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# Towards mainstream partial nitritation/anammox in four seasons: Feasibility of bioaugmentation with stored summer sludge for winter anammox assistance

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**Abstract**

The strong effect of low temperatures on anammox bacteria challenges its application over the winter in temperate climates. Winter bioaugmentation with stored summer surplus sludge is a potential solution to guarantee sufficient nitrogen removal in winter. Firstly, the systems for which nitrogen removal deteriorated by the temperature decrease (25°C→20°C) could be fully restored with 118–220% stored sludges (6-month) bioaugmentation. Secondly, the reactivation of these stored sludges was tested in the lower temperature systems (15/10°C). Compared to the activity before storage, between 56%-41% of the activity of granular sludge was restored within one month, and 41%-32% for floccular sludge. Additionally, 85-87% of granules and 50-53% of flocs were retained in the systems. After reactivation (15/10°C), a more specialized community was formed (diversity decreased) with Candidatus *Brocadia* still dominant in terms of relative abundance. Capital and operating expenditures (CAPEX, OPEX) were neglectable compared to sewage treatment costs (0.19-0.36%).

**Keywords:** Deammonification; Preservation; Nitrification; Wastewater; Planctomycetota

# 1. Introduction

Partial nitritation/anammox (PN/A) is a commonly applied wastewater treatment technology for ammonium removal in the sidestream (i.e., sludge line) (Lackner et al., 2014). It is based on the conversion of ammonium to nitrogen gas through the activity of aerobic and anoxic ammonium-oxidizing bacteria (AerAOB and AnAOB). For mainstream (i.e., water line) applications, PN/A can reduce the COD-supplementation requirements, oxygen demand, and excess sludge production by respectively 100%, 60%, and 80% compared to traditional nitrification/denitrification (Cao et al., 2017).

In temperate climate regions, the temperature of the mainstream fluctuates between 5 – 20 ℃ (Hendrickx et al., 2014). Specifically, in winter, temperatures can below 10℃, which affects the microbial activity. The effluent quality of the PN/A reactor is sensitive to low temperatures due to the low growth rates of AnAOB (the maximal specific growth rates are 0.02 $d^{-1}$ at 20℃ and only 0.005 $d^{-1}$ at 10℃ (Lotti et al., 2014)), leading to effluent quality deterioration. Low temperatures, therefore, remain a major challenge for applying PN/A in sewage treatment plants (STP).

Low temperatures in winter reduce the activity of AnAOB. Current STP uses several operational strategies to mitigate the reduced activity such as sludge retention times (SRT) and hydraulic retention times (HRT) adjustment (Guo et al. 2013; Ha et al. 2010). Prolonging the HRT is an effective strategy yet requires larger reactor volumes. Merely extending the sludge retention time (SRT) in winter will not be sufficient as it requires a roughly 2.5 times increase in SRT (Arrhenius, $\theta = 1.10$). The problem might be solved by bioaugmentation. That strategy has been applied in the nitrification/denitrification process to mitigate the suppression of low temperature (Head and Oleszkiewicz, 2004;

Figdore et al., 2018). For AnAOB, this bioaugmentation technology was already explored as a remedy to recover system performance from inhibition or shock (e.g., high COD concentration) (Tang et al., 2014). To overcome low-temperature effects on the AnAOB system by bioaugmentation, only the sidestream sludge was used.

However, the temperature differences (> 30°C in the sidestream versus < 15°C in the mainstream) between the two systems would strongly affect the activity of the bioaugmented biomass (Head and Oleszkiewicz, 2004). Biomass loss from the sidestream reactor would also be harmful to the STP as it contributes to 15-25% of the total ammonium removal. Podmirseg et al. (2010), for example, showed that bioaugmentation influenced the microbial community composition of both systems (sidestream and mainstream). Bioaugmentation would promote the relative abundance of the expected genera (e.g., Candidatus *Brocadia*) (Chen et al., 2015; Figdore et al., 2018). Although the reactor performance improves after bioaugmentation (Chen et al., 2015), the positive effects are only maintained for a short period (Patureau et al. 2001). This might be attributed to competition, inhibition, predation, or the presence of bacteriophages caused by the addition of exogenous microorganisms (Herrero and Stuckey, 2015).

Stored anammox biomass was applied to start up the bioreactor or to restore the biological process after a disturbance or inhibition (Wenjie et al., 2014). The 'anammox bioaugmentation of stored AnAOB biomass to relieve the effect of low temperature' concept, to the authors' knowledge, has not been explored thus far. The reactivation of stored sludge at low temperatures is critical as the stored sludge is directly exposed to low temperatures during winter bioaugmentation. To verify that, the long-term reactivation of stored biomass in bioreactors at low temperatures, which has also never been tested before, was carried out in the present research.

The overall objective of this research was to provide evidence for AnAOB bioaugmentation as a remedy for temperature drops on winter days. To achieve this, three parts were examined: i) validation of the stored biomass bioaugmentation mitigation concept at a moderate temperature difference (25 to 20°C), ii) the effects of different reactor temperatures (15, 10, and 4℃) on the stored summer sludge's activity recovery, biomass retention, and community shift, iii) concept evaluation through a cost estimation (capital (CAPEX) and operating (OPEX) expenditures) based on the data from Nieuwveer STP (Breda, the Netherlands) and compared to normal operation. The findings present a new strategy to facilitate the implementation of high-performance nitrogen removal through winter.

# 2. Materials and methods

## 2.1. Characteristics of the preserved AnAOB sludge

Sidestream sludge was used as inoculum for the experiments because it is a good proxy for mainstream sludge due to species (e.g., the AnAOB, AerAOB, and NOB which were dominated by Candidatus *Brocadia*, *Nitrosomonas*, and *Nitrospira,* respectively) (Laureni et al., 2016) and composition similarities (e.g., heterotrophs dominated 67-84% of the total community in this study and 80-90% in mainstream studies) (Lotti et al., 2015b; Yang et al., 2018). Both floccular (harvested from the 990 $m^3$ sidestream PN/A installation in Breda (the Netherlands) with the particle size 0 – 0.45 mm) and granular (harvested from the 600 $m^3$ potato-processing wastewater line in Olburgen (the Netherlands) with the particle size 0.2-3.0 mm) are exploited AnAOB sludges were dominated by Candidatus *Brocadia*, and their initial AnAOB activity was 119 ± 6 and 79

± 6 mg $NH_4^+$-N $g^{-1}$ VSS $d^{-1}$ at 20 ℃, respectively for the floccular and granular sludges. The sludges were stored for 6-month at cost-effective conditions: without cooling and no nitrogen additives conditions (without nitrate and nitrite)) (Zhu et al., 2022).

Before bioaugmentation, the sludge was washed four times with a buffer solution which consisted of tap water spiked with $NaHCO_3$ (0.4 g $L^{-1}$) and trace elements solution A/B (1 ml $L^{-1}$) to remove the COD, phosphorus, and ammonium produced by biomass decay during sludge preservation. The composition of trace element solution A/B was based on Van de Graaf et al. (1995).

**2.2. Setups for validation of the stored biomass bioaugmentation mitigation concept**

The stored biomass bioaugmentation mitigation concept is the use of sludge stored from the reactor that is bioaugmented when the reactor (the same reactor as the sludge harvested) temperature is decreased, thus restoring the nitrogen removal performance to the state before the temperature drops.

Two cylindrical sequencing batch reactors (SBR), inoculated with either floccular (R1) or granular (R2) sludge, were operated for 140 days. The working volume of the reactors was 2.25 L (total volume of 2.5 L). The targeted nitrogen loading rate and volume exchange ratios were 320 mg N $L^{-1}$ $d^{-1}$ and 33%, respectively, leading to a cycle time of 2 h and an HRT of 6 h. Each cycle included 80 minutes of continuous feeding (reaction), 30 minutes of settling, 6 minutes of decantation, and 4 minutes of idle time. The sequencing batch mode was achieved through timers (EverFlourish EMT757-F, Germany) which controlled influent/effluent pumps (SEKO R1/R7, United States) and overhead stirrers (200 rpm) (Velp Scientific ES, Italy). There was no sensor control during the experiments.

The overall experiment was divided into three stages. During stage – I (Days 1-27), the reactors were operated at 25.2 ± 0.3℃. During stage – II (Days 28-62 for R1); Days 28-86 for R2), the temperature was decreased to 20.1 ± 0.4℃ to get a steady state. During stage – III (Days 63-140 for R1; Days 87-140 for R2), bioaugmentation with stored summer AnAOB biomass (two times for R1 and one time for R2), aiming to offset the performance deterioration caused by temperature decrease in stage – II.

The initial biomass concentrations were 1.0 ± 0.1 and 1.3 ± 0.1 g VSS $L^{-1}$ for R1 (floccular sludge) and R2 (granular sludge), respectively. After each bioaugmentation, the biomass concentration in the reactors increased by a percentage of about 100%. Steady state was achieved before each new bioaugmentation. The SRT was not controlled but monitored by measuring volatile suspended solid (VSS) concentration in the effluent during the whole test.

**2.3. Stored-sludge low-temperature reactivation**

Stored-sludge reactivation contains two main parts: AnAOB activity recovery and the bioaugmented biomass retention in the system. Multiple cylindrical SBRs, with a working volume of 1.2 L (volume exchange ratio is 33%), were operated for about 35 days to reactivate the stored sludge under different temperatures (No biological replicates were included, yet variation was accounted for by sampling at different time points 30-35 days in a row). The cycle composition, reactor operation, and targeted nitrogen loading rate were the same as R1 and R2. After preserving for six months, the stored floccular and granular sludge (same to Section – 2.2) were bioaugmented into SBRs which operated at 15.3 ± 0.4 °C, 10.4 ± 0.4 ℃, and 3.9 ± 0.2 ℃, respectively. Their initial biomass concentrations were 2.3 ± 0.3, 4.0 ± 0.6, and 6.4 ± 0.2 g VSS $L^{-1}$, respectively. The higher biomass concentration was chosen at a lower temperature since that could improve the tolerance and resilience of biomass to some content (Jin et al., 2013).

### 2.4. Reactors' operation and synthetic wastewater composition

Except the $NH_4^+$-N and $NO_2^-$-N, synthetic feed consisted of tap water spiked with $KH_2PO_4$-P (3.2 mg $L^{-1}$), $NaHCO_3$ (350 mg $L^{-1}$), $MgSO_4 \cdot 7H_2O$-Mg (2.2 mg $L^{-1}$), $CaCl_2 \cdot 2H_2O$ (2 mg $L^{-1}$), and trace element solution A/B (0.5 ml $L^{-1}$). The influent was deoxygenated by $N_2$ purging, followed by manually pH adjusting to 6.9-7.0 with the addition of 3 M HCl. The pH of the reactors was not controlled, yet the influent low pH lowered the pH of the reactors to 7.2-7.5 (pH rises due to anammox). An $N_2$ gas balloon was installed on the influent vessel to balance the pressure inside since it was always air-tight.

### 2.5. Analytical procedures

Liquid (6 ml) and microbial samples (6 ml) were taken periodically from the influent, effluent, and reactors. After filtering by 0.2 μm syringe filters (CHROMAFIL Xtra PVDF, Germany) and storing at 4°C, $NH_4^+$-N, $NO_2^-$-N, and $NO_3^-$-N were measured with a San$^{++}$ Automated Wet Chemistry Analyzer (SKALAR, the Netherlands). The biomass concentration was followed over time using VSS measurements (APHA, 1998). A handheld meter was used to monitor pH (Hach HQ30d, USA).

### 2.6. Microbiome analysis

To test the microbial community shift during the sludge reactivation period at different temperatures, the V4 region of the 16S rRNA gene was used after the genomic DNA was extracted using Powerfecal kit (Qiagen, Germany) following the manufacturer's instructions. The extracted DNA samples were stored at -20℃ until shipped to a commercial company (Novogene Europe, United Kingdom) for amplicon sequencing analysis. The set of forwarding 515f (GTGCCAGCMGCCGCGGTAA) and reverse 806r (GGACTACHVGGGTWTCTAAT) primers were used to amplify the V4 hypervariable region of the 16S rRNA gene by polymerase chain reaction (PCR) (Kozich et al., 2013). The amplicon sequencing libraries were pooled and sequenced in an Illumina paired-end platform. After sequencing, the raw reads were quality filtered, chimeric sequences were removed and ASVs were generated. Subsequently, microbial community analysis was performed by Novogene using Qiime software (V1.7.0). For phylogenetical determination, the SSURef database from SILVA (http://www.arb-silva.de/) was used. Relative abundances of ASVs were reported as % total sequencing reads count. To compare the community diversity, Shannon, Chao1, and Coverage indices were calculated. The data have been deposited with links to BioProject accession number PRJNA778735 in the NCBI BioProject database.

## 2.7. Calculations

### 2.7.1. Specific AnAOB activity, nitrogen conversion rate, and sludge retention time

The nitrogen removal rate (NRR, mg N $L^{-1}$ $d^{-1}$), specific AnAOB activity ($SAA_T$, mg $NH_4^+$-N $g^{-1}$ VSS $d^{-1}$), and sludge retention time (SRT, d) were calculated according to the following equations.

$$NRR = \frac{Q_{in}}{V} \times (N_{in} - N_{out}) \qquad (Eq. 1)$$

$$SAA_T = \frac{ARR}{VSS_{reactor}} \qquad (Eq. 2)$$

$$SRT = \frac{VSS_{reactor} \times V}{VSS_{effluent} \times Q_{out}} \qquad (Eq. 3)$$

$Q_{in}$ and $Q_{out}$ are the flow rate of influent and effluent, respectively [L $d^{-1}$]; V is the reactor volume [L]; $N_{in}$ and $N_{out}$ are the nitrogen concentration of influent and effluent, respectively [mg N $L^{-1}$]; ARR is the ammonium removal rate [mg N $L^{-1}$ $d^{-1}$]; $VSS_{reactor}$ and $VSS_{effluent}$ are the biomass concentration in reactor and effluent, respectively [g VSS $L^{-1}$].

### 2.7.2. Arrhenius equation

The microbial temperature effect was normally described by a simplified Arrhenius equation (Eq.4).

$$\mathrm{SAA_T} = \mathrm{SAA_{20°C}} \times \theta_{\mathrm{AnAOB}}^{(\mathrm{T}-20°\mathrm{C})} \qquad (\mathrm{Eq.}\,4)$$

θ is the temperature coefficient [unitless]; T is the respective temperature of the measured system [°C]. A θ value of 1.10 was chosen based on previous work performed by the authors (Vandekerckhove et al., 2020).

### 3.2.7.3 The percentage of activity recovery and biomass retention in reactors

The activity recovery percentage (p) is defined as the measured specific AnAOB activity ($SAA_T$) divided by the expected specific AnAOB activity ($SAA_{T,excepted}$, normalized to T°C based on Arrhenius) (Eq.5 and Eq.6). The biomass retention percentage is the ratio of biomass concentration in the end (average the last three samples) divided by the initial value (Day 0).

$$\mathrm{p} = \frac{\mathrm{SAA_T}}{\mathrm{SAA_{T,excepted}}} \qquad (\mathrm{Eq.}\,5)$$

$$\mathrm{SAA_{T,excepted}} = \mathrm{SAA_{20,initial}} \times \theta_{\mathrm{AnAOB}}^{(\mathrm{T}-20°\mathrm{C})} \qquad (\mathrm{Eq.}\,6)$$

$SAA_{20,initial}$ the initial specific AnAOB activity at 20°C before the sludge storage [mg $NH_4^+$-N $g^{-1}$ VSS $d^{-1}$]. The values were respectively 119 ± 9 and 79 ± 11 mg $NH_4^+$-N $g^{-1}$ VSS $L^{-1}$ for floccular and granular sludge.

# 3. Results and discussion

### 3.1. Validation of stored AnAOB bioaugmentation at moderate temperature differences

This section had the objective to validate the bioaugmentation mitigation concept at a moderate temperature difference (25 to 20°C). After 6-month preservation, the AnAOB activity in stored flocs and granules decreased to 62.5 ± 3.9 and 45.8 ± 4.2 mg $NH_4^+$-N $g^{-1}$ VSS $d^{-1}$, respectively (Zhu et al., 2022). R1 (flocs bioaugmentation) and R2 (granules bioaugmentation) were run for 140 days to verify the feasibility of stored summer sludge bioaugmentation to restore the nitrogen removal performance of the reactor after temperature reduction. The nitrogen removal performance and sludge retention properties were compared throughout the experiment (Fig.1).

#### 3.1.1. 220% flocs bioaugmentation mitigated the effect of 5.1C° decrease

Before reducing the temperature from 25.2 ± 0.3℃ to 20.1 ± 0.4℃, the effluent quality of $NH_4^+$-N, $NO_2^-$-N, and $NO_3^-$-N remained stable (4.4 ± 1.3 mg $L^{-1}$, 5.3 ± 1.2 mg $L^{-1}$, and 9.0 ± 1.2 mg $L^{-1}$) with removal ($NO_3^-$-N production) rates of respectively 122 ± 3.8, 160 ± 4.2, and 32 ± 1.8 mg N $L^{-1}$ $d^{-1}$. The temperature change on Day 28 resulted in a sudden drop of the total inorganic nitrogen (TIN) removal rate from 250 ± 7.8 to 173 ± 24.0 mg N $L^{-1}$ $d^{-1}$ (Fig.1C). The SRT was increased from 9.8 ± 0.6 to 16.0 ± 0.4 d. To restore the effluent quality to the level before the temperature decrease, 1.2 g VSS $L^{-1}$ stored flocs were bioaugmented into R1 (106% of the initial biomass concentration, leading to the increase from 1.1 to 2.3 g VSS $L^{-1}$) (Fig.1E). The TIN removal rate improved significantly, peaking at 259 mg N $L^{-1}$ $d^{-1}$ on Day 73. In the following days, the TIN removal rate gradually decreased due to biomass washout (2.3 to 1.6 g VSS $L^{-1}$). To further mitigate the influence of temperature drop, a second bioaugmentation (114% of the initial biomass concentration, leading to the increase from 1.6 to 2.9 g VSS $L^{-1}$)

was conducted on Day 89. Biomass washout still occurred, yet adaptation to the receptor system appeared after 30 days (TIN removal rate stable at 246 ± 8.2 mg N $L^{-1}$ $d^{-1}$). After bioaugmentation, the rapid SRT decrease was followed by a gradual increase (e.g., SRT from 9.3 to 13.6 d after the first bioaugmentation). The impacts caused by temperature decrease were offset by a two times stored flocs bioaugmentation.

#### 3.1.2. 118% granules bioaugmentation mitigated the effect of 5.1C° decrease

Acclimatization has been regarded as a promising solution to mitigate the inhibition of low temperatures (De Cocker et al., 2018). The reactor was, therefore, operated for a longer period at 20.1 ± 0.4℃ (60 days in R2 versus 36 days in R1) to study the potential of adaptation to low temperatures before bioaugmentation. After approximately 60 days of operating at the relatively low temperature (20℃ versus 25℃), no obvious increase of the TIN removal rate was found (stabilized at 147 ± 9.4 (222 ± 5.8 mg N $L^{-1}$ $d^{-1}$ at 25℃). The SRT increased to 22.0 ± 0.4 from 12.8 ± 0.3 d. On Day 87, 1.6 g VSS $L^{-1}$ stored granules (118% of the initial biomass concentration, leading to the increase from 1.3 to 2.9 g VSS $L^{-1}$) were supplemented into R2 (Fig. 1F). The TIN removal rate immediately increased to 236 ± 15.0 mg N $L^{-1}$ $d^{-1}$. As opposed to the R1 (flocs bioaugmentation), the bioaugmented granules could be successfully retained in the reactor with a low biomass washout within 40 days, resulting in a stable TIN removal rate. The SRT change after the stored granules bioaugmentation was the same as flocs (increased immediately followed by the gradual decrease).

#### 3.1.3. Stored sludge bioaugmentation restored the performance deterioration

A 5.1℃ temperature drop resulted in a sudden TIN removal rate reduction for the R1 (flocs bioaugmentation) and R2 (granules bioaugmentation) by respectively 31% and 34%. That was consistent with previous research (Lotti et al., 2014; Lotti et al., 2015c).

It is might a consequence of the higher energy barrier for enzymatic reactions (Tian et al., 2019). Because of the temperature drop, the nitrogen removal rate decreased rapidly, thereby, exceeding the nitrogen discharge limits. As shown above, exogenous bioaugmentation with stored biomass is a promising solution to mitigate the impacts of temperature drop. The biomass adaptation to the low temperature can be excluded since more than two months of steady state was observed before bioaugmentation (R2). Furthermore, more than one month of stable performance after the inoculation suggested the enhancement could last a certain period.

A high sludge retention time was essential to maintain AnAOB in the reactor (low growth rate and biomass yield (Lotti et al., 2014)). Compared to the R2, almost twice the number of stored flocs (220% of the initial flocs in R1 vs. 118% of the initial granules in R2) were required to mitigate the effect of the 5.1℃ temperature drop. That was mainly due to higher floc washout in the effluent relative to the R2 (13.3 ± 0.3 d of SRT in R1 vs. 30.3 ± 2.3 d of SRT in R2). Two possible reasons explained that: i) Granules have a better sedimentation performance (higher biomass density) and settling properties than flocs, especially after storage (Abma et al., 2007; Xu et al., 2020), and ii) flocs were more susceptible to low temperature (more activity lost) since the higher activity was recovered in granules (Fig.3A). That is probably attributed to the existence of the polymeric matrix that has a protective role in granular sludge to improve the bacterial cold tolerance (Jin et al., 2013).

After bioaugmentation, the SRT dropped at first, followed by an increase and a final stabilization. Pei et al. (2015) also reported that a bioaugmentation could reduce the SRT of nitrifiers. A solids balance analysis demonstrated that the SRT values of R1 and R2 were stabilized at 13 ± 0.3 and 30 ± 2.3 days, respectively. Thus, the granules (with a longer SRT than flocs) achieved a higher mass of sludge in the reactor. The SRT in R2 after bioaugmentation was higher than before, which was essential for the newly bioaugmented biomass to ensure long-term retention in the received system. The results were in line with the finding of R1, indicating a certain percentage of the bioaugmented biomass could eventually remain and grow in the receptor reactors.

## 3.2. Stored-sludge could reactivate (activity recovery and biomass retention) at low-temperature systems

Whether the inoculants can show activity and be retained directly in the low-temperature conditions after the storage is another challenge to the success of the novel stored summer biomass bioaugmentation concept. This section had the objective to assess the reactivation performance of the stored biomass (flocs and granules) at three different temperatures (15℃/10℃/4℃) reactors (Fig.2).

### 3.2.1. AnAOB activity recovered in 15℃/10℃ flocs-/granules- based reactors

The stored flocs and granules were reactivated rapidly (five days) for the reactor run at 15.3 ± 0.4°C. After five days, the specific AnAOB activity was already stable, showing a value of 28.3 ± 2.5 mg $NH_4^+$-N $g^{-1}$ VSS $d^{-1}$ for the granules. For the flocs, on the other hand, the AnAOB activity decreased from 39.9 mg $NH_4^+$-N $g^{-1}$ VSS $d^{-1}$ on Day 7 to 27.9 mg $NH_4^+$-N $g^{-1}$ VSS $d^{-1}$ on Day 33. The reactor operated at 10.4 ± 0.4℃ required a longer reactivation time (defined as the time from Day 0 to the day when the maximum specific AnAOB is reached) for both sludge types (seven days at 10℃ vs. five days at 15°C). For the floc-based reactor, the specific AnAOB activity followed the same trend as the 15°C reactors, which a gradual decrease of activity after seven days. For the granules, the specific AnAOB activity decreased after 20 days of reactivation. The

AnAOB activity was neglectable in the initial ten days in reactors at 3.9 ± 0.2℃. In the following 20 days, nearly no specific AnAOB activity was detected.

Rapid recovery of the AnAOB activity is one of the essential factors for successful bioaugmentation. With decreasing temperature, the recovery percentage (i.e., the measured AnAOB activity divided by the expected activity (after Arrhenius-based temperature correction, $\theta = 1.10$)) decreased. For the flocs, 41 ± 5.7% of AnAOB activity could be recovered at 15℃ and 32 ± 6.7% at 10℃ within a month (Fig.3). The granules showed a similar trend with a recovery percentage of 56 ± 4.9% to 41 ± 3.0% at respectively 15℃ and 10℃.

As opposed to the research reactivating the stored anammox sludge at high temperature (33 – 37℃) where the specific AnAOB activity could be completely recovered within a month (Ali et al., 2014; Magrí et al., 2012), only a certain percentage of AnAOB activity was restored at a lower temperature in the current study.  Several reasons attributed to that. Firstly, the key enzyme activities in AnAOB (e.g., nitrite reductase (*nir*)) were probably suppressed at low temperatures which might contribute to the inhibition of the anammoxosome and the cell nucleus, which in turn limit the specific AnAOB activity (Zhang et al. 2019). Secondly, the AnAOB growth rate at 10 – 15℃ had a ± 8-time decrease compared to that at 33 – 37℃ (after Arrhenius-based temperature correction with the temperature difference of 22℃). There was a lack of sufficient growth at the reduced temperatures. Thirdly, due to the decay during the sludge storage (biomass and AnAOB activity decay rates were 0.0041 and 0.002 $d^{-1}$, respectively for floccular sludge, and the values were 0.003 and 0.0013 $d^{-1}$ for granular sludge (Zhu et al., 2022)), the longer biomass preservation time of the present research compared with other studies (180 days vs. 30 – 150 days in Ali et al.

(2014) and Magrí et al. (2012)) might result in the lower recovery percentage. Moreover, the Arrhenius coefficient might not be constant at low temperature (< 15℃) due to possibly two rate-determining enzymes with different temperature optima (Lotti et al., 2015c), which could also affect the expected AnAOB activity calculated in the present research.

**3.2.2. Higher biomass retention was achieved in granules-based reactors**

As shown in Fig. 2B, at 15℃°, the biomass concentration in the flocs-based reactor dropped from 2.3 to 1.2 g VSS $L^{-1}$ over 30 days compared to 1.7 to 1.5 g VSS $L^{-1}$ for the granular-based reactor. At 10℃, ± 49% of the bioaugmented (on Day 0) flocs were washed out within a month. But for the granules, the biomass concentration remained stable (3.0 ± 0.2 g VSS $L^{-1}$). Reactivation at 4℃ caused more sludge washout from the reactors compared to 15℃ and 10℃. That was consistent with Guo et al. (2010), who found the cold temperature could impact the settling characteristics of biomass during the research at varying temperatures (5-30℃). Regarding the biomass retention percentage (Fig.3B), for granules, 84.9 ± 3.4% to 86.6 ± 4.1% of sludge could be retained, whereas the values were only 49.7 ± 5.4% to 53.1 ± 1.5% for flocs. The stable biomass concentration and AnAOB activity (Section – 3.2.1) after 30 days reactivation suggested partial inoculated bacteria acclimated and proliferation in the receptor system successfully.

Compared with the pre-adapted strain or consortia used in other bioaugmentation studies (El Fantroussi and Agathos, 2005), the stored AnAOB sludge (both flocs and granules) could probably be bioaugmented directly into the receptor system without any pretreatment steps, which is then easy-operation and economic-effective.

Negligible AnAOB activity detected at 4℃ would hinder this concept using at lower temperatures (e.g., lower than 5℃) since the AnAOB activity could not be recovered even though enough biomass was retained in the system (49.7% and 84.9% respectively for floccular and granular sludge).

### 3.3. The abundance of AnAOB genera can be promoted at 15℃ and 10℃

This section had the objective to assess the microbial community shift as the relative AnAOB abundance is essential for biomass reactivation under low temperatures. Approximately 95% of the metabolically active community abundance was classified into 8 phyla (Fig. 4A/B), of which Planctomycetota, Proteobacteria, Bacteroidetes, and Chloroflexi were the most abundant phyla in both floccular and granular sludges. For both stored sludges, the relative abundance of Planctomycetota in the reactors increased at higher reactivation temperature (e.g., 49%, 40%, and 38% for floccular sludge at 15, 10, and 4℃, respectively). Hence, too low temperatures (e.g., 4℃) could not achieve reactivation of the stored anammox sludge. The relationship between the relative abundance of Planctomycetota and reactor temperature was in line with Akaboci et al. (2018).

Three representative AnAOB genera (Candidatus *Brocadia*, Candidatus *Kuenenia*, and Candidatus *Jettenia*) were detected in the stored sludge. Candidatus *Brocadia* dominated the community in floccular and granular sludge, with a relative abundance (expressed relatively over the total community) of 37% and 24%, respectively, representing approximately 99% of Planctomycetota (Fig. 4C/D). After 30 days' reactivation, Candidatus *Brocadia* was still dominant at 15℃ with a relative abundance of 48% and 54% for flocs and granules, respectively (versus 38% and 42% at 10℃, respectively). The relative abundance of the other AnAOB genera also increased in all systems but still with a low level (e.g., Candidatus *Kuenenia* increased to 0.7% from almost 0 in 15℃ flocs-based reactor). For the 4°C reactors, the relative abundance of three AnAOB genera was similar to the values before reactivation for both sludges. This contrasted with Reeve et al. (2016), who found no clear community shifts during the reactivation period. The difference might attribute to the recovery temperature (28℃ vs. 4 – 15°C in the present research) and the biomass source (pilot-scale system with relatively simple influent vs. full-scale system with complex influent composition). The relative abundance of the denitrifier-related genus, *Denitratisoma*, was present in reactors. This is likely due to the occurrence of endogenous respiration, releasing COD for the heterotrophic bacteria (Contreras et al., 2011).

Even though the reactivation temperature (15 and 10℃) was much lower than their optimum (35°C) (Hu et al., 2013), AnAOB could still be enriched in the system compared to the other bacteria present in sludge. That was probably attributed to the low DO and COD concentration conditions (compared to the influent of their parent reactors) which decreased the growth rates of certain bacteria (e.g., heterotrophic bacteria). The higher AnAOB recovery percentage at 15℃ compared to 10℃ (Section – 3.2) was in line with the higher relative abundance of the dominant genus. Even after 30 days' reactivation, the community in all four reactors was still dominated by Candidatus *Brocadia*. This competitive advantage of Candidatus *Brocadia* over other AnAOB at low temperatures was also reported by Hendrickx et al. (2014). At 4℃, the community composition was relatively stable in both floccular- and granular- based reactivation reactor. All the bacteria present in the sludge might have a lower metabolic activity at that temperature, which led to the slow changes in the most abundant genera in the short term.

The Shannon, Chao1, and coverage indices were used to reflect microbial diversity. The goods coverage of all samples was above 0.995, indicating that the sequences in the samples were detected with high probability and the results of diversity analysis had high reliability and authenticity. In addition, Shannon and Chao1 indices were generally showed the same trend of decreasing as dropping off the reactivation temperatures, which demonstrated the higher microbial diversity. Under relatively high-temperature conditions, there is more opportunity to reconstitute bacteria that favor the settlement of AnAOB. This was in line with Wang et al. (2022), who reported that the community could resist the effect of low temperature by increasing the diversity of microbes. But the dominant species were weakened. Compared to the stored sludge, microbial diversity decreased after reactivation in the 10°C and 15°C reactors. Certain species were probably removed from the system by selection (e.g., washout due to lower SRT and worse settling properties) and competition caused by temperature (metabolism and growth rate), thereby, triggering changes in diversity. Moreover, compared to the complex influent in a full-scale STP system, the simple influent composition might eliminate certain bacteria (e.g., heterotrophic bacteria, due to the absence of COD). That possibly explained the decrease of the diversity in the process of anammox sludge reactivation. Therefore, the enhanced AnAOB activity due to activity reactivation was accompanied by a more specialized (less diversity) and dominant community for anammox, which promoted a more efficient anammox process.

### 3.4. The stored summer sludge bioaugmentation concept is economic-effective

The proposed ‘bioaugmentation with stored summer sludge for winter anammox assistance’ concept was shown in Fig.5A.

The average temperature of the mainstream wastewater changed between 11.6℃ and 21.3℃ during the whole year (Nieuwveer STP). We assume to harvest sludge when temperatures are higher than 20℃ and bioaugment sludge when temperatures are below 13℃. A year, thus, consists of four periods: (P – 1) sludge harvest (July, August, and September), (P – 2) sludge storage (October, November, and December), (P – 3) sludge bioaugmentation (January, February, and March), and (P – 4) idle (April, May, and June) (Fig.5B). Thus, the average biomass preservation period was assumed to 6-month on average.

The biomass concentration in the mainstream system is assumed to be the same as that in summer (2.3 g TSS $L^{-1}$), due to the low growth rate of AnAOB sludge in the winter period (0.02 $d^{-1}$ at 20 ℃ and only 0.005 $d^{-1}$ at 10 ℃ (Lotti et al., 2014)). The increase in biomass concentration in winter could only be attributed to bioaugmentation. In winter, it was assumed that no biomass growth and loss occurred in mainstream. The cost assessment of this concept was shown in Table – 1. The estimated OPEX was 0.031-0.040 € $IE^{-1}$ $year^{-1}$. This was mainly attributed to the base consumption during storage. According to our previous research, the pH during biomass storage should be maintained within 7.2-8.0 to avoid enhanced decay rates due to low pH (Sun et al., 2020).

The CAPEX, which was the cost for the construction of the storage tank (cement tank (Meerburg, 2016)), was 0.025-0.069 € $IE^{-1}$ $year^{-1}$. Thus, the total cost of this concept was 0.056-0.109 € $IE^{-1}$ $year^{-1}$. That was neglectable (0.19-0.36%) compared to sewage treatment cost (30 € $IE^{-1}$ $year^{-1}$ in high-income EU countries) (Zessner et al., 2010). Due to the generic unit '€ $IE^{-1}$ $year^{-1}$' being proposed in the present research, the cost assessment results could be extrapolated to other STPs located in the high-income EU countries in temperate regions (e.g., the Netherlands). Additionally, to extrapolate the concept to other temperate regions (avoid the influences of socioeconomic status), the percentage of concept cost to the total cost of STP was also put forward in this study, which showed the cost-effectiveness of the concept more visually.

The present research assumptions are based on a system where mainstream PN/A is already implemented full-scale. The incoming bCOD/N ratio is expected lower than 2, which is in line with the expected ratio of pre-treated sewage (e.g., high-rate activated sludge) (Laureni et al., 2019; Malovanyy et al., 2015). That means maximally 50% of the total nitrogen could be removed by denitrification, and the residual nitrogen removal (to meet the discharge standards) might be attributed to anammox. Lower bCOD/N ratios mean even more total nitrogen removal via PN/A. The produced sludge in summer (436 ton TSS) is enough for the 'winter bioaugmentation concept' (require 239-306 ton TSS) (Table – 1). Based on that, 3677-10200 $m^3$ of excess sludge needs to be stored (this range comes from the different biomass concentrations during storage: 30 g TSS $L^{-1}$ (the tested value in pre-test) versus 65 g TSS $L^{-1}$ (the feasible B-sludge thickening level in Nieuwveer STP)).

### 3.5. Outlook

Maintaining sufficient AnAOB at low temperatures is still one of the main challenges (Kumwimba et al., 2020; Liu et al., 2020). Even though some studies achieved high nitrogen removal efficiency at relatively low temperature, e.g., Ma et al. (2013) (e.g., 2.3 kg N $m^3$ $d^{-1}$ under 16°C in up-flow anaerobic sludge blanket reactor), there was still a drop in temperature during winter which would result in the decrease of AnAOB activity. So, more biomass is needed in winter (e.g., 77% more sludge is required at 10 °C versus 16°C based on Arrhenius with $\theta = 1.10$) to treat the incoming loading rate.

Due to the low growth rate of AnAOB (Strous et al., 1998), the ‘winter bioaugmentation concept’ proposed in the present research should be a feasible strategy to solve that. Except for the low temperature, achieving stable partial nitritation (provide stable nitrite for anammox) is another challenge for mainstream anammox application, which also needs to be tested next. In addition, rainy seasons are also challenging for STP due to the low nitrogen concentration, high flow rate, and low HRT. In this case, the sewage treatment efficiency is reduced and there is a risk of biomass washout. This might also be mitigated with sludge supply to the mainstream with stock biomass.

According to the authors’ knowledge, this was the first time that this novel concept was studied. Overall, the present study provided a new insight to alleviate the lower activity and slower metabolism in winter. Nonetheless, further additional studies on substantial areas are required before it is globally used in the application. First, different bioaugmentation dosage percentages (amount of the biomass in the receptor system) and inoculation frequencies are also interesting to explore. Repeating bioaugmentation (gradually increasing the sludge concentration in the reactor) on a weekly or monthly basis could be an alternative strategy to guarantee the ‘critical biomass concentration’ in the reactor since the temperature decreased gradually during autumn and winter. Second, avoiding the supplemented sludge loss from the system was another challenge (e.g., immobilization, flocculant). According to the present research, granular sludge was a good option, but it is not always available in the application. The use of a screen might be the option to retain granules in the mainstream. Third, after this first proof of concept at 20°C, it is recommended to test this at 15°C or lower temperature in future experiments.

## 4. Conclusions

The potential for winter bioaugmentation with the stored summer sludge was demonstrated for the first time. The effect of a 5.1℃ temperature decrease could be alleviated effectively by the stored sludge bioaugmentation (118-220% of initial biomass). Moreover, the stored-sludge could be reactivated efficiently (AnAOB activity recovery and biomass retention) after bioaugmented into low-temperature reactors. Additionally, a specialized community was formed in the system (less diversity with a higher relative abundance of dominant genera (Candidatus *Brocadia*)). In the end, this concept revealed the economic feasibility of the application. It presents a new countermeasure to enhance the nitrogen-removal performance on winter days.

E-supplementary data of this work can be found in the online version of the paper.

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# Table and Figure Captions

**Fig. 1** – Reactor performance in flocs system (A, C, E) and granules system (B, D, F). A, B: Effluent nitrogen concentrations and temperature; C, D: Nitrogen conversion rate; E, F: Biomass concentration and SRT.

**Fig.2** - Reactivation of stored sludge (six months) under different temperatures. A, B: 15 ℃; C, D: 10 ℃; E, F: 4 ℃.

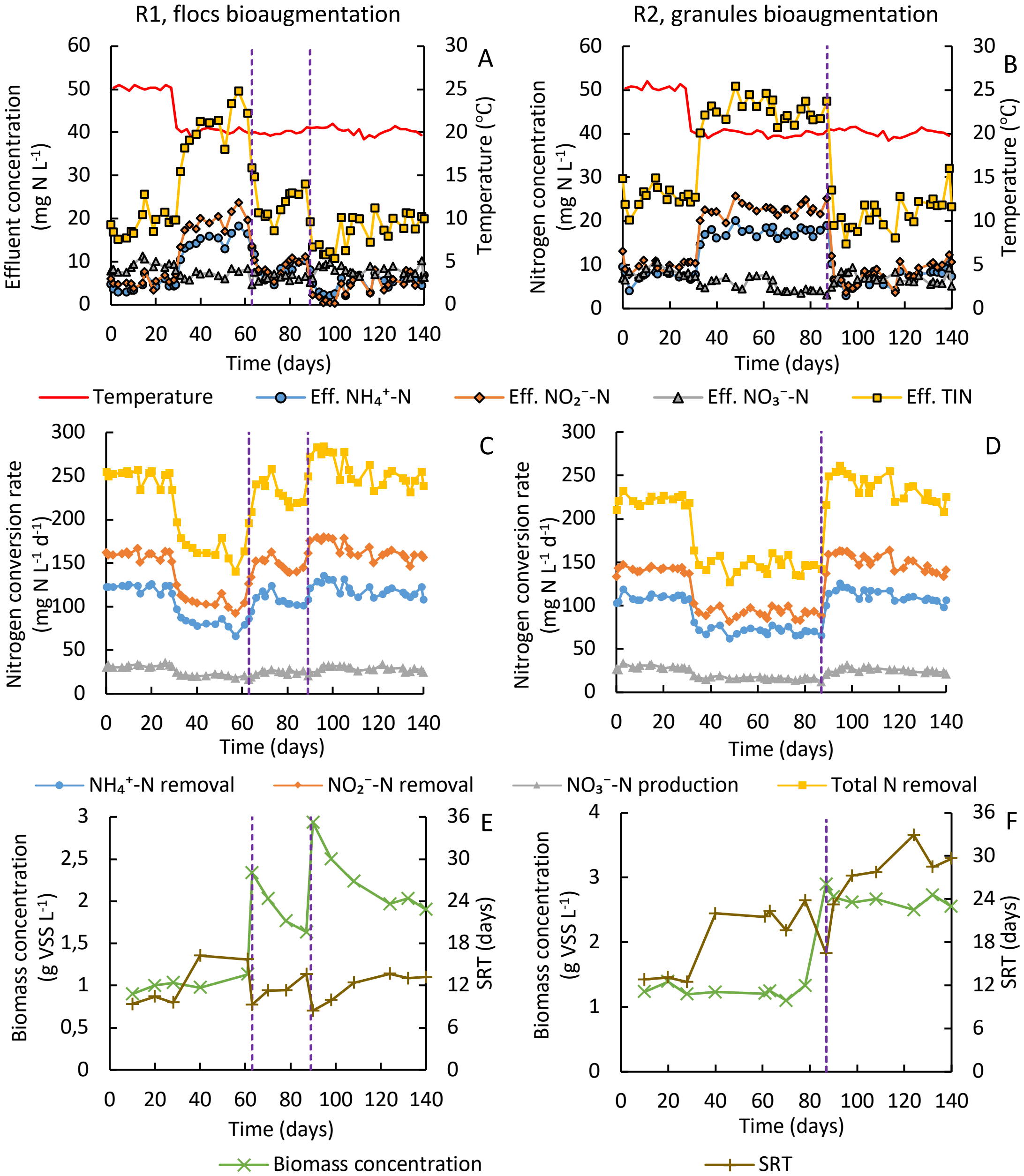
**Fig.3** - The percentage of AnAOB activity recovery and biomass retention under different temperatures. A, AnAOB activity recovery percentage; B, biomass retention percentage.

**Fig.4** - The microbial community during the stored sludge reactivation at phyla (A, B) and genus levels (C, D), expressed relatively over the total community (A, C: Flocs-based reactor; B, D: Granules-based reactor). Only the dominant nitrogen removal related bacteria (AnAOB (orange), AerAOB (green), NOB (blue), and denitrifying bacteria (purple)) were shown.

**Fig.5** - Schematic of the winter bioaugmentation concept (A) and the potential harvest/preservation/bioaugmentation period (B). P – 1, sludge harvest; P – 2, sludge preservation; P – 3, sludge bioaugmentation; P – 4, idle.

**Table – 1** The cost assessment of the concept ‘bioaugmentation with stored summer sludge for winter anammox assistance’.

**Fig. 1**



R1, flocs bioaugmentation
R2, granules bioaugmentation
A
B
C
D
E
F
Effluent concentration (mg N L-1)
Nitrogen concentration (mg N L-1)
Temperature (°C)
Time (days)
Temperature
Eff. NH4+-N
Eff. NO2−-N
Eff. NO3−-N
Eff. TIN
Nitrogen conversion rate (mg N L-1 d-1)
NH4+-N removal
NO2−-N removal
NO3−-N production
Total N removal
Biomass concentration (g VSS L-1)
SRT (days)
Biomass concentration
SRT

**Fig.2**

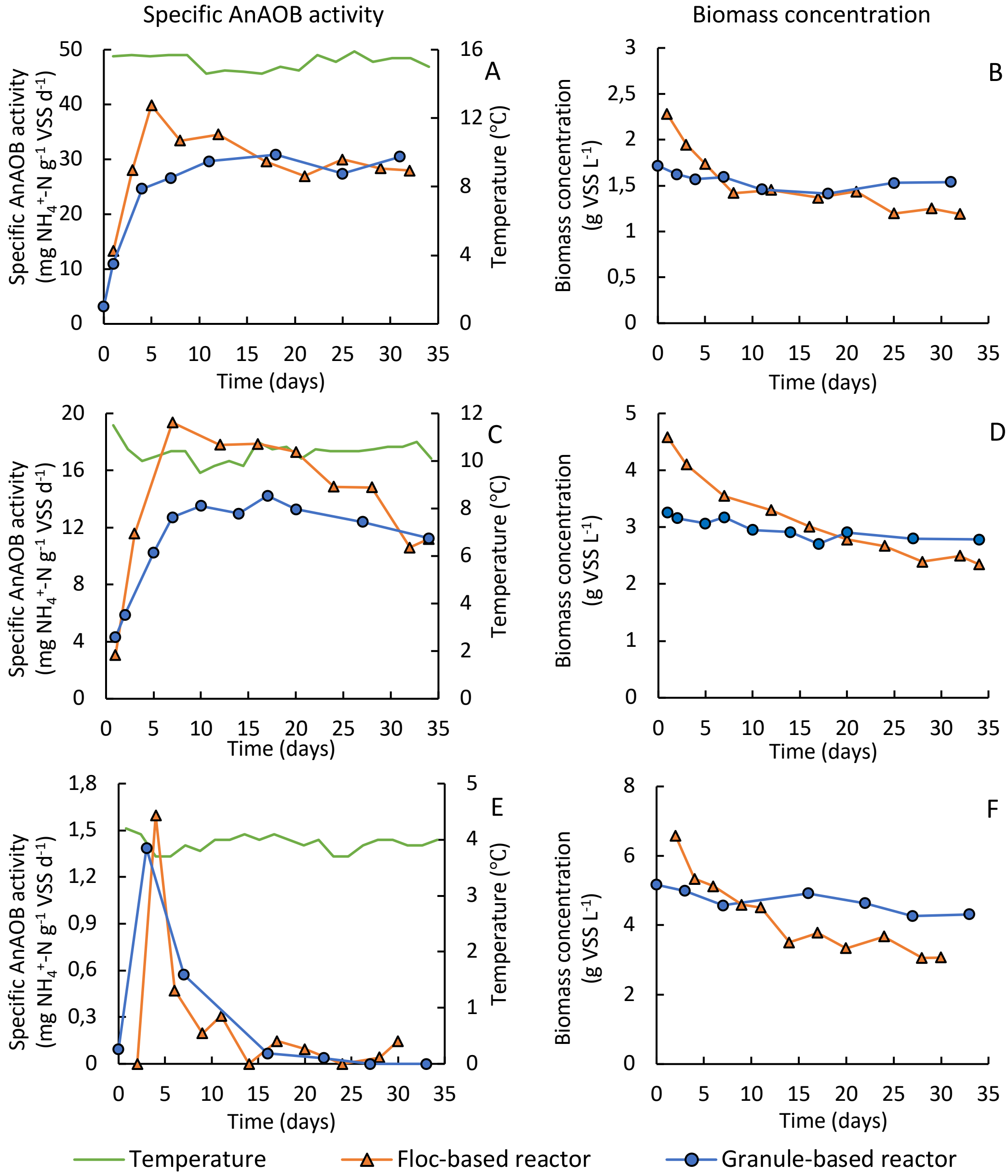

Specific AnAOB activity
Biomass concentration
A
B
C
D
E
F
Specific AnAOB activity
(mg NH$_4^+$-N g$^{-1}$ VSS d$^{-1}$)
Temperature (°C)
Biomass concentration
(g VSS L$^{-1}$)
Time (days)
Temperature
Floc-based reactor
Granule-based reactor

**Fig.3**

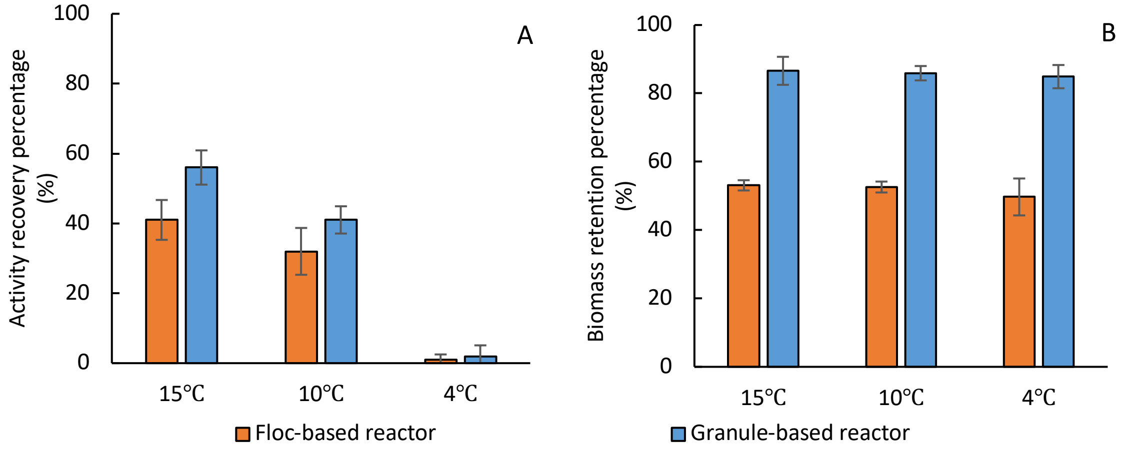

A
Activity recovery percentage (%)
100
80
60
40
20
0
15°C
10°C
4°C
B
Biomass retention percentage (%)
100
80
60
40
20
0
15°C
10°C
4°C
Floc-based reactor
Granule-based reactor

**Fig.4**

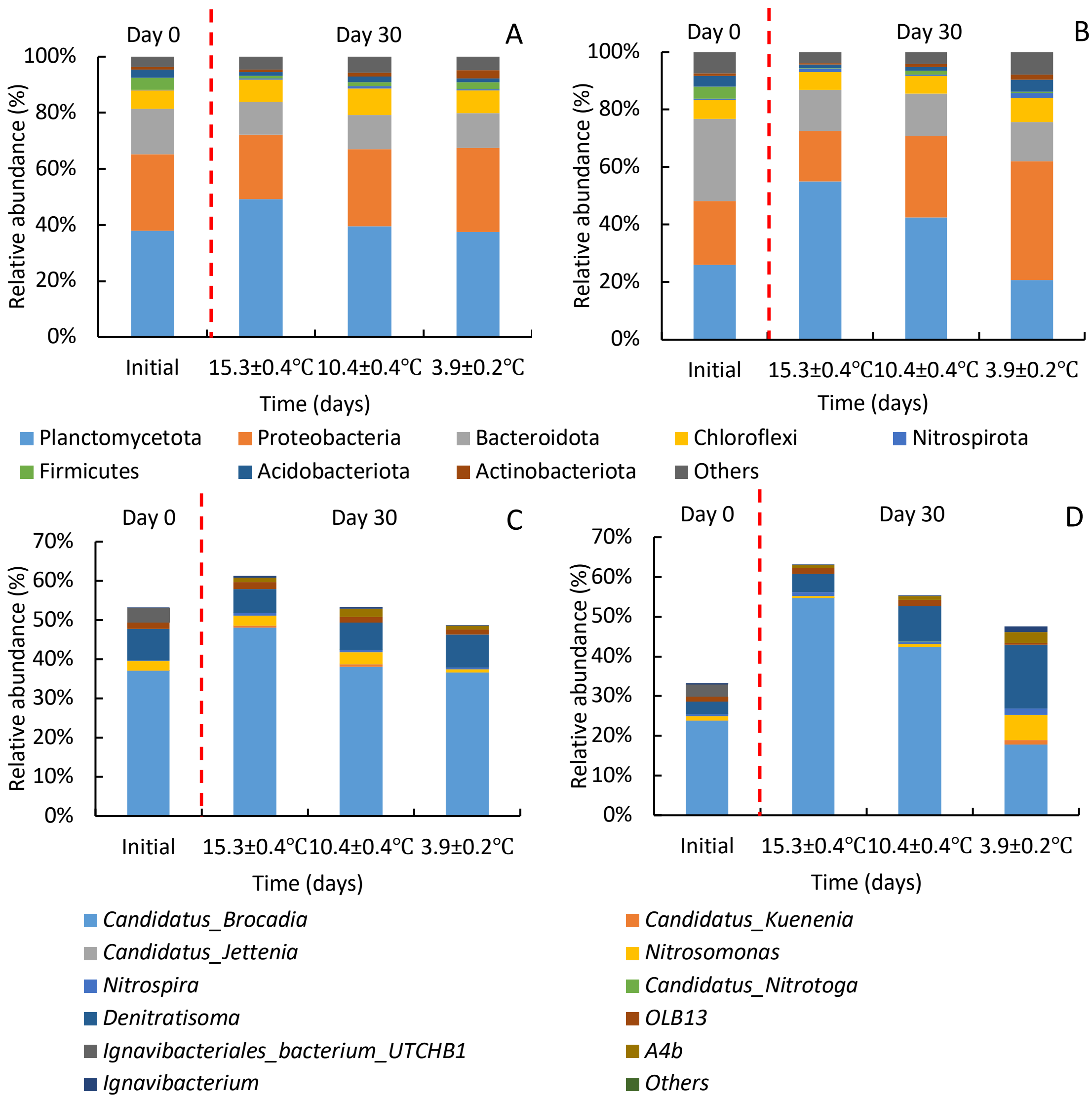
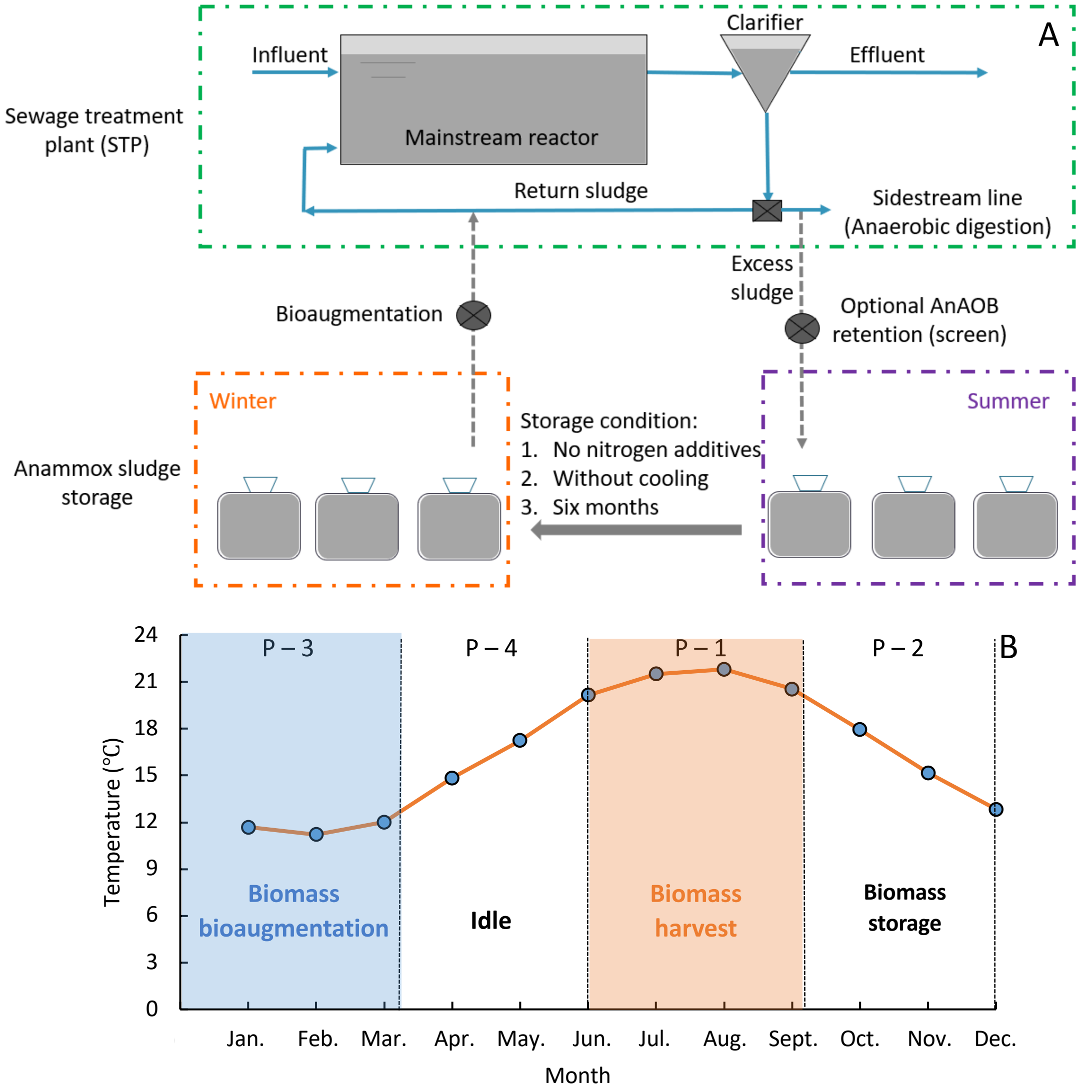

A
B
C
D
Day 0
Day 30
Relative abundance (%)
100%
80%
60%
40%
20%
0%
70%
50%
30%
10%
Initial
15.3±0.4°C
10.4±0.4°C
3.9±0.2°C
Time (days)
Planctomycetota
Proteobacteria
Bacteroidota
Chloroflexi
Nitrospirota
Firmicutes
Acidobacteriota
Actinobacteriota
Others
*Candidatus_Brocadia*
*Candidatus_Kuenenia*
*Candidatus_Jettenia*
*Nitrosomonas*
*Nitrospira*
*Candidatus_Nitrotoga*
*Denitratisoma*
*OLB13*
*Ignavibacteriales_bacterium_UTCHB1*
*A4b*
*Ignavibacterium*
*Others*

**Fig.5**



A
Influent
Mainstream reactor
Clarifier
Effluent
Sewage treatment plant (STP)
Return sludge
Sidestream line (Anaerobic digestion)
Excess sludge
Bioaugmentation
Optional AnAOB retention (screen)
Winter
Summer
Anammox sludge storage
Storage condition:
1. No nitrogen additives
2. Without cooling
3. Six months
B
P – 3
P – 4
P – 1
P – 2
Biomass bioaugmentation
Idle
Biomass harvest
Biomass storage
Temperature (°C)
0
3
6
9
12
15
18
21
24
Jan.
Feb.
Mar.
Apr.
May.
Jun.
Jul.
Aug.
Sept.
Oct.
Nov.
Dec.
Month

**Table – 1**

| | Parameters | Value | Unit |
|---|---|---|---|
| Stored sludge | Required sludge | 239-306 | ton TSS |
| | Available sludge in summer (90 days) [a] | 436 | Ton TSS |
| | Storage volume | 3,677-10,200 | $m^3$ |
| Cost | OPEX (NaOH) | 0.031-0.040 | € $IE^{-1}$ $year^{-1}$ [b] |
| | CAPEX (Cement tank) | 0.025-0.069 | € $IE^{-1}$ $year^{-1}$ |
| | Total cost | 0.056-0.109 | € $IE^{-1}$ $year^{-1}$ |
| | Percentage of concept cost to the total cost of STP [c] | 0.19-0.36 | % |

Note: a, AnAOB yield is 0.122 g VSS $g^{-1}$ $NH_4^+$-N (Lotti et al., 2015a)

b, IE represents ‘inhabitant equivalents’.

c, The sewage treatment cost in STP is ~30 € $IE^{-1}$ $year^{-1}$ in high-income countries (e.g., Austria)