fractionation during transport and storage. Granule size also decreased over the time span of the experiment in both reactors, especially below 12 °C. The average granule size reduced from approximately 3 to 1–2 mm in reactor B, while the granules in reactor A became even smaller constituting fine particles by approximately 0.5 mm of granules size towards the end of the period. Wu et al. (2016) and Owusu-Agyeman et al. (2019) observed large anaerobic granules (3–3.5 mm) and claim higher mass transfer due to their internal structure, including big pore size, high porosity and short diffusion distances compared to medium and small granules. Small granules (<1 mm) appeared to be weaker and more easily washed-out from the system (Wu et al., 2016). Moreover, Singh et al. (2019) investigated UASB reactor operation treating dairy wastewater at 20 °C and found that LCFA-containing feed stimulated granule flotation and wash out from the reactors due to LCFA-encapsulated granular sludge (Singh et al., 2019). This could also explain the frequent granule expansion in the system as parts of the wastewater inlet at IVAR Grødaland originates from a dairy and a slaughterhouse.

3.1. Transient time and COD removal efficiencies

UASB reactors were started-up at 25 °C at a low OLR of 1.0 g $COD_{dissolved}\cdot l^{-1}\cdot d^{-1}$, and an acclimatization period over the first five days was monitored until the reactors reached a quasi-steady state condition. The adaptation time of the system (transient time) increased with decreasing temperature in both reactor (Fig. 2). At 25, 16, and 12 °C, the transient time at different OLR were in the same range at 5, 9, and 14 days, respectively. This suggests the granules to adapt relatively quickly to decreasing operating temperatures, adaptations that would not imply community structure changes. The ability of the UASB system to recover rapidly from temperature and loading perturbations demonstrates the robustness of the system, which is an important consideration for full-scale applications.

At lower temperatures (8.5, 5.5, and 2.5 °C), longer transient times were required to reach a new steady-state, at the higher loadings up to 68 days. Noticeably, during operation at these temperatures, lower inlet dissolved COD concentration at high OLRs affected the hydrodynamic condition by increasing the inlet flow rate to achieve the desired OLR, resulting in lower HRT in the system and reduced dissolved COD removal efficiency. Decreasing HRT leads to increased granule areal loading which for a biofilm system leads to higher effluent concentrations (Henze et al., 2008; Zhang et al., 2015). Following an increase in OLR, indeed, the effluent dissolved COD concentration increased every first day after increasing the OLR. Additionally, during acclimatization periods, observed dissolved COD removal efficiency was affected by bulk phase VFA accumulation up to 600 mg as acetic acid$\cdot l^{-1}$ accompanied by decreasing alkalinity, and pH dropped to 5.9–6.7 (see supplementary materials). This non-steady state response, where dissolved COD efficiencies showed high levels of fluctuation (±greater than3%), was more pronounced during lower temperatures. Hence, additional buffer (1000 $mg\cdot l^{-1}$ $NaHCO_3$) was added occasionally, especially during acclimatization periods, to assure process stability during low temperature loading perturbations. As the reactors stabilized, the effluent dissolved COD and VFA accumulation decreased until the next steady-state condition was established (see supplementary materials).

Suspended COD effluent concentrations fluctuated throughout reactor operation with 3–48% removal efficiencies, and no systematic trend was observed, regardless of operating temperatures and OLRs (data not shown). Fig. 3 presents dissolved COD removal efficiencies at steady-state for the different temperatures and OLRs. At higher temperatures, 16 and 25 °C, the dissolved COD removal efficiency for all OLRs remained the same in the 60–70% range. Even at temperatures as low as 12 °C, at all operating OLRs, the methanogenic capacity of the UASB reactor was sufficient to maintain a dissolved COD removal efficiency above 50%. There was a significant change in dissolved COD removal efficiency at 8.5, 5.5 and 2.5 °C. At 8.5 °C, COD removal efficiency was also above 50% for OLR up to 8 g $COD_{dissolved}\cdot l^{-1}\cdot d^{-1}$, however, a general decrease of dissolved COD removal efficiency was observed when increasing the OLR to 10–15 g $COD_{dissolved} l^{-1}\cdot d^{-1}$. Low temperatures (5.5 and 2.5 °C) accompanied by higher OLR resulted in overloading the UASB reactor and higher dissolved COD effluent concentrations resulted.

At steady-state, alkalinity and VFA were stable in both reactors, and external buffering was not necessary. However, at low-temperatures (<8.5 °C) and OLRs above 12 g $COD_{dissolved}\cdot l^{-1}\cdot d^{-1}$, VFA accumulation and decreasing alkalinity occurred (see supplementary materials), indicating the reactors to become overloaded and the dissolved COD removal efficiencies decreased below 30%. This is comparable to the result by Dague, Banik, and Ellis (1998), whereby lower temperatures resulted in reduced rates of substrate removal when treating synthetic wastewater at 5–25 °C (Dague, Banik, and Ellis, 1998). Similar findings have also been reported in the literature by Mahmoud et al. (2004) and Bandara et al. (2012) using UASB reactors treating real municipal wastewater at lower temperatures and relatively low OLR < 3 g

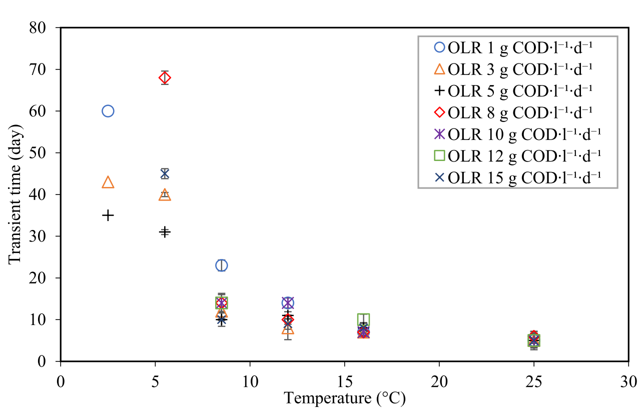

Fig. 2. Averaged transient time to steady state at different temperatures and OLRs in reactor A and B. The student *t*-test revealed no significant difference (p greater than 0.05) between reactor A and B transient times.

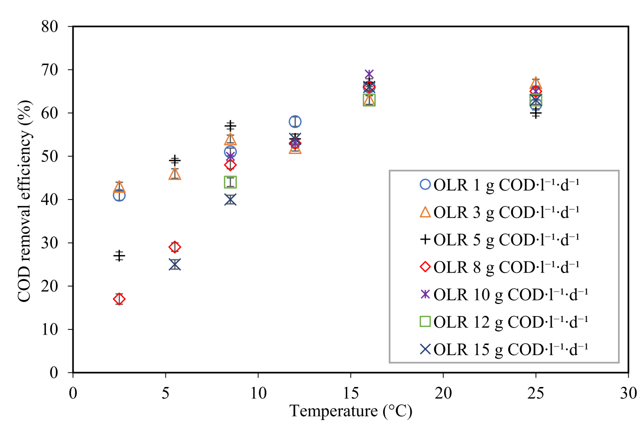

Fig. 3. Dissolved COD removal efficiencies at steady state as depending on t temperature and OLRs. Error bars represent standard errors from measurements taken during steady state conditions in reactor A and B. The student *t*-test revealed no significant difference (p greater than 0.05) between reactor A and B COD removal efficiencies.

$COD_{dissolved} \cdot l^{-1} \cdot d^{-1}$. Using single stage UASBs, observed COD removal efficiencies were 44% at 15 °C (Mahmoud et al., 2004) and 40% during winter time down to 6 °C (Bandara et al., 2012). The presented results were relatively lower than the aforementioned long-term anaerobic granular reactor operation (1243 days) at 4–15 °C and OLR up to 10 g $COD_{dissolved} \cdot l^{-1} \cdot d^{-1}$ (McKeown et al., 2009a, McKeown et al., 2009b), who demonstrated greater than 80% COD removal efficiencies with VFA-based synthetic wastewater which consisted of acetate, butyrate, propionate and ethanol. Compared to UASB reactor substrate in this study, the VFA-based wastewater used by McKeown et al. (2009a,b) is more easily degradable than complex municipal wastewater used in this study with approximately 10% suspended COD inlet, indicating hydrolysis and/or fermentations could be rate-limiting. Petropoulos et al. (2017) investigated the intrinsic capacity of cold-adapted communities to treat domestic wastewater at 4, 8, and 15 °C in batch systems and indeed demonstrated hydrolysis/fermentation to be the limiting step, and hydrolysis to be twice as temperature sensitive as methanogenesis (Petropoulos et al., 2017).

3.2. Methane production

UASB reactor performance may also be evaluated by methane production. At each temperature, methane production increased with increasing OLR as expected, consistent with the amount of organic matter removed in the UASB reactors. Overall, biogas composition was mainly methane (above 70%) at all operating temperatures and OLRs. A slight decrease in methane fraction from above 80% at 25 °C to approximately 75% at 5.5 °C was observed, however, methane fractions increased to above 80% at 2.5 °C. The high methane contents (%) in this study, especially at lower temperatures, were likely due to high ratio of dissolved CO_2 in the liquid phase.

Fig. 4a and 4b show biomass volume specific methane production rates (as COD equivalents per granular sludge volume) in reactor A and B depending on temperatures and OLRs. At each temperature, methane production rates increased as expected following increasing OLR, however, significant decreasing at lower temperatures (8.5, 5.5 and 2.5 °C). On the other hand, methane productions rate at 25, 16 and 12 °C were relatively comparable and not significantly affected by increasing OLR, indicating the loading capacity could be higher than 15 g $COD_{dissolved} \cdot l^{-1} \cdot d^{-1}$. At the lowest temperature (2.5 °C) and OLR of 1.3 ± 0.1 g $COD_{dissolved} \cdot l^{-1} \cdot d^{-1}$, the specific methane production rate was 0.55 ± 0.04 g COD CH_4·l $biomass^{-1} \cdot d^{-1}$ and then increased slightly as the OLR was increased to 8.1 ± 0.1 g $COD_{dissolved} \cdot l^{-1} \cdot d^{-1}$ giving 1.70 ± 0.03 g COD CH_4·l $biomass^{-1} \cdot d^{-1}$. These findings suggests that anaerobic granules are capable of adapting to reduced temperatures and maintain system performances (COD removal and methane production) over long terms of operation at UASB loading rates up to around 8 g $COD_{dissolved} \cdot l^{-1} \cdot d^{-1}$ at psychrophilic conditions.

Observed methane yields are presented in Fig. 4c and 4d. Methane yield was calculated as g COD methane per g COD removed. The average methane yield obtained in reactor A and B were 0.86 ± 0.01, 0.91 ± 0.01, 0.85±, 0.91 ± 0.00, 0.93 ± 0.01, and 0.90 ± 0.00 g COD CH_4.g $COD_{removed}^{-1}$ at 2.5, 5.5, 8.5, 12, 16, and 25 °C, respectively. In general, more than 90% of COD removed was converted to methane, and the methane yield did not change significantly with respect to temperatures. The overall COD balance closed at above 90% of the inlet COD at all operating temperatures and OLRs. Analytical uncertainty, gas leakages, and the inaccuracy of the gas counter at low gas flow rates are possible explanations of the minor shortage (3–10% unaccounted COD). Henze et al. (2008) emphasized that fat or LCFA-containing substrates typical of dairy wastewater results in high COD removal efficiencies, but not equivalent CH_4 production rates leading to considerable COD balance gaps (Henze et al., 2008). Singh et al. (2019), Singh et al. (2020) found that this occurrence could be explained by lipid and/or LCFA accumulation in the granules which is also associated with granules flotation and occasional wash out. Entrapment or accumulation of slowly

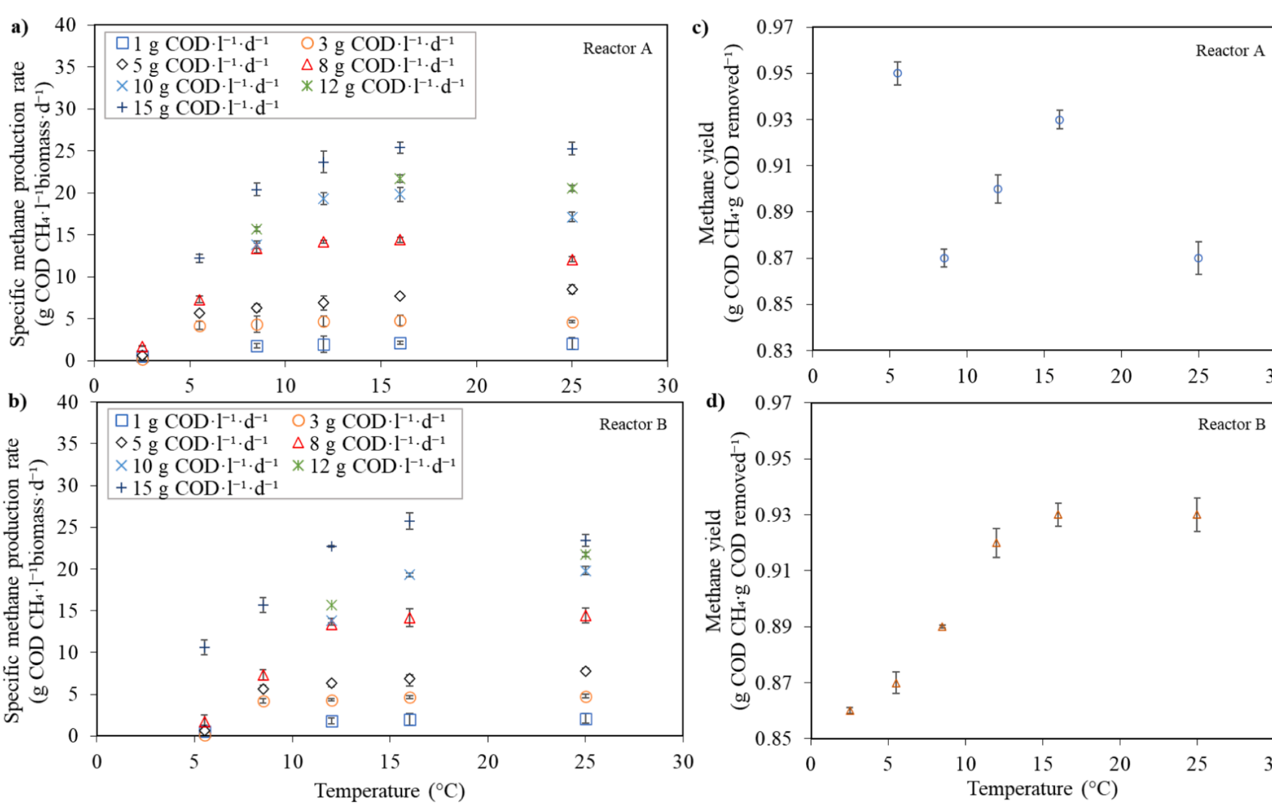

Fig. 4. Specific methane production rate per volume biomass (a and b) and overall COD specific methane yield (c and d) at steady state conditions in reactor A and B. Error bars represent standard errors from measurements taken during steady state conditions.

biodegradable polymeric, colloidal and residual particulate COD in the sludge blanket have a similar effect on the COD balance (Zhang et al., 2018). Significant fractions of lipids and complex protein substrates (like casein) is typical of the original wastewater studied herein. Another cause for a COD gap at low-temperatures is a significant amount of dissolved methane at the effluent lost (by gas transfer) during sample analysis. In this study, dissolved methane in the liquid effluent was compensated by Henry's law. However, Souza et al. (2011) and Wu et al. (2017) found that dissolved methane was oversaturated in the liquid phase of an anaerobic bioreactor effluent (saturation factor of 1.03–1.67), increasing with the increased methane solubility at decreasing temperatures. Hence, several putative explanations for the missing COD are possible, but regardless of cause, the limited amount suggests a limited significant effect on the overall inferences in this study.

3.3. Nitrogen and phosphorous variability

Besides organic conversion and methane production, nitrogen and phosphorous availability in the anaerobic reactors was also important for bioreactor performance evaluation. The results show that the applied wastewater had nitrogen mainly in particle-bound nitrogen (60–80%), while phosphorous was predominantly dissolved, mainly orthophosphate (60–90%). Some nitrogen and phosphorous concentration decrement were observed in the UASB reactor effluent with no systematic trend at different temperatures and OLRs. The total nitrogen and total phosphorous concentration decreased in the range of 10–33% and 4–20 %, respectively (see supplementary materials), slightly beyond the expected effect of assimilation by anaerobic cell growth. Particle-bound nitrogen and phosphorous could be removed by UASB reactor through conversion to dissolved forms, sedimentation, and granule entrapment (Elmitwalli & Otterpohl, 2011). Compared to the inlet orthophosphate concentration, effluent orthophosphate concentration slightly decreased

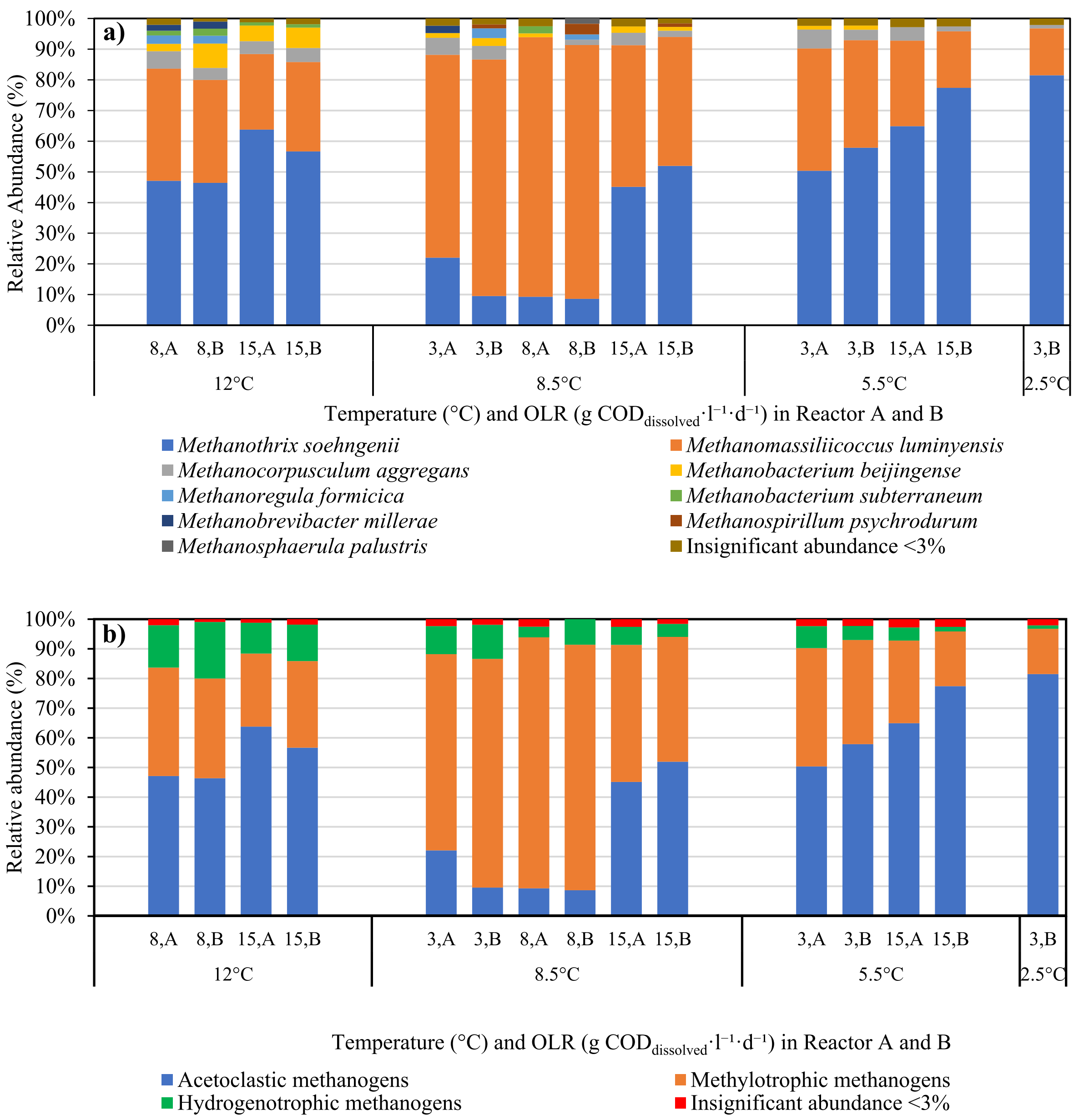

Fig. 5. The relative abundances of microbial communities in UASB granules at different operating temperatures and OLRs at (a) archaeal species level and (b) methanogen groups. A and B on the x-axis refer to microbial samples taken from reactor A and B, respectively. Numbers beside A and B represent OLR in g $COD_{dissolved}\bullet l^{-1}\bullet d^{-1}$.

by approximately 10% (see supplementary materials), at all operating temperatures and OLRs, probably due to cell assimilation. Over time, net NH_4 increased over the reactors (ammonia accumulation) which is typical of ammonification during fermentation of amine groups (as found in amino acids, amino sugars, urea, and nucleotides).

3.4. Microbial community analysis

Stable long term UASB performance at low-temperatures and at significant OLRs indicate the existence of well-balanced and stable interacting communities in the granules. Temperature compensation could result from physiological adaptations, and/or evolved community changes, and this study included microbial community analysis in order to assess potential adaptations. The MiSeq amplicon sequencing produced high quality data by more than 89% of coverage on average, representing the percentage of sample sequences aligned to a deposited sequence in National Center for Biotechnology Information (NCBI) gene bank. The relative abundances of microbial communities in UASB granules at different operating temperatures and OLRs at (a) archaeal species level and (b) methanogen groups are presented in Fig. 5.

The archaeal community structure was dynamic, with shifts at the methanogen species level following decreasing temperatures and increasing OLRs. Fig. 5a shows predominant species in the archaeal community contributing to at least 97% relative abundance. The most predominant methanogen species in all granule samples were: The obligate acetoclastic methanogen *Methanothriix soehngenii* belongs to *Methanosarcinales* order which decarboxylates acetate (Huser, Wuhrmann, and Zehnder, 1982); The H_2-dependant methylotrophic methanogen *Methanomassiliicoccus luminyensi* belongs to *Methanomassiliicoccales* order which reduces the methyl-groups of methylated compounds to methane with H_2 as electron donor (Söllinger & Urich, 2019); The two autotrophic hydrogen oxidizer *Methanocorpusculum aggregans* (heterotype *parvum*) and *Methanobacterium beijingense* belong to *Methanomicrobiales* and *Methanobacteriales* order, respectively, using hydrogen and carbon dioxide or formate as substrates (Ma et al., 2005; Oren, 2014). These four species made up more than 90% of the relative abundance in the archaeal communities regardless of operating temperatures and OLRs. However, systematic shifts in the methanogen composition were observed under increasing OLR and as a response to lower temperatures, suggesting a concurrent shift of the predominant community methanogenic pathways (Fig. 5b). At 12 °C, *M. soehngenii* and *M. luminyensis* fractions of the archaeal community were 46–64% and 29–47%, respectively. A significant shift was observed upon temperature reduction to 8.5 °C in both reactors. At OLR 3 and 8 g $COD_{dissolved}{\cdot}l^{-1}{\cdot}d^{-1}$, the relative abundance of *M. luminyensis* increased up to 85%, and the relative abundance of *M. soehngenii* decreased down to<10%. However, after further decrement of the operating temperatures, *M. soehngenii* abundance gradually increased to more than 82% at 2.5 °C. The relative abundance of *M. aggregans,* and *M. beijingense* fluctuated regardless of operating temperatures and OLRs in the range 2–10%. Grouped according to major methanogenic pathway (Fig. 5b), the archaeal community could be divided into three methanogen guilds, as presented in Fig. 5b, corresponding to archaeal dominant species in Fig. 5a. At 12 °C, acetoclastic methanogen abundance in the granule archaeal community were 46–64% and 29–47%, respectively.

During operating temperature of 12 °C and low OLR, acetoclastic methanogens were relatively more abundant in both reactors and acetoclastic methanogens slightly increased at high OLR (Fig. 5b). Similar observations were reported by Zhang et al., (2018) whereby acetoclastic *Methanosaetaceae* were abundant after 300 days of operation at 10–20 °C in a UASB reactor. By decreasing operating temperature to 8.5 °C, a significant methanogenic composition shifts were observed, especially at low to medium OLRs, probably due to adaptation to temperature change. Methylotrophic methanogens, specifically *Methanomassiliicoccales*, became more dominant contributing to more than 70% relative abundance. Interestingly, *Methanomassiliicoccales* is known as a methylotrophic methanogens lacking the Wood-Ljungdahl pathway and therefore cannot oxidize methyl-groups to CO_2 (Söllinger & Urich, 2019). Consequently, they are dependent on an external electron donor (i.e. H_2) and compete with autotrophic methanogens. However, with increasing OLRs and decreased temperatures to 2.5 °C, the relative abundance of acetoclastic methanogen reappear as the dominant guild contributing to more than 70% of the methanogenic abundance. These shifts have also been observed by Nozhevnikova et al. (2007) reporting about 95% methane to originate from acetate at 5 °C. In this study, acetoclastic methanogens became increasingly dominant under low-temperature (2.5 °C), indicating acetoclastic growth and acetate to be the main precursor of methanogenesis at the very low temperatures.

In many studies, hydrogenotrophic methanogens played an essential role in anaerobic treatment at low-temperatures (McKeown et al., 2009a, McKeown et al., 2009b; Bandara et al., 2012; Smith, Skerlos, and Raskin, 2015; Petropoulos et al., 2017). However, in this study, the results showed that the relative abundances of hydrogenotrophic methanogens were low and reduced with the decreasing temperatures to approximately 14%, 7.3%, 4.5% and 1.1% at 12, 8.5, 5.5, and 2.5 °C, respectively. Inferring from the high abundance of acetoclastic methanogens, homoacetogenesis is possibly the main hydrogen consuming reaction at low temperature. Caution must be made at this stage as the inference is based on relative abundance only and shifts in predimonant methane formation pathways must be confirmed by flux analysis. An increased acetate production was also observed during UASB operation, especially at 2.5 °C (see supplementary materials). Furthermore, bacterial community analysis showed an increased relative abundances of genus Acetoanaerobium (homoacetogen) in UASB granules at the decreasing temperatures. Homoacetogens convert H_2 and CO_2 to acetate outcompeting hydrogenotrophic methanogens at low-temperatures (Kotsyurbenko et al., 2001; Nozhevnikova et al., 2007). Kotsyurbenko et al. (1996) showed that homoacetogens grow two times faster at 6 °C and were less temperature sensitive in the entire psychrotolerant range as indicated by experimentally estimated Q_{10} values of 2.2 and 4.1, respectively (Kotsyurbenko et al., 1996). In line with these studies, the presented findings in this study suggested temperature and organic loading to not only affect the UASB bioprocess performances but also results in a change in the microbial community composition and potentially the dominant degradation pathway of organic matter. Slow temperature changes, at the scale of seasonal variations, may therefore allow for selection of psychrotolerant, and even psychrophilic methanogens, and this indicates that low temperature anaerobic granular wastewater treatment is a viable treatment alternative even at high latitudes.

Low-temperatures anaerobic bioreactor operation offers economic advantages due to reduce heating requirement and bioenergy production potential, especially for high latitude countries. Even though the temperatures of municipal wastewater at Grødaland WWTP during the coldest period is around 12–15 °C, the UASB investigation down to 2.5 °C demonstrated the robustness and capacity limit at the very low temperature. Despite the stable and robust reactor performance, a significant fraction of organic matter still remained in the effluent, and secondary effluent quality cannot be achieved without further treatment, obvious in case of nutrient removal Furthermore, the methane loss in the dissolved phase could also offset the positive effect on carbon footprint from anaerobic wastewater treatment (Liu et al., 2014). For field applications, post-treatment capable of removing residual COD, methane and allow for nutrient recovery is required, and comprehensive reviews on putative post-treatment technologies have been published recently (Chernicharo, 2006; Mai, Kunacheva, and Stuckey, 2018). UASB system may prove valuable for retrofitting wastewater treatment plants overloaded by organic carbon. The rather low footprint and high COD removal at high OLR (even at low temperatures) makes it an interesting option for pre-treatment of secondary aerobic reactors. This would allow for reduced aeration requirements and increased energy recovery turning existing facilities more sustainable.

4. Conclusions

Anaerobic wastewater treatment using low temperature-adapted granules was demonstrated at psychrophilic temperature ranges. No significant temperature effects were observed on specific methane production rates and methane yield down to 12 °C at an OLR up to 15 g $COD_{dissolved} \cdot l^{-1} \cdot d^{-1}$, while reduced but sustained COD removal was demonstrated even at 2.5 °C. Microbial community analysis demonstrated distinct community adaptations and intricate methanogen dynamics indicative of the low-temperature performance. The results suggest that anaerobic wastewater treatment is possible at low temperatures and normal but variable organic loading rates, if adaptations times follow seasonal variations.

CRediT authorship contribution statement

Anissa Sukma Safitri: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Krista Michelle Kaster:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Roald Kommedal:** Conceptualization, Methodology, Formal analysis, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.127616.

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