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Impact of low and high temperatures on aerobic granular sludge treatment of industrial wastewater

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ABSTRACT

The goal of this study was to unravel the impact of high and low temperatures (*T*) on glycogen-accumulating microorganisms (GAOs) which were stimulated in an aerobic granular sludge plant fed with industrial wastewater, which is derived from the cleaning of trucks transporting chocolate and beer. Among GAOs, *Candidatus* Competibacter (*Ca*. Competibacter) was the most abundant. The long-term impact on (1) anaerobic dissolved organic carbon (DOC) uptake, (2) sludge morphology, and (3) microbial community composition was investigated. In addition, the short-term impact of *T* changes on the anaerobic uptake rate was evaluated. High *T* (above 38 °C) and low *T* (below 11 °C) had a negative impact on the relative read abundance of *Ca*. Competibacter and the anaerobic DOC uptake. Nevertheless, the carbon removal efficiency and the settleability of the biomass were not affected. Denitrifiers such as *Thauera* and *Zoogloea* were promoted over *Ca*. Competibacter under high *T* and low *T*, respectively, indicating their positive contribution to granulation maintenance.

Key words: aerobic granulation, anaerobic selector, feast famine regime, microbial community, sequencing batch reactor (SBR), substrate storage

HIGHLIGHTS

- The long-term exposure to high *T* completely inhibited *Ca*. Competibacter.
- The long-term exposure to low *T* decreased the *Ca.* Competibacter population but not at an alarming level.
- The short-term exposure of sludge enriched with *Ca.* Competibacter to high *T* did not affect the carbon uptake rate.
- The short-term exposure of sludge enriched with *Ca.* Competibacter to low *T* negatively affected the carbon uptake rate.

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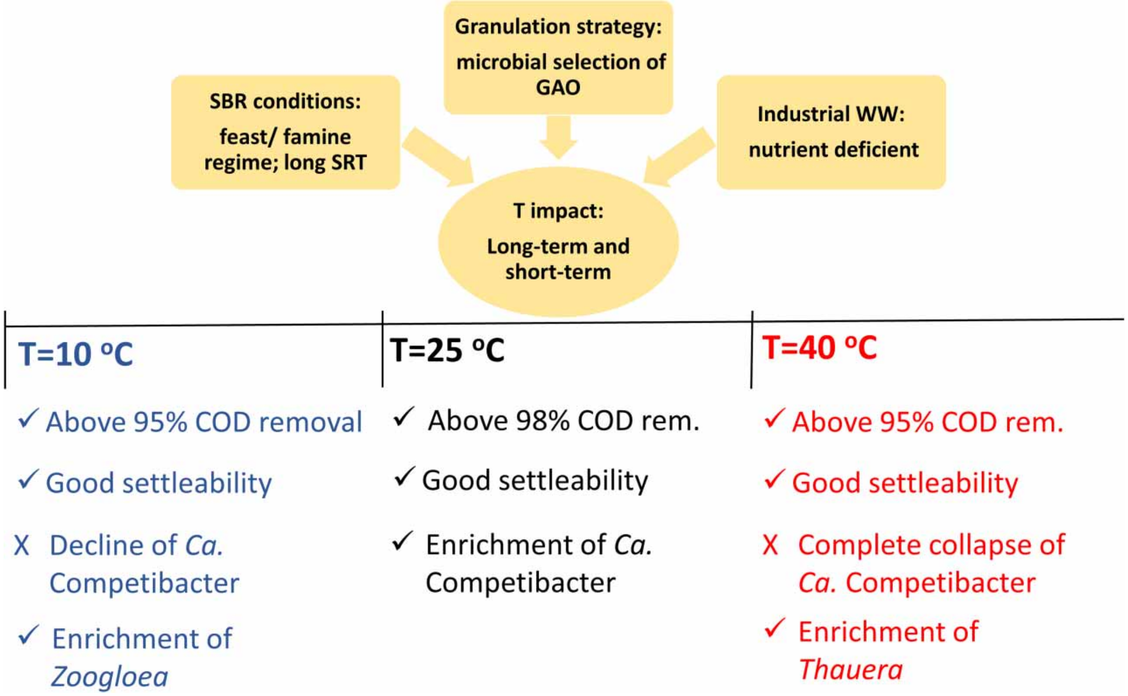
GRAPHICAL ABSTRACT



INTRODUCTION

Aerobic granular sludge (AGS) has numerous advantages. Aerobic granulation in conventional sequencing batch reactors leads to excellent settling and less sludge production (de Kreuk & van Loosdrecht 2004; Chen *et al.* 2020). However, operational disturbances may lead to granule disintegration. Since temperature is an important operational condition, AGS stability has been studied under a wide range of temperatures. Pronk *et al.* (2015) set the temperature at 35 °C, because temperatures above 30 °C are common in industrial wastewaters, and cultivated AGS successfully with different substrates and conventional activated sludge as inoculum sludge. Araújo *et al.* (2022) studied the impact of 20 and 30 °C on AGS formation and stability and reported that temperature affects the structure and the size of the granules, but not the reactors' performance and the microbial population. However, the microbial community analysis was based only on one operating moment. Wan *et al.* (2014) found that the microbial community shifted when the temperature dropped from 25 to 15 to 6 °C, entirely losing the ammonium oxidizing bacteria at 6 °C. de Kreuk *et al.* (2005) showed that low temperatures negatively influenced the time needed for granulation, AGS stability, and conversion rates. In fact, the formation of the granules at 8 °C needed twice as much time in comparison with 20 °C and failed when the fraction of aerobic granules in the inoculum was low. Furthermore, nitrification was completely inhibited at the lower temperature and the anaerobic substrate uptake hardly took place (most acetate leaked to the aerobic phase). Jiang *et al.* (2016) reported successful granulation at 12 °C, pointing out the positive contribution of the polyvalent cations (i.e. Ca^{2+}) in the influent. Martinez *et al.* (2017) reported successful granulation at even lower temperatures (7 °C), highlighting that the inoculum sludge was cold-acclimated. As far as the biological conversions, chemical oxygen demand (COD) removal was comparable with AGS systems at 30 °C but the nutrient removal was approximately 20% less. All in all, some biological conversions seem to be more vulnerable to temperatures such as nitrification. On the other hand, granulation is achieved either at low or high temperatures.

Regarding the selection of slow-growing organisms such as polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs), it is believed that GAOs outcompete PAOs at higher temperatures (above 25 °C) (Panswad *et al.* 2003; Lopez-Vazquez *et al.* 2009). A more recent study showed that PAOs are stable even at higher temperatures (30–35 °C) (Qiu *et al.* 2022). Qiu *et al.* (2022) proved that at higher temperatures, PAOs lose the competitive advantage due to substrate deficiency, because the substrate utilization is higher at higher temperatures. However, the above-mentioned outcome was possible to be reversed when the ratio of carbon to phosphorus was increased.

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GAOs are best known due to their impact on the biological phosphorus removal process. GAOs have a similar metabolism as PAO but without contributing to the removal of phosphorus, as the latter do. Therefore, in a suitable environment for the development of PAOs, GAOs will inevitably coexist (Panswad *et al.* 2003; Lopez-Vazquez *et al.* 2008). However, their presence is not always undesirable. When the wastewater is poor in nutrients but rich in organic matter, the stimulation of GAO is a successful strategy to combat bulking sludge, even leading to granular sludge formation (de Kreuk & van Loosdrecht 2004; Tsertou *et al.* 2022, 2023).

When the feast (anaerobic)/famine (aerobic) regime is applied in biological units, in which the ultimate goal is to remove only organic pollutants and not nutrients, then GAO will compete successfully with the other bacteria, reaching high read abundancies (Caluwé *et al.* 2022; Tsertou *et al.* 2022). The metabolism of GAOs includes (i) substrate (mainly VFAs, volatile fatty acids) uptake, biopolymer (PHA, polyhydroxyalkanoates) production, and glycogen consumption as an energy source during the anaerobic phase, and (ii) PHA consumption, glycogen replenishment, and biomass growth during the aerobic phase (Lopez-Vazquez *et al.* 2009).

Given the importance of the long-term stability of AGS, it is needed to evaluate factors that could have a strong impact on GAO. Temperature is one of these factors, as it acts on biological systems in two important ways, first, the growth rate of microorganisms and secondly, the solubility of oxygen in the liquid phase. High temperatures accelerate the growth and reduce the dissolved oxygen (DO) concentrations. Conversely, low temperatures slow down the growth of microorganisms.

Needless to say that climate change is more noticeable than ever. Longer heatwaves and heavier winters have been the norm in the last few years. Therefore, it is important to define both the upper and lower temperature limits for stable granulation (Schulte 2015).

The majority of the studies, regarding the temperature impact on biological systems, is performed with synthetic wastewater with acetate or propionate as the main substrates (de Kreuk *et al.* 2005; Qiu *et al.* 2022). The goal of this study is to quantify the temperature effect on the granulation process in terms of sludge morphology, anaerobic dissolved organic carbon (DOC) uptake, and microbial composition of AGS treating real industrial wastewater.

METHODS

Seed sludge and wastewater

Both the inoculum sludge and the wastewater were derived from a tank truck cleaning company (TTC). Its activities are transporting and storage of food products such as chocolate and beer. The cleaning process of the trucks produces wastewater rich in biodegradable organic content. The low content of nutrients demands the dosing of nitrogen (N) and phosphorus (P) in order to meet the growth needs of the microorganisms. The COD/N/P ratio was kept at 100/3.1/0.4 by adding NH_4Cl and K_2HPO_4. Furthermore, the influent pH was adjusted to 7.0 by adding $NaHCO_3$ (Caluwé *et al.* 2022; Tsertou *et al.* 2022). Detailed information about the wastewater characteristics can be found in the supplementary material (Table i).

Reactors configuration and operational conditions

The investigation of the temperature impact was performed in two stages. The first experiment (stage I) investigated the impact of high temperatures (*T*) (ranging from 25 to 40 °C) and the second experiment investigated the impact of low temperatures (stage II) (ranging from 25 to 10 °C). In each stage, two parallel sequencing batch reactors (SBR) were set up; the reference (REF) and the experimental (EXP). The temperature was held at 25 °C for the reference reactor and was changed for the experimental reactor (Figure 1). The reactors used an anaerobic feast/aerobic famine regime. The 24 h SBR cycle consisted of prolonged anaerobic feeding (1 L in 4 h slow feeding) followed by 0.5 h mixing, aeration (approximately 18 h), settling (1 h), and discharge. In that way, the settings of the biological treatment of the full-scale wastewater treatment plant were simulated (Caluwé *et al.* 2022). Each reactor had a working volume of 11.5 L and was equipped with a pH sensor (Jumo Techline), oxygen and temperature sensors (Hach Lange LDO), an air disc (AQUADISTRI), a mechanical mixer (Heidolph RZR 2020), a dosing pump (Iwaki metering pump), a discharge valve (Eriks ER 10.X33.S00), and a thermostatic bath (VWR). The data were collected, processed, and plotted by LabView software (National Instruments). The SBR configuration is included in the supplementary material. The DO was maintained between 1 and 2 $mg{\cdot}L^{-1}$ in the REF reactor. The DO set point for the EXP reactor was changed to 1–4 $mg.\ L^{-1}$ when *T* increased in order to maintain sufficient DO conditions. The organic loading rate (OLR) was slightly different in the two stages because the industrial wastewater has a variable composition. As a result, during stage I (high *T*), the average OLR was 0.4 ± 0.04 kg COD $(m^3.\ day)^{-1}$ and, during stage II (low *T*), it was 0.5 ± 0.07 kg COD $(m^3.\ day)^{-1}$.

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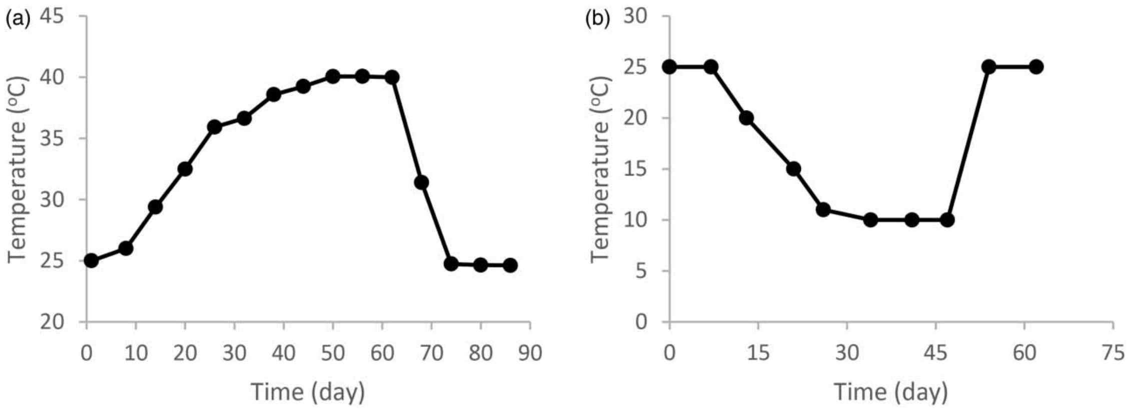

Figure 1 | Temperature profile for the experimental reactor. Experimental design of high (left) and low (right) temperatures.

Mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), and sludge volume index (SVI) were performed in accordance with Standard Methods (APHA 1998).

NH_4^+-N, NO_2^--N, NO_3^--N, PO_4^{3-}-P, sCOD, and COD were measured with Hanna Instruments photometers by use of instruments test kits HI93715, HI93707, HI93766, HI93717, and HI93754B medium range, respectively. DOC was measured by a Sievers innovox TOC analyzer. VFAs were measured with a Hach DR 2800 Portable Spectrophotometer by using the test kit LCK-365 (Hach). pH and conductivity of wastewater were measured manually with a Hanna HI-98194 Multiparameter Water Quality Meter. 16S rRNA gene amplicon sequencing is the molecular technique that was used for the identification of the microbial community. A detailed description can be found in the study by Tsertou *et al.* (2022). Sludge morphology was observed using an Olympus B310 light microscope with phase contrast illumination (Ph1) and a total magnification of two times.

Anaerobic uptake capability

The anaerobic DOC uptake was measured on a weekly basis both *in situ* (long-term exposure to temperature changes) during an SBR cycle (as described by Tsertou *et al.* 2022) and *ex situ* (short-term exposure to temperature changes) in separate batch tests with sludge sampled from the two SBRs. For the *ex situ* measurement, endogenous sludge was collected at the end of the SBR cycle as described by Tsertou *et al.* (2023). The collected sludge (400 mL) from both reactors was transferred to a beaker on a magnetic stirring plate and was exposed to 40 and 25 °C during stage I, and 10 and 25 °C during stage II. The anaerobic DOC uptake test started only when the endogenous sludges reached the temperature setting. Then 100 mL of wastewater was added. During the test, mixed liquor samples were taken (1) before adding the wastewater, (2) after adding the wastewater, (3) after 1 h of anaerobic mixing, after 2 h of anaerobic mixing, and after 3 h of anaerobic mixing.

RESULTS

Part A

This section includes the impact of high temperatures on settleability (Figure 2(a)), morphology (Figure 2(b)), bacteria abundancies (Figures 3(b) and 4), anaerobic DOC uptake (Figure 3(a), 3(c) and 3(d)), and COD removal efficiency.

Reactor performance

The carbon removal was assessed for the two SBRs. The %COD removal was 97.4 $\pm$ 1.5 for the EXP and 98.2 $\pm$ 0.3 for the REF reactor. Despite the high COD removal efficiency of both reactors, only the REF reactor always complied with the Flemish discharge limits (COD $<$125 $mg{\cdot}L^{-1}$). Effluent total suspended solids (TSS) were low for both reactors (27.2 $\pm$ 17.9 $mg{\cdot}L^{-1}$ and 15.1 $\pm$ 9.3 $mg{\cdot}L^{-1}$ for EXP and REF reactors, respectively). Other parameters such as NH_4^+-N, NO_3^--N, PO_4^{3-}-P, sCOD, and DOC of the effluent were measured during the operation of the SBR. The data are shown in Table ii in the supplementary material.

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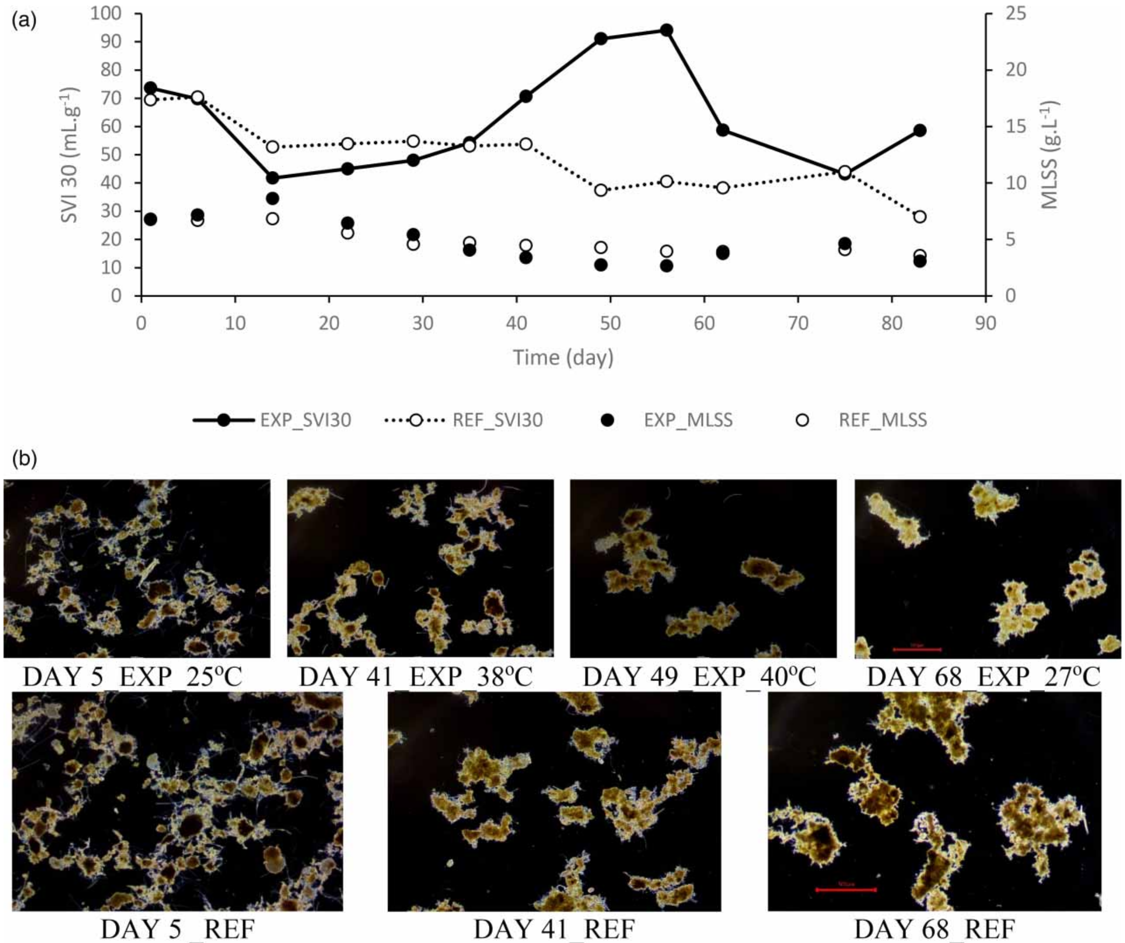

Figure 2 | (a) Sludge concentration and sludge volume index after 30 min (SVI 30). (b) Morphology evolution for the EXP reactor (on the top) and for the REF reactor (on the bottom). Bar indicates 500 μm.

Granular sludge characteristics

MLSS tended to increase during the first 21 days in both reactors (Figure 2(a)). Afterwards, it was decided to manipulate the SRT. The targeted SRT was 30 days. Then, there was a continuous drop in biomass concentration. In order to maintain sufficient biomass, sludge wasting was stopped after day 49. The average MLVSS/MLSS ratio was 0.75 ± 0.12 for the EXP reactor and 0.77 ± 0.08 for the REF reactor. The SVI 30 improved steadily for the REF reactor (avg. SVI 30 was 49.7 ± 12.7 mL·g^{-1}). On the other hand, the SVI 30 increased slightly up to 94 mL·g^{-1} for the EXP reactor from day 35 until day 56. During that period, the applied temperatures increased stepwise from 37 to 40 °C. SVI 30 improved again after day 56 when *T* was still at 40 °C. The overall settling performance was very good for the EXP reactor. SVI 30 never exceeded 100 mL·g^{-1}. The settling performance was in line with the structure of the sludge. Although the actual particle size was not measured, the microscopic examination showed that the granule size increased in both reactors (Figure 2(b)). Furthermore, filaments were absent from both reactors.

Anaerobic metabolism of *Ca.* Competibacter

Ca. Competibacter was the most abundant genus in the REF reactor and accounted for 90 ± 3% (median value) of the total GAO abundance. Therefore, the anaerobic metabolism is attributed mainly to *Ca.* Competibacter (Figure 3(b)). *In situ* and *ex situ* anaerobic substrate uptake measurements were performed in order to evaluate the impact of temperature changes on the anaerobic metabolism.

The high anaerobic DOC uptake capacity of the biomass during the first days of the operation (Figure 3(a)) indicated that the biomass was already enriched in GAO since the seeding sludge originated from a full-scale industrial WWTP applying a

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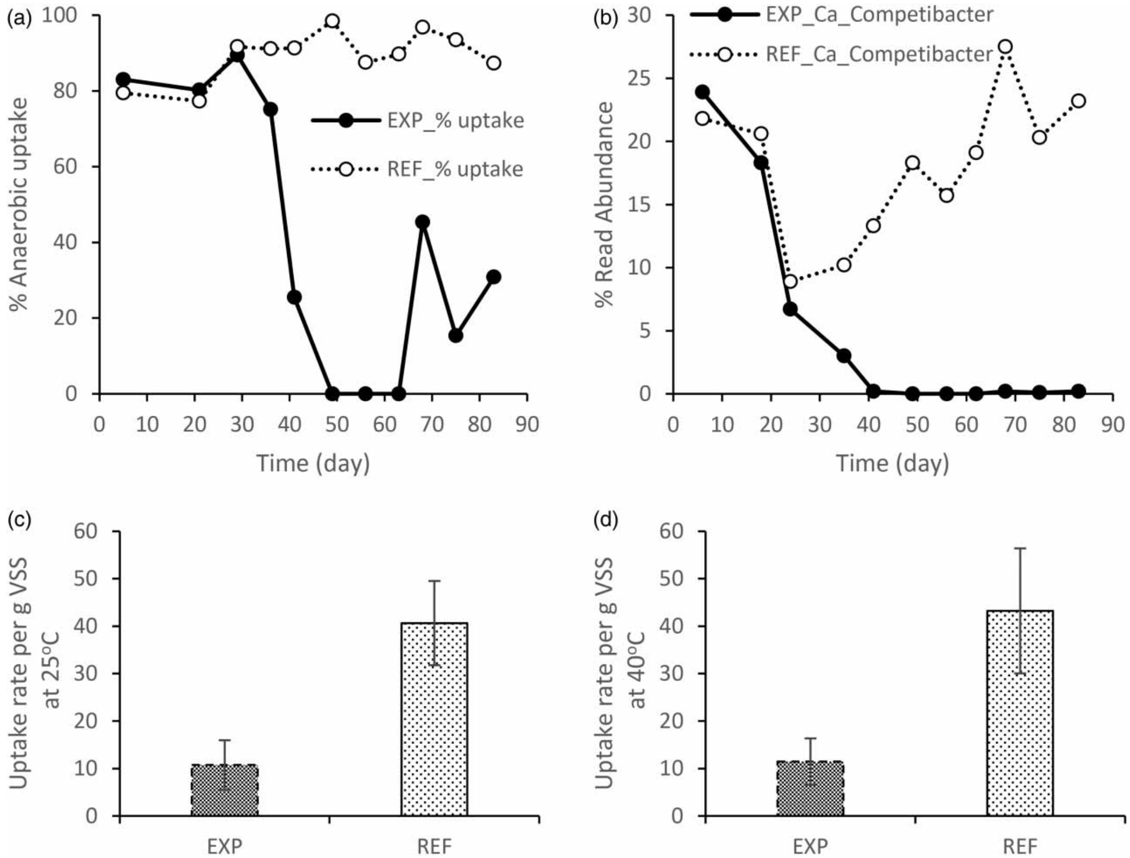

Figure 3 | (a) Percentage of anaerobic DOC uptake in *in situ* measurements, (b) % read abundance of *Ca.* Competibacter, (c) anaerobic DOC uptake rate (mg DOC·(gVSS·h)$^{-1}$) in *ex situ* measurements at 25 °C, (d) anaerobic DOC uptake rate (mg DOC·(gVSS·h)$^{-1}$) in short-term/*ex situ* measurements at 40 °C. Error bars indicate the standard deviation ($n = 7$).

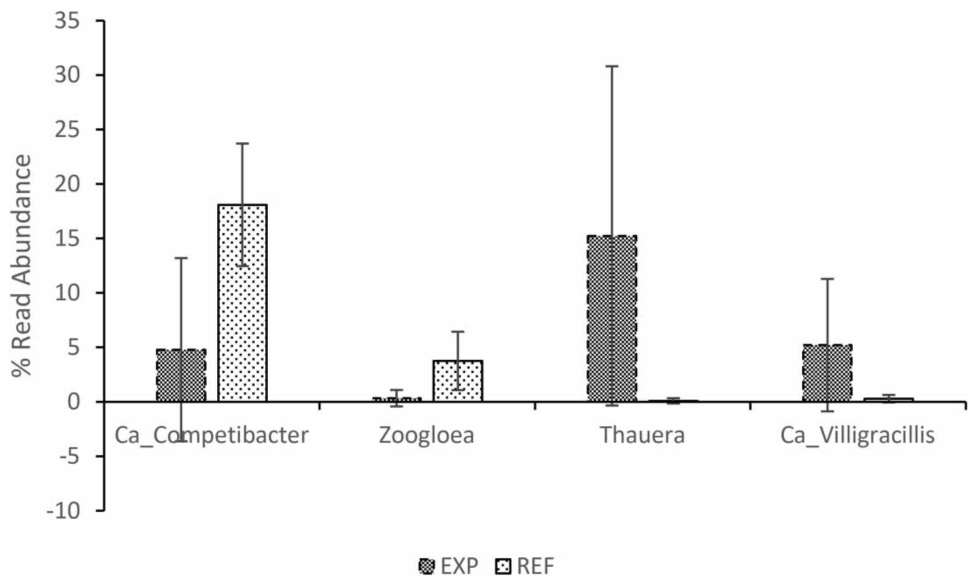

Figure 4 | Average read abundance of *Ca*. Competibacter, *Zoogloea*, *Thauera*, *Ca.* Villigracillis during the whole operation of the SBRs. Error bars indicate the standard deviation ($n = 11$). Bacteria diversity was identified by 16S rRNA gene amplicon sequencing.

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feast (anaerobic)/famine (aerobic) regime (Caluwé *et al.* 2022). In addition, the industrial wastewater was poor in nutrients. These conditions are proven drivers for GAO proliferation at both laboratory scale and full scale (Tsertou *et al.* 2022, 2023).

In the long-term operation at 25 °C, the REF reactor presented stable anaerobic uptake (av. 89.5 $\pm$ 6.5%). This order of magnitude is expected in systems fed with industrial wastewater. Almost complete anaerobic uptake is observed only in studies with acetate or propionate as the sole carbon source (Dockx *et al.* 2021). The anaerobic DOC uptake capacity of the EXP reactor presented large variations in the function of time during the long-term operation. From the beginning until day 29 (from 25 to 36 °C), the average anaerobic DOC uptake was 84.2 $\pm$ 4.7%. Afterwards, the anaerobic DOC uptake dropped to 75% on day 36, when the temperature was around 38 °C. Further deterioration (up to 66% decrease in anaerobic uptake) was observed within only 5 days. On day 41, the anaerobic DOC uptake decreased to 25.5%. The average temperature in the reactor between day 36 and day 41 was 38.5 $\pm$ 0.4 °C. Complete loss of the anaerobic uptake was detected in the following days (from day 49 until day 63) when the average temperature was 40.1 $\pm$ 0.3 °C. Consequently, all the substrate leaked into the aerobic phase where it could be consumed by ordinary heterotrophic organisms. The temperature was set back to 25 °C on day 68 in order to check if the inhibition of the anaerobic metabolism could be reversed. The average % of anaerobic uptake was 30.5 $\pm$ 15.0% and the average temperature was 24.8 $\pm$ 0.6 °C during the final operational period (between day 68 and day 83). Although the application of cooler temperatures positively affected the anaerobic uptake, the complete recovery of the anaerobic DOC uptake capacity probably needs more time.

The *ex situ* measurements (Figure 3(c) and 3(d)) started on day 41 when the anaerobic metabolism was stable for the REF reactor but significantly worse for the EXP reactor, indicating different GAO enrichment and activity levels in the sludges from both reactors. Under short-term exposure to temperature changes, the maximum uptake rate is observed within the first hour of the anaerobic uptake test. For REF reactor, the average substrate uptake rate was 40.6 $\pm$ 8.9 mg DOC·g^{-1}VSS·h^{-1} at 25 °C and 43.2 $\pm$ 13.2 mg DOC·g^{-1}VSS·h^{-1} at 40 °C. The uptake rate was slightly higher for the REF reactor at 40 °C, indicating that the short-term exposure to high temperatures will not affect the DOC uptake capacity of microorganisms with storage capability. For EXP reactor, the average substrate uptake rate was 10.7 $\pm$ 5.2 mg DOC·g^{-1}VSS·h^{-1} at 25 °C and 11.4 $\pm$ 5.3 mg DOC·g^{-1}VSS·h^{-1} at 40 °C.

Microbial community analysis

Ca. Competibacter, *Defluviicoccus*, and *Propionivibrio* were the GAO identified. For the REF reactor, the average % read abundances were 18.09 $\pm$ 5.62, 1.63 $\pm$ 0.47 and 0.03 $\pm$ 0.04, respectively.

For the EXP reactor, the average % read abundances were 0.33 $\pm$ 0.31 for *Defluviicoccus* and 0.02 $\pm$ 0.03 for *Propionivibrio.* For *Ca.* Competibacter, the average % read abundance was 21.07 $\pm$ 3.95 when *T* ranged from 25 to 31 °C, 4.86 $\pm$ 2.66 when *T* ranged from 31 to 37 °C, 0.08 $\pm$ 0.09 when *T* ranged from 37 to 40 °C and 0.16 $\pm$0.10 when *T* returned back to 25 °C. *Zoogloea* and *Thauera* were among the top 20 identified bacteria in this study. Average % read abundances for *Zoogloea* and *Thauera* in the REF reactor were 3.75 $\pm$ 2.68 and 0.08 $\pm$ 0.24, respectively. High temperatures seem to affect significantly both *Zoogloea* and *Thauera* but in a different way. The average % read abundance for *Zoogloea* and *Thauera* was 0.93 $\pm$ 1.09 and 0 $\pm$ 0, respectively, when *T* ranged from 25 to 38 °C. When the *T* increased further from 38 to 40 °C, the read abundance of *Zoogloea* dropped to zero. Furthermore, it did not recover when *T* returned back to 25 °C. In contrast, the % read abundance of *Thauera* significantly increased at high temperatures, reaching the average value of 23.93 $\pm$ 12.70. The upward trend of *Thauera* read abundance continued even when the temperature was returned to 25 °C. In addition, *Ca.* Villigracilis and *Lewinella* were the most abundant filamentous organisms present. Regarding *Lewinella,* a similar evolution was observed for the two reactors. Regarding *Ca*. Villigracilis, the read abundance remained low (0.29 $\pm$ 0.34) for the REF reactor. In the EXP reactor, the average read abundancies were 0.10 $\pm$ 0.08 when *T* ranged from 25 to 38 °C, 7.08 $\pm$ 7.87 when *T* ranged from 38 to 40 °C, and 9.50 $\pm$ 2.27 when *T* returned back to 25 °C. The evolution of *Ca*. Villigracilis seems to be affected by the increase of *T*. Despite the high-measured read abundancies of the filamentous *Ca*. Villigracilis, no filaments were detected during the microscopic examination suggesting that *Ca*. Villigracillis are found inside the flocs. The temperature ranges and the % abundances as a function of time are presented in the supplementary material.

Part B

Impact of low temperatures on settleability (Figure 5(a)), morphology (Figure 5(b)), bacteria abundancies (Figures 6(b) and 7), anaerobic DOC uptake (Figure 6(a), 6(c) and 6(d)), and COD removal efficiency.

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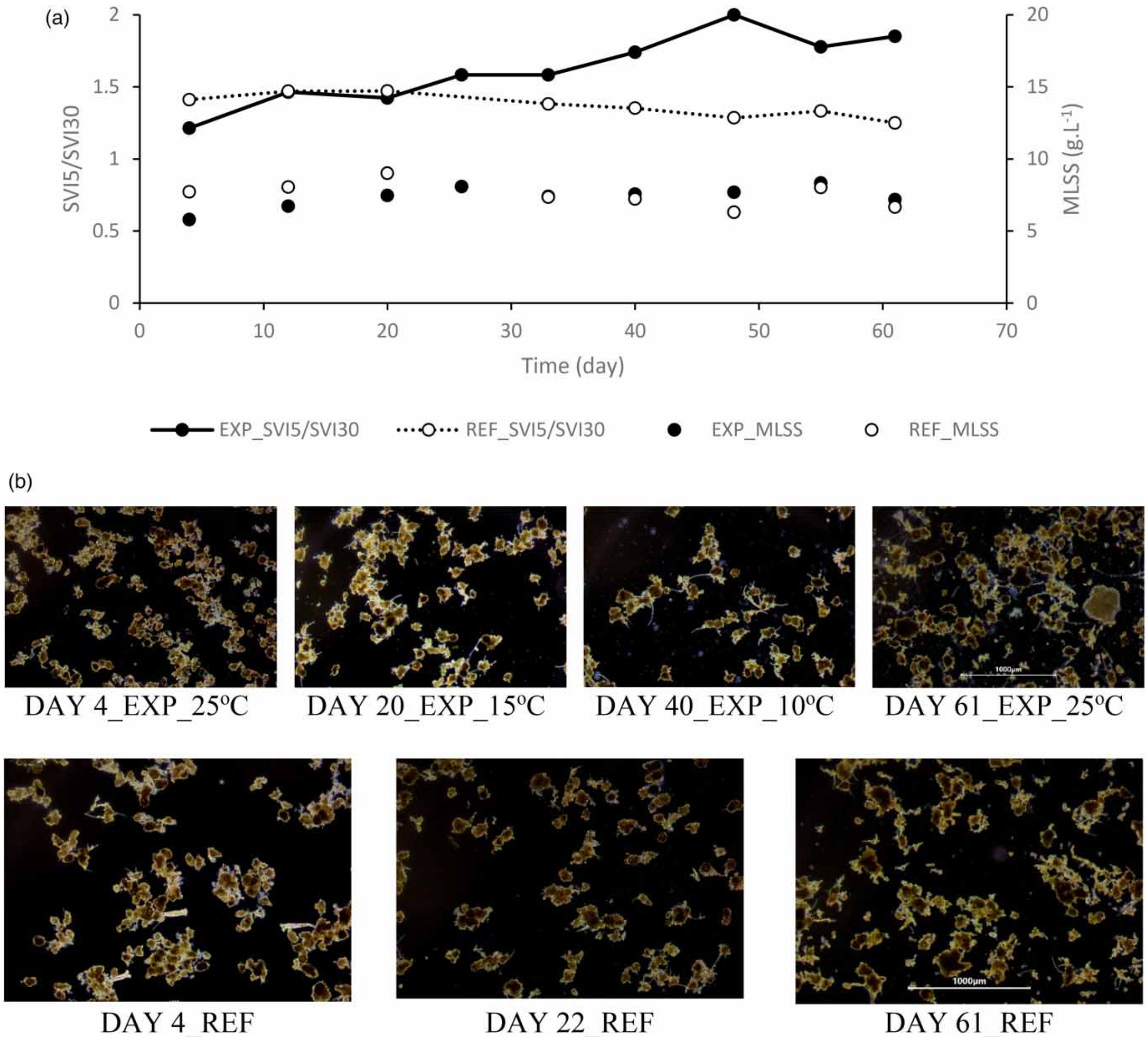

Figure 5 | (a) Sludge concentration and SVI 5/SVI 30. (b) Morphology evolution for EXP reactor (on the top) and for the REF reactor (on the bottom). Bar indicates 1,000 μm.

Reactor performance

The carbon removal was assessed in the two SBRs. The average % COD removal was 96.8 ± 1.7 and 98.2 ± 0.6 for EXP and REF reactors, respectively. The average COD value of the effluent was 180 ± 91 and 102 ± 33 mg·L^{-1} for EXP and REF reactors, respectively. The average TSS value for the effluent was 69.74 ± 63.48 and 11.89 ± 4.48 mg·L^{-1} for EXP and REF reactors, respectively. COD and TSS values started to exceed the discharge limits only when *T* was at 10 °C. When *T* returned back to 25 °C, COD and TSS removal improved. Other parameters such as NH_4^+-N, NO_3^--N, PO_4^{3-}-P, sCOD, and DOC of the effluent were measured during the operation of the SBR. The data are shown in Table ii in the supplementary material.

Granular sludge characteristics

The average MLSS was 7.36 ± 0.75 and 7.55 ± 0.86 g·L^{-1} for EXP and REF reactors, respectively. The sludge concentration remained relatively high during the whole operation since there was no extra sludge wasting applied. The average MLVSS/MLSS ratio was 0.85 ± 0.01 and 0.84 ± 0.01 for EXP and REF reactors, respectively. The average SVI 30 was 20.41 ± 5.42 and 22.99 ± 2.34 mL·g^{-1} for EXP and REF reactors, respectively. Figure 5(a) shows the SVI 5/SVI 30 ratio instead of the SVI 30 because it is the only different trend that is observed between the two systems. The difference in the ratio between the two reactors became more significant when lower temperatures were applied in the EXP reactor, suggesting deterioration of the

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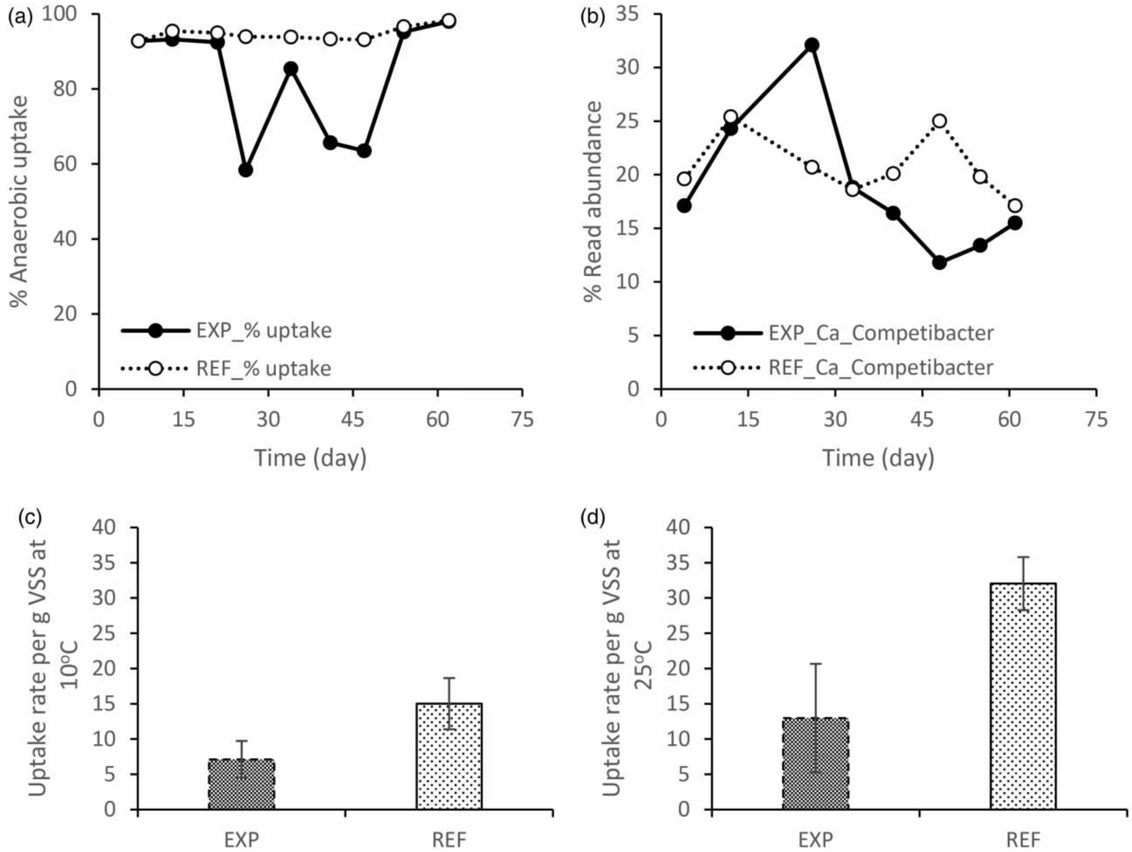

Figure 6 | (a) Percentage of anaerobic carbon uptake in long-term/*in situ* measurements, (b) % read abundance of *Ca*. Competibacter, (c) substrate anaerobic uptake rate (mg DOC·g^{-1}VSS·h^{-1}) in short-term/*ex situ* measurements at 10 °C, (d) substrate anaerobic uptake rate (mg DOC·g^{-1}VSS·h^{-1}) in short-term/*ex situ* measurements at 25 °C. Error bars indicate the standard deviation (REF $n = 7$, EXP $n = 4$).

settling properties. The morphology of the granules did not change during the whole operation. Small and compact granules were present in both reactors, resulting in good settleability.

The particle size was not measured but the morphological examination showed that the granules had sizes of less than 200 μm (Figure 5(b)).

Anaerobic metabolism of *Ca.* Competibacter

Ca. Competibacter had the highest relative abundance among all identified microorganisms in both reactors. *Ca.* Competibacter read abundancies were 95.0 ± 1.9% of all GAOs identified in the EXP reactor and 96.0 ± 1.2% in the REF reactor (median values). It can be concluded that *Ca*. Competibacter (Figure 6(b)) was responsible for the overall anaerobic uptake metabolism. Long-term anaerobic substrate uptake (Figure 6(a)) and short-term anaerobic substrate uptake (Figure 6(c) and 6(d)) measurements were performed in order to evaluate the impact of the temperature changes on the anaerobic metabolism.

The average anaerobic uptake was 94.7 ± 1.8% for the REF reactor. Regarding the EXP reactor, the average anaerobic uptake was 92. 80 ± 0.4% when T ranged from 25 to 15 °C, 68.2 ± 11.9% when T ranged from 11 to 10 °C and 96.6 ± 2.0% when T returned back to 25 °C. In this study, low temperatures (T at 10 °C) were applied for 28 days. The *ex situ* measurements started for the REF reactor from the beginning of the operation. The *ex situ* measurements for the EXP reactor started when the anaerobic uptake was getting worse. The short-term exposure to 10 °C had the same effect for the sludge from both reactors. The uptake rate at 10 °C was lower compared to the uptake rate at 25 °C. The uptake rate was 7.12 ± 2.59 mg DOC (gVSS·h)$^{-1}$ for the EXP reactor and 15.02 ± 3.64 mg DOC (gVSS·h)$^{-1}$ for the REF reactor at 10 °C, whereas the uptake rate was 12.97 ± 7.70 mg DOC (gVSS·h)$^{-1}$ and 32.04 ± 3.76 mg DOC (gVSS·h)$^{-1}$ at 25 °C, respectively. Furthermore, it was observed that the longer the sludge of EXP reactor was exposed to low T, the lower the uptake rate was during the batch tests at 25 °C. In general, the sludge from REF reactor had higher DOC anaerobic uptake rates than the sludge from

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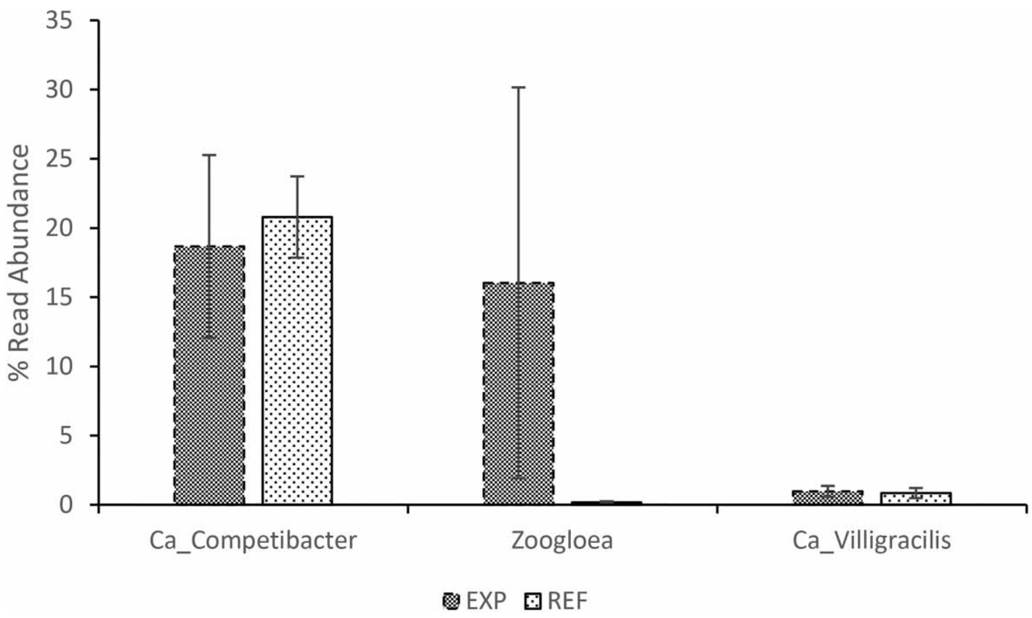

Figure 7 | Average read abundance during the whole operation of the SBRs. Error bars indicate the standard deviation ($n = 8$). Bacteria diversity was identified by 16S rRNA gene amplicon sequencing.

the EXP reactor, both at low temperatures (10 °C) and high temperatures (25 °C). These results show that the short-term exposure of *Ca*. Competibacter to low temperatures (10 °C) affected the uptake rate.

Microbial community analysis

Ca. Competibacter and *Defluviicoccus* were the most abundant GAO. For the REF reactor (25 °C) the average % read abundances were 20.79 ± 2.94 and 0.93 ± 0.22, respectively. For the EXP reactor, the average % read abundances were 18.66 ± 6.59 and 0.94 ± 0.25, respectively. The most remarkable change of read abundance was for *Zoogloea* in the EXP reactor. The average % read abundance was 16.04 ± 14.12 for the EXP reactor and 0.18 ± 0.09 for the REF reactor. The % read abundance for *Zoogloea* rose from 0.5 to 7.2 when the temperature changed from 15 to 11 °C. Maintaining cold temperatures resulted in further increases in the abundance of *Zoogloea*. Ca. Villigracillis was the only known filamentous organism present in the list of the 20 most abundant bacteria. The average % read abundance was 0.98 ± 0.40 for the EXP reactor and 0.85 ± 0.37 for the REF reactor. The temperature ranges and the % abundances as a function of time are presented in the supplementary material.

DISCUSSION

The temperature has a great impact on the activity of microorganisms. It affects the microorganisms in two different ways. First, *T* affects the chemical and enzymatic reactions of the cells. Faster reactions are happening when *T* rises, resulting in an increase in the growth rate as well. However, there is a critical *T* above which the growth ceases completely because of the deactivation of proteins and nucleic acids in the cells. Second, *T* has an impact on oxygen solubility in the bulk liquid. More specifically, the increase of *T* will lead to a decrease in DO. In this study, the DO concentration was always sufficient.

GAOs dominate the microbial community at 25 °C, when a specific feeding strategy is applied and the goal of the biological process is carbon removal only. The first condition is the microbial selection through the feast (anaerobic)/famine (aerobic) feeding regime. The second condition is the composition of the wastewater, which in this case is poor in phosphorus (Lopez-Vazquez *et al.* 2009; Bin *et al.* 2015; Wang *et al.* 2021; Christiaens *et al.* 2022; Tsertou *et al.* 2022). However, in full-scale WWTPs, temperature drops during winter and increases during summer. Therefore, whereas the average wastewater composition is known and the feast (anaerobic)/famine (aerobic) feeding strategy is easily applicable, temperature could be unpredictable. In this study, *Ca.* Competibacter was the most dominant organism when the reactor ran continuously at

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25 °C, making the REF reactor a point of reference and a source of GAO-enriched sludge for *in situ* and *ex situ* measurements of the anaerobic DOC uptake capacity.

Among the selection pressures for granulation (microbial selection, short settling time, high shear stress, feeding strategy, substrate type), the microbial selection of slow-growing bacteria such as GAOs is considered the most important (Chen *et al.* 2020). Nonetheless, the co-growth of fast-growing aerobic heterotrophic bacteria cannot be avoided when only microbial selection is applied and a complex substrate is fed, resulting in the coexistence of floccular and granular sludge (Tsertou *et al.* 2022).

Long-term exposure to high temperatures

Long-term operation under high temperatures (above 38 °C) did not significantly affect the granulation in terms of settleability performance and morphological structure. However, long-term operation affected granulation in terms of microbial composition. Specifically, *Ca.* Competibacter and *Zoogloea* disappeared and were replaced by *Thauera* when the temperature increased up to 40 °C. The anaerobic DOC uptake was completely inhibited, resulting in a high substrate gradient at the start of the aeration phase. In essence, the anaerobic feast phase was transformed into an aerobic feast phase. The elevated *T* favored the proliferation of the denitrifier *Thauera* since *Thauera* has an optimum temperature for growth between 28 and 40 °C (Sagastume *et al.* 2008). Furthermore, as the easily biodegradable substrate escaped to the aerobic phase, *Thauera* seemed to perform substrate uptake under aerobic conditions. This ecophysiological response of *Thauera* has been described before. Thomsen *et al.* (2007) reported the consumption of VFA with oxygen as an electron acceptor by *Thauera*. Eventually, the enrichment of *Thauera* (amyloid producer) was in favor of granulation maintenance, because the amyloid adhesins are components of extra polymeric substances (EPS) and contribute positively to the granulation. The positive role of denitrifiers in the granulation process was shown before. Christiaens *et al.* (2022) reported the promotion of the denitrifier *Zoogloea* over the GAO *Ca*. Competibacter under partially aerobic feeding without impairing the granulation process. Wang *et al.* (2021) also reported *Zoogloea* enrichment over *Ca.* Competibacter at 30 °C, when carbon source leaked into the aerobic phase. *Ca.* Villigracillis seem to help granules' structural stability as well. *Ca.* Villigracillis, which proliferated under high temperatures, were almost exclusively located inside the flocs.

Studies about the direct impact of high temperatures on GAO are limited. There are more studies about the impact of high temperatures on PAO. Conclusions about the impact of high temperatures on GAO could be drawn from these studies in an indirect way. The majority of them reported that higher temperatures affected the PAO population and the anaerobic phosphorus release. Panswad *et al.* (2003) showed that the anaerobic carbon uptake did not change between 20 and 30 °C despite the fact that phosphorus release and uptake declined at 30 °C in comparison with 20 °C, indicating the stimulation of GAOs at higher temperatures. The authors furthermore found that the anaerobic carbon uptake by PAO-enriched sludge stopped at 35 °C, indicating that 35 °C was a critical *T* for GAO-type microorganisms as well. Lopez-Vazquez *et al.* (2008) studied the impact of high temperatures on biomass enriched with GAOs. They found that the acetate uptake rate decreased above 35 °C and stopped around 40 °C. In this study, the anaerobic carbon uptake at 36 °C (EXP reactor) was comparable with anaerobic uptake at 25 °C (REF reactor). A difference between the two reactors regarding the anaerobic uptake capacity was observed when *T* increased around 37 °C, but a significant anaerobic uptake drop was observed at 38 °C, followed by the complete inhibition of the anaerobic uptake around 40 °C. These findings are in line with the results of Lopez-Vazquez *et al.* (2008) and suggest that the critical temperature, in which the anaerobic uptake is inhibited, is higher than 35 °C for a sludge enriched in GAOs.

All in all, it is believed that the anaerobic metabolism was probably inhibited due to enzyme denaturation. This conclusion was made because other potential inhibiting factors were excluded such as acidic conditions (pH was monitored during the SBR operation), lack of nutrients, inhibitory gases (such as sulfide), toxic compounds and salinity, substrate deficiency, and short SRT.

Short-term exposure to high temperatures

Both the *Ca.* Competibacter-enriched sludge (from the REF reactor) and the *Ca*. Competibacter-poor sludge (from the EXP reactor) were exposed to 40 °C in the anaerobic DOC uptake batch tests. As was expected, the GAO-enriched sludge performed better (Figure 3(c) and 3(d)). Lopez-Vazquez *et al.* (2009) reported complete anaerobic acetate depletion at 10, 15, 30, and 35 °C with enriched GAO sludge cultivated at 20 °C. However, complete substrate uptake was never achieved in this study (data not shown) even for the GAO-enriched sludge at 25 °C, probably because the added substrate was industrial

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wastewater (the same used for the long-term operation of the reactors) which contains a mixture of easily and slowly biodegradable compounds. Furthermore, the GAO-enriched sludge exhibited a slightly higher substrate uptake rate at 40 °C in comparison with 25 °C. Our finding is in contradiction with the findings of Lopez-Vazquez *et al.* (2007) who reported complete collapse and no uptake rate at 40 °C, when they performed batch tests with GAO-enriched sludge. By taking into account the negative impact of long-term exposure to high temperatures on *Ca*. Competibacter, we expected also to observe very low anaerobic substrate uptake not only for the GAO-poor sludge but also for the GAO-enriched sludge as Lopez-Vazquez *et al.* (2007) did. This result indicates that trusting only short batch tests could lead to misleading conclusions about the impact of high temperatures on GAOs.

Long-term exposure to low temperatures

The long-term implementation of low temperatures had no impact on granulation in terms of settleability and morphological structure, but it resulted in a drop in both the anaerobic DOC uptake and the *Ca.* Competibacter read abundance. An explanation can be the partial inhibition of the glycolysis pathway, which results in less produced energy for the anaerobic uptake, which has been reported before at low *T* (Lopez-Vazquez *et al.* 2009). Regarding the microbial transition, the low temperatures favored the proliferation of *Zoogloea*, which was also identified during the microscopic examination because of the characteristic 'finger-like' structure (Figure 8 in the supplementary material shows a higher magnification figure of microscopic examination on day 40). According to the Midas Field Guide (Microbial Database for Activated Sludge), *Zoogloea* is an EPS and PHA producer. Furthermore, its excessive growth could lead to viscous bulking. It was not the case in this study. The leakage of some of the substrate into the aerobic phase could be the cause of its enrichment in this study. Weissbrodt *et al.* (2013) reported the selection of *Zoogloea* over *Ca*. Accumulibacter and *Ca*. Competibacter, when VFA leaked to the aerobic phase at 23 $\pm$ 2 °C.

Short-term exposure to low temperatures

The short-term exposure to low temperatures decreased the anaerobic DOC uptake rate for the sludge of both reactors. Lopez-Vazquez *et al.* (2007) measured the lowest substrate uptake rate of GAO-enriched biomass at 10 °C in comparison with other batch tests performed at higher temperatures (from 15 to 35 °C). The study of Lopez-Vazquez *et al.* (2007) and this study confirms that conversion rates slow down significantly when temperature decreases. In this study, the substrate uptake rate was 53% less in the batch test at 10 °C in comparison with 25 °C in *Ca*. Competibacter-enriched sludge. In the study of Lopez-Vazquez *et al.* (2007), the maximum acetate uptake at 10 °C was approximately 67% lower than at 25 °C.

CONCLUSIONS

In this study, we show the impact of *T* on granular sludge stability. Filamentous bacteria extending from the flocs were not observed. The settleability of the granules was very good. *T* significantly affected the microbial composition. In fact *Ca*. Competibacter was outcompeted by *Thauera* at high *T* and *Zoogloea* at low *T*. Both the high temperatures and the low temperatures negatively affected the abundance of *Ca*. Competibacter. Long-term exposure to high temperatures seems more detrimental in comparison with low temperatures. Long-term exposure to high temperatures above 38 °C completely inhibits the anaerobic metabolism probably due to enzyme denaturation. Long-term exposure to low temperatures (below 11 °C) leads to less efficient anaerobic metabolism, resulting in a lower growth rate of *Ca*. Competibacter. The results of this study have important implications for the application of the AGS technology in industry. Due to climate change, the temperature of industrial wastewater and biological treatment can be high. This study shows that this can have a significant impact on microbial activity and composition in AGS systems, requiring appropriate measures to be taken to prevent it.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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