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Abstract: Bioaugmentation with summer harvested sludge during winter could compensate for the bacterial activity loss but requires that the sludge activity can be restored after storage. This study assesses the effect of temperature and redox addition during the storage of partial nitritation/anammox (PN/A) granular and floccular sludge over 180 days. Anoxic storage conditions (in the presence of nitrite or nitrate and the absence of oxygen) resulted in a loss of 80 – 100% of anammox bacteria (AnAOB) activity capacity at 20℃ and 4℃, while anaerobic conditions (without oxygen, nitrite, and nitrate) lost only 45 – 63%. Storage at 20℃ was more cost-effective compared to 4°C, and this was confirmed in the sludge reactivation experiment (20℃). Furthermore, AnAOB activity was negatively correlated with the electrical conductivity level ($R^2 > 0.85$, $p < 0.05$). No significant differences were found in the activity capacity of aerobic ammonia-oxidizing bacteria (AerAOB) under different storage conditions ($p > 0.1$). The relative abundance of dominated AnAOB (Candidatus *Brocadia*) and AerAOB genera

(*Nitrosomonas*) have remained in both sludges. Concluding, preserving PN/A biomass without cooling and nitrite or nitrate addition proved to be a cost-effective strategy.

Keywords: Deammonification; Decay; Mainstream; Redox; Sewage treatment; Sludge characteristics

1. Introduction

Around 25 years after conceptually proposing the importance of anaerobic ammonia oxidation (anammox) bacteria (AnAOB) in energy-positive sewage treatment (Mulder et al., 1995), a current hot topic in wastewater treatment is the implementation of so-called mainstream partial nitritation/anammox (PN/A) or deammonification. Due to the lower energy and carbon demand as well as the lower N_2O emission compared to conventional nitrification/denitrification, it is an economical and environmental-friendly process (Agrawal et al., 2018; Ali et al., 2016) which consists of aerobic ammonium-oxidizing bacteria (AerAOB), that oxidize roughly half of the NH_4^+ to NO_2^- ('partial nitritation') and AnAOB, that oxidize the produced NO_2^- and residual NH_4^+ to N_2 ('anammox') (Agrawal et al., 2018).

PN/A systems have been successfully implemented in the reject water line (sidestream) all over the world (Lackner et al., 2014). Compared to the sidestream, which has a higher temperature (15 – 20℃ higher) and nitrogen concentration (more than 20 times), achieving efficient nitrogen removal in the mainstream is much more challenging. Especially during the winter period, as the mainstream temperature could drop to about 10 – 12℃ in western Europe (20 – 22℃ in summer) according to the data in Nieuwveer sewage treatment plant (STP) (Breda, the Netherlands). This temperature decrease

45 significantly reduces the activity of functional bacteria, especially for the AnAOB. Lotti
46 et al. (2014) reported a growth rate of 0.02 d^{-1} at 20°C and only 0.005 d^{-1} at 10°C. As an
47 extended sludge retention time in winter will not be sufficient because of the low growth
48 rates, this problem may be tackled by storing excess PN/A sludge, mainly harvested over
49 summer, and reinoculated this in winter.

50 Long-term preservation to maintain enough and activated sludge for at least 180 days
51 is essential for the successful application of this novel concept. Different commonly used
52 storage methods were deemed unfeasible for this bioaugmentation concept.
53 Cryopreservation (-20 ℃, -80 ℃ and -200 ℃) with various cryoprotective agents (e.g.,
54 dimethyl sulfoxide) that prevent activity loss during sludge or culture collections storage
55 has been extensively described (Rothrock et al., 2011; Viancelli et al., 2017), the cooling
56 process and cryoprotective agents are expensive which limit its application at full-scale.
57 Likewise, immobilizing technique (Ali et al., 2014), consortia transformation strategy (Shi
58 et al., 2020), and protective agents addition (e.g., hydrazine, glycerol, skim milk)
59 (Ganesan and Vadivelu, 2020; Rothrock et al., 2011; Vlaeminck et al., 2007) is also not
60 feasible for full-scale application because of the operational complexity and high cost,
61 even though many of them have proved useful.

62 In contrast, substrates or redox spikes are expected to be applicable for the bio-
63 augmentation concept since they could well retain anammox activity and low operation
64 cost. The addition of redox buffers (nitrite or nitrate) during sludge preservation was
65 investigated, as it may be effective in preventing sulfate reduction, which produces H_2S,
66 harmful to biomass (Vlaeminck et al., 2007). Wang et al. (2016) demonstrated that about

30% of AnAOB activity was maintained after 180 days of preservation at 35℃ with weekly supplying NH_4^+-N and NO_2^--N (50 mg N L^{-1} respectively). Ali et al. (2014) stored anammox sludge with 3 mM molybdate (inhibitor of sulfate reduction) and a regular supply of NH_4^+ and NO_2^- at room temperature and maintained 65% of the activity after five months of storage. Considering that both NH_4^+ and NO_2^- are substrates for AnAOB, periodical adding them could well avoid starvation.

Besides redox stabilization, the low temperature has also been proved beneficial for anammox sludge preservation. Vlaeminck et al. (2007) reported that 4°C without nitrate storage addition was a recommended strategy for PN/A biofilm storage over five months (maintained 55% AnAOB activity). Xing et al. (2016) also revealed that anammox granules preserved at 4 ℃ without substrate addition had a lower decay rate and higher nitrogen removal capacity after storage compared to granules stored at 20°C.

To evaluate the feasibility of the novel winter bioaugmentation concept, essential information about PN/A sludge storage and reactivation is currently still lacking. Firstly, various preservation methods have been applied in several studies, but there is no study comparing all these different preservation methods, which is essential for a fair comparison, as results can be influenced by the microbial community composition and sludge types. Secondly, most studies only assess the preservation of anammox or nitrifying sludge, not PN/A sludge. Thirdly, room or lower temperature reactivation is important since it has more implementation potential for its lower required amount of energy (heat), whereas sludge reactivation has only been widely studied at the optimum temperature. The preserved sludge in previous studies could be reactivated at the

optimum temperature (30 – 40 ℃) from days to weeks (Ali et al., 2014; Viancelli et al., 2017).

The overall objective of this study is to find a cost-effective, and simple operation strategy to store PN/A sludge on a large scale. Therefore, three parts were examined: i) the effect of temperature and redox stabilization over 180 days' storage of PN/A granules and flocs derived from full-scale STPs, ii) the potential of the stored biomass reactivation after 180 days, and iii) the OPEX (operating expenses or expenditure) and cost-effective analysis of different preservation strategies. Over the biomass storage, physicochemical characteristics and community composition are closely monitored. The finding will have good guiding significance for the storage of PN/A sludge on a large scale and provide the cost-effective biomass source for the concept of winter bioaugmentation with the stored summer sludge.

2. Materials and methods

2.1. PN/A sludge source and characterization

To verify that the strategy proposed in this study was feasible and stable for different sludge, floccular sludge (with a biomass concentration of 9.0 ± 0.2 g VSS L^{-1}) and granular sludge (13.6 ± 0.9 g VSS L^{-1}) were collected from full-scale sidestream (990 m^3, Breda, The Netherlands) and potato-processing wastewater (600 m^3, Olburgen, The Netherlands) PN/A installations, respectively. The characteristics of both sludges are shown in Table – 1.

2.2. Sludge storage procedure and strategies

The sludge was stored under different storage conditions for 180 days in 5 L plastic

containers (0.4 L of headspace, with a small opening to avoid pressure build-up by gas (e.g., N_2 and H_2S) production). Four preservation strategies were tested (Table – 2): 1) no N spike, i.e., sludge storage under anaerobic conditions (without oxygen and redox buffer), 2) NO_2^-+NH_4^+ spikes (substrates for AnAOB), 3) NO_3^-+NH_4^+ spikes (Due to the ammonium produced by biomass decay was always consumed in nitrate strategy from our pre-test. In the present research, ammonium was added to ensure adequate supply.), and 4) NO_3^- spikes (redox buffer to alleviate sulfate reduction). Two preservation strategies (i.e., 'no N spike' and 'NO_2^-+NH_4^+) were also duplicate tested at 4°C for floccular sludge. Only floccular sludge was chosen to test the strategy of 4℃ because the activity maintenance had already been proved in the previous study (Vlaeminck et al., 2007), and the high cooling cost was also not expected to be applied in the biomass storage on the large scale. The concentration of ammonium, nitrite, and nitrate was maintained between 30 – 180 mg N L^{-1} by regular addition of NH_4Cl, $NaNO_2$, and $NaNO_3$, respectively. The determination of NO_2^--N concentration is based on previous research (Talan et al., 2021). For NH_4^+-N and NO_3^--N, their concentrations were consistent with NO_2^--N since no inhibition occurred at the concentration lower than 200 mg N L^{-1} (Strous et al., 1999; Zhu et al., 2017). All storage experiments were performed in duplicate, and the mean values are reported.

During storage, the pH was controlled manually within 7.2 – 8.0 by 1 M HCl and NaOH addition. The dissolved oxygen (DO) concentration was lower than 0.01 mg O_2 L^{-1} in all the storage containers. The storage vessels were mixed five times per week.

2.3. Single and consecutive batch activity tests

133 **2.3.1. Single batch test: the maximum potential activities determining**

134 Batch activity tests were performed to determine the maximum potential activities of
135 AnAOB every 30 days. After mixing, about 20 mL of sludge was harvested from each
136 container and stored at 20 ± 1℃ for one day to make sure that all the batch tests were
137 carried out at the same temperature. The sludge was washed four times and diluted to
138 a biomass concentration of 1.0 g VSS L^{-1} with a buffer solution containing only $NaHCO_3$
139 (0.4 g L^{-1}) and trace elements (1 ml L^{-1}) (Van de Graaf et al., 1995). Ammonium and nitrite
140 (50 mg N L^{-1}) were spiked in all Erlenmeyer flasks. The AerAOB and NOB activity was
141 determined in flasks exposed to the air, whereas the flasks to determine AnAOB activity
142 were sealed with rubber stops after 15 min flushing with N_2 (to provide anoxic
143 conditions). The flasks were incubated on a shaker (200 rpm) at 20 ± 1℃, and pH was
144 adapted to 7.5 (with HCl) at the start of the tests. The biomass concentration was only
145 measured at the start of each test. DO and pH levels were also followed during the whole
146 test. The DO concentrations for aerobic tests were higher than 7.0 mg O_2 L^{-1} and pH
147 values were around 7.5. All the batch tests were performed in duplicates, and the
148 average values were reported. To monitor the nitrogen conversion, samples were taken
149 periodically (every hour) and stored at 4°C until analysis. The maximum specific
150 anammox activity was determined from the maximum slope of the curve indicated by
151 the decrease of NH_4^+-N over time, divided by the biomass concentration in the flask.

152 **2.3.2. Consecutive batch test: bacterial activity reactivation**

153 After 180 days, consecutive batch tests (i.e., 20 spikes over 7 days) were performed to
154 assess the activities' reactivation. Sludge was pre-treated and spiked every 12h (after

155 washing) to avoid substrate limitation or accumulation. The substrate conversion rate
156 was quantified every other cycle (7 in total) by measuring the NH_4^+-N, NO_2^--N, and NO_3^--
157 N concentration. The biomass concentration was only determined at the start of each
158 test. The activity recovery percentage was defined as the maximum activity obtained in
159 these seven days divided by the initial activities (before storage).

160 **2.4. Analytical procedures**

161 Liquid and microbial samples were taken periodically from the storage containers
162 (mixed before sampling). In addition, during the batch tests, liquid samples were taken
163 from the Erlenmeyer flasks regularly. After filtering by 0.2 μm syringe filter (CHROMAFIL
164 Xtra PVDF) and storing at 4°C, NH_4^+-N, NO_2^--N, and NO_3^--N were measured with a San^{++}
165 Automated Wet Chemistry Analyzer (SKALAR, the Netherlands). H_2S in liquid was
166 measured using Spectroquant test kits (Merck, Germany). The biomass concentration
167 was followed over time using volatile suspended solids (VSS) measurements (APHA,
168 2005). Handheld meters were used to monitor pH, electrical conductivity (EC), and DO
169 concentration (Hach HQ30d, USA). The detailed information about extracellular
170 polymeric substances (EPS) extraction and heme c measurement is shown in S – 1
171 (Supporting Information). The calculation of free nitrous acid (FNA) and free ammonia
172 (FA) are shown in S – 2 (Supporting information). The V4 region of the 16S rRNA gene
173 was sequenced for the following-up analysis by Novogene Europe (United Kingdom)
174 after the genomic DNA was extracted using a Powerfecal kit (Qiagen, Germany) in the
175 lab. The detailed method is presented in S – 3 of the Supporting Information.

176 **2.5. Bacterial activity capacity calculation**

Sludge decay during storage can be attributed to activity decay and biomass loss. As the objective of the present research is to maintain enough and activated sludge after 180 days, an accurate parameter that combines these two factors is proposed to analyze the effects of storage on the sludge: the bacterial activity capacity R_c, which is calculated using the following equation.

$$R_c = r_b * C_s * V_t \quad (Eq. 1)$$

R_c is the bacterial activity capacity [mg NH_4^+-N d^{-1}] for AerAOB/AnAOB and [mg NO_3^--N d^{-1}] for NOB; r_b is the maximum bacterial (AnAOB, AerAOB, and NOB) activity [mg NH_4^+-N g^{-1} VSS d^{-1}] for AerAOB/AnAOB and [mg NO_3^--N g^{-1} VSS d^{-1}] for NOB; C_s is the biomass concentration [g VSS L^{-1}]; and V_t is the sludge volume [L].

The decay rate (d^{-1}) of bacterial activity capacity depends on activity decay (decrease in sludge-specific activity) and cell death (decrease in biomass concentration), and is calculated according to Hao et al. (2009). The bacterial decay rates are calculated respectively as the slopes of logarithmic plots of the activity capacity of AnAOB by linear regression ($p < 0.5$).

2.6. Cost assessment

As far as the author knows, this is the first research to assess the cost-effectiveness during the PN/A sludge preservation process. Room temperature is assumed stable at 20°C, thus only the 4°C conditions need to consume electricity to cool down. During the whole biomass preservation period, the main OPEX is nitrogen compounds addition (NH_4Cl, $NaNO_2$, and $NaNO_3$), pH control (HCl and NaOH), and potential cooling (20°C →

4℃). Mixing cost is negligible compared to the other operational factors (< 0.5% of the total cost). All the OPEX is calculated based on the in-suit sludge storage.–

OPEX per specific AnAOB activity (€ d kg NH_4^+-N^{-1}) is put forward to analyze the cost-effectiveness of different biomass preservation strategies. Its calculation process is the whole OPEX (over 180 days, € ton VSS^{-1}) divided by the AnAOB activity of Day-180 (kg NH_4^+-N ton VSS^{-1} d^{-1}). The strategy which has the lowest value is regarded as the most cost-effective preservation method.

3. Results and discussion

3.1. Anaerobic conditions maintained higher bacterial activity capacity than anoxic conditions

3.1.1 AnAOB activity capacity

Over time, the AnAOB activity capacity decreased in both sludge types and all storage conditions due to a strong decrease in AnAOB activity and a mild decrease in biomass concentration in most conditions (Figure. 1).

Considering only the temperature, sludge stored at 4℃ outperformed sludge stored at 20℃ in AnAOB activity capacity retention. Comparing the 'no N spike' at 20°C and 4°C, the AnAOB activity capacity was respectively 1.95 and 2.98 kg NH_4^+-N d^{-1} on Day-180 in floccular sludge. For the 'NO_2^-+NH_4^+', this difference was even more pronounced: 0.05 and 1.15 kg NH_4^+-N d^{-1} at 20°C and 4°C, respectively. According to Adav et al. (2007) and Gao et al. (2012), the endogenous respiration and cell lysis process that microorganisms use to sustain activity during storage decelerates at low storage temperatures, which probably could explain a lower drop in biomass concentration. Taking 'no N spike' as an

example, only 7.5% of biomass concentration loss was observed at 4℃ compared to 30.7% loss at 20℃ in floccular sludge. Although there was little variation between activity preservation (59.47% at 4℃ versus 52.36% at 20℃), 'no N spike (4℃)' still maintained much higher activity capacity than that at 20℃.

For the storage strategies at 20℃, sludge stored without N spike ('no N spike (20℃)') retained a higher AnAOB activity capacity (37 – 49%) compared to sludge with redox (nitrite or nitrate) addition (0 – 20%) in both floccular and granular sludges. This is contrary to many previous studies, that is, at 20°C, regular NO_3^--N addition is the best strategy (Engelbrecht et al., 2016; Vlaeminck et al., 2007). Firstly, pH control under anaerobic conditions in the present research might benefit the AnAOB activity capacity maintenance. The sludge consumed alkalinity during biomass decay and starvation, which led to the pH decrease (Anjali and Sabumon, 2014). The low pH values could increase FNA concentration (Figure. S3) that probably inhibited AnAOB activity (Strous et al., 1999). Secondly, the glycogen (one of the intracellular polymers that microbial store in cells when there was an adequate supply of substrates) might serve only as a maintenance energy source for microorganisms (Ma and Wang, 2018). However, it possibly also be used by the dissimilatory nitrate/nitrite reduction to ammonium process (as electron donor) to generate energy under anoxic condition stress, which increased the decay rate of biomass. That could promote it to have much better resistance, tolerance, and self-adaptation to starvation.

Specific for 'NO_2^-+NH_4^+ (20℃), the addition of substrates during storage resulted in the loss of almost all AnAOB activity even though AnAOB preferentially oxidizes both

ammonium and nitrite into N_2 when they are present (Ganesan and Vadivelu, 2020). On one hand, only nitrogen compounds were provided during the preservation process while their metabolic activity requires many other elements (e.g., inorganic carbon, trace elements, etc.) (Ma et al., 2015; Van de Graaf et al., 1995). With nitrogen as the only substrate, the starvation of microbes possibly is more serious than that without nitrogen addition (Lu et al., 2018). On the other hand, the EC levels increased rapidly (e.g., 17.53 mS cm^{-1} in 'NO_2^-+NH_4^+ (20°C)' versus 5.09 mS cm^{-1} in 'no N spike (20°C)' on Day-180 for floccular sludge), which was also harmful to AnAOB (Section – 3.4). However, the higher nitrite concentration range (30 – 180 mg N L^{-1}) compared to previous studies (e.g., 50 – 70 mg N L^{-1} (Ali et al., 2014; Wang et al., 2016)) was unlikely to be the main cause for the failure of 'NO_2^-+NH_4^+' since this strategy was still unsuitable for biomass preservation even at the concentration of 50 mg N L^{-1} as studied by Ganesan and Vadivelu (2020).

In the present research, NO_3^--N was supplied as a redox buffer to prevent sulfate reduction. Low temperature (4°C) and NO_2^--N could also play the same role to suppress sulfate reduction (Vlaeminck et al., 2007). A higher H_2S concentration after 180 days of preservation was detected in the 'no N spike (20°C)' compared to the other conditions (i.e., 'no N spike (4°C)' and anoxic conditions) for both floccular (0.23 versus < 0.19 mg S L^{-1}) and granular (0.08 versus < 0.06 mg S L^{-1}) sludge (Figure. 2). This confirmed the inhibition of sulfate reduction under low temperature conditions or in the presence of NO_3^--N and NO_2^--N. However, this H_2S concentration probably did not affect the AnAOB activity according to the previous research (Jin et al., 2013).

In short, for both sludges, the AnAOB activity decay rates under anaerobic conditions

(i.e., 'no N spike') (0.0028 – 0.0041 d^{-1}) were more than twice as low compared to under anoxic conditions (i.e., 'NO_2^-+NH_4^+', 'NO_3^-+NH_4^+', and 'NO_3^-') (0.0094 – 0.0239 d^{-1}). The results were in line with Ma and Wang (2018), who got an AnAOB activity decay rate of 0.0049 d^{-1} in anaerobic conditions and 0.0129 d^{-1} in anoxic conditions after 60 days of storage. The biomass decay was much less critical than activity decay during the sludge storage under different storage strategies (3 – 20 times lower, Figure. 1G/H).

3.1.2 AerAOB and NOB activity capacity

The floccular sludge stored at 4°C ('no N spike (4°C)' and 'NO_2^-+NH_4^+ (4°C)') maintained the highest AerAOB activity capacity (75 % and 66% of the initial value, respectively) after 180 days of storage (Figure. 3). Floccular sludge stored at 20°C without N spike ('No N spike (20°C)') or with nitrate (and ammonium) spikes ('NO_3^-+NH_4^+ (20°C)' and 'NO_3^- (20°C)') also retained more than 50% of the AerAOB activity capacity. Sludge spiked with nitrite and ammonium ('NO_2^-+NH_4^+ (20°C)'), in contrast, lost more than 70% of its activity capacity. Similarly, in the granular sludge, about ~65% of the AerAOB activity capacity could be retained in all conditions, except for 'NO_2^-+NH_4^+ (20°C)' (28%).

The difference in AerAOB activity capacity between different storage strategies was similar to that on AnAOB activity capacity retention which might attribute to the change of EC levels, FNA and/or FA concentrations, and nitrite concentration range mentioned above (Section – 3.1.1). Specific for low-temperature, a high AerAOB activity capacity could be retained at 4°C (3.36 and 2.81 kg NH_4^+-N d^{-1} for 'no N spike (4°C)' and 'NO_2^- +NH_4^+ (4°C)' compared to that 2.40 and 1.30 kg NH_4^+-N d^{-1} at 20°C), which was confirmed

285 by Gao et al. (2012) who reported that 4℃ was significantly better to store aerobic
286 granules compared to room temperature.

287 NOB was more sensitive to starvation compared to AerAOB. A very low NOB activity
288 capacity was obtained after 180 days of storage except for 'no N spike (4℃)' (floccular
289 sludge) and 'NO_3^- (20℃)' (floccular and granular sludge). 'NO_2^-+NH_4^+ (20℃)' had the
290 lowest NOB activity capacity, which was followed by 'NO_2^-+NH_4^+ (4℃)' and 'NO_3^-+NH_4^+
291 (20℃)' for both floccular and granular sludge. The high FA (0 – 4 mg L^{-1}, Figure. S1) in
292 the storage vessels might lead to the low NOB activity capacity maintenance. According
293 to Vadivelu et al. (2007), a concentration of 1 – 6 mg FA-N L^{-1} eliminated the NOB in
294 PN/A sludge during reactor treatment. That was also proved by the higher NOB decay
295 rate than AerAOB at the same preservation conditions (e.g., 0.003 d^{-1} versus 0.015 d^{-1} in
296 'no N spike (20℃)' of floccular sludge). NOB suppression appeared during storage was
297 beneficial for PN/A application.

298 Compared to the decay rate of AnAOB and NOB, the AerAOB was lower. That was
299 likely because AerAOB promoted cellular adaptation to starvation by stabilizing
300 intracellular macromolecular levels (Ma et al., 2017). Apart from that, three
301 physiological advantages might also be attributed to (Geets et al., 2006): i) AerAOB has
302 stable catabolic cellular components (e.g., energy-generating enzymes), which
303 corresponded to their fast reactivation (Section-3.2) ii) AerAOB has low maintenance-
304 energy demand than other bacterial, and iii) signaling pathways involved in starvation
305 survival would be initiated in AerAOB during storage.

306 **3.2 Both AnAOB and AerAOB activity of sludge stored under anaerobic conditions**
307 **were reactivated**

308 **3.2.1 AnAOB activity reactivation**

309 The AnAOB activity was increased during the first 3 – 4 days but decreased afterward
310 (Figure. 4). The decline can be attributed to the biomass loss during the reactivation in
311 the flasks (the medium was replaced every day, resulting in an estimated biomass loss
312 of 10% – 15% per day).

313 The anaerobically preserved sludge (i.e., 'no N spike') could recover faster (activity
314 doubled after 2 – 3 days) than the anoxically preserved sludge and showed a higher
315 recovery percentage (87 – 114% versus 6 – 50%) at 20℃ (Figure. 4E/F). Without
316 substrate supply during preservation (i.e., 'no N spike'), AnAOB might still maintain
317 activity through the internal endogenous metabolism and intercellular substrates from
318 biomass decay by inducing starvation proteins (especially enzymes related to the PN/A
319 process) (Ma and Wang, 2018). In addition, the anoxic storage conditions changed
320 rapidly (pH adjustment and nitrate or nitrite addition periodically, EC increased gradually)
321 could probably harm AnAOB that affected its reactivation. Ma et al. (2017)
322 demonstrated that AnAOB sludge, starved in anaerobic conditions (during 40 h), almost
323 completely recovered after 6 h whereas sludge stored in anoxic conditions could only be
324 reactivated for 34%.

325 Even though the preserved biomass at both 4°C and 20°C could be reactivated, 4°C
326 conditions had a higher recovery percentage than 20℃ (114% versus 99% in 'no N spike'
327 and 17% versus 6% in 'NO_2^-+NH_4^+' for floccular sludge). That was in line with the

328 performance during the biomass storage that 4℃ could maintain higher AnAOB activity
329 after 180 days of preservation.
330 Sludge reactivation is essential for the full-scale application of stored sludge in the
331 concept of 'winter bioaugmentation with stored summer sludge'. Mainstream
332 inoculation and high sludge retention time (SRT) applied may be sufficient to achieve
333 AnAOB reactivation which is in order of days (i.e., 3-4 days) while the SRT is in order of
334 weeks (i.e., ~4 weeks, based on the original reactors of both sludges). So, this is a good
335 chance that dedicated the reactivation tank would not be needed in practice. Even if the
336 reactivation reactors are applied, their volume is limited. Because several batches of
337 sludge can be sequentially reactivated, not only one, which can save space.
338 **3.2.2 AerAOB activity reactivation**
339 Even though the AerAOB in principle can be obtained through the activated sludge
340 inoculating with activated sludge from other conventional mainstream sewage
341 treatment plants, the AerAOB preservation and reactivation together with AnAOB could
342 still have some benefits. Because the activated sludge inoculation would introduce high
343 levels of potential NOB activity, which could connect the NOB to AerAOB rather than the
344 AerAOB to AnAOB. That will add extra competition for nitrite. NOB suppression remains
345 the main challenge for mainstream PN/A application (Peng et al., 2020), and the yearly
346 introduction of new NOB could hamper the overall performance by disturbing the
347 microbial balance.
348 AerAOB has higher activity recovery percentage than AnAOB at 20℃ (56 – 110% vs. 5
349 – 114%) in total (Fig. 4E/F). Except for the sludge stored in ‘no N spike’ which recovered

93 – 100% of activity, more than 57% of activity was reactivated for the other sludges. For the recovery of AerAOB activity, that sensitivity difference compared to AnAOB could be explained by the 10-times higher growth rate of AerAOB (0.04 h^{-1} for AerAOB and 0.003 h^{-1} for AnAOB at 32-33℃ (Jetten et al., 2001)). Similar to the present research, Gao et al. (2012) reported that AerAOB activity could be fully restored within ten days after eight months of storage from almost 0 for all different storage conditions. The present research results indicated that even when AerAOB was stored together with AnAOB (PN/A sludge), they were still easy to be reactivated.

3.3. Dominant genera retained their relative abundance over the storage period

Since ammonium was always present in the sludge stored with only nitrate spikes (i.e., 'NO_3^- (20℃)') due to biomass decay, the storage conditions resembled the conditions in 'NO_3^-+NH_4^+ (20℃)' which resulted in a similar bacterial activity capacity. Therefore, only the samples from the 'NO_3^-+NH_4^+ (20℃)' strategy were analyzed.

The Shannon index decreased over time in the floccular sludge (e.g., from 7.125 on day-0 to 5.498 on day-180 in 'no N spike (20℃)') that the diversity of the communities decreased during storage (Table – S1). The DO levels during the whole experiments were lower than 0.01 mg O_2 L^{-1}, some species related to aerobic processes might be not adapted to the conditions were eliminated. In addition, a salinity condition was created due to a gradual increase of the EC value during preservation (Section – 3.4), which possibly also affected the diversity according to the finding of He et al. (2019). That was also the possible explanation for the higher diversity at 4℃ (lower EC levels) than 20℃. The index exhibited opposite results in granular sludge (e.g., from 6.096 on day-0 to

372 6.777 on day-180) (Table – S1). In both sludges, 'NO_2^-+NH_4^+ (20°C) groups had the lowest
373 diversity than others, which probably indicated the substrates addition strategy was not
374 suitable for biomass preservation at the community level.

375 The microbial community composition at the phylum level is shown in Figure. S2A/B.
376 There were twenty main microbial phyla (relative abundance ≥ 0.1 % in at least one
377 sample) detected in all samples, Planctomycetes, Proteobacteria, Bacteroidetes, and
378 Chloroflexi among them accounted for more than 87 % and 83% in floccular and granular
379 sludge, respectively. For floccular sludge, the relative abundance of Planctomycetes (a
380 subset of AnAOB) (14.7 – 38.2%), increased under all different strategies except 4°C
381 groups and 'NO_3^-+NH_4^+ (20°C)', suggesting that low temperature or nitrate addition
382 could help AnAOB to tolerate starvation to some extent. Proteobacteria (27.1 – 40.8%),
383 containing nitrite reductase genes (nir) and possibly almost all AerAOB (Wu et al., 2020),
384 showed a trend of decrease under all the conditions. Contrary to that, Chloroflexi (2.2 –
385 7.0%), facultative anaerobes widely existed in autotrophic systems (Chen et al., 2016),
386 increased with increasing preservation time. Different from floccular sludge, granular
387 sludge has the stable Planctomycetes (~30%) and Proteobacteria (~22%). It was likely
388 due to the microbial community of granules being more resistant to the external effects
389 than flocs according to Wang et al. (2018). In addition, for the heterotrophic bacterium,
390 Firmicutes (2.4 – 5.1%) kept stable in different groups which were the same as floccular
391 sludge.

392 The taxonomic results of dominant nitrogen removal-related bacteria in genus level
393 are shown in Figure. S2C/D. The common AnAOB genera, Candidatus *Brocadia*,

Candidatus *Kuenenia*, and Candidatus *Jettenia* were identified in floccular and granular sludge. Representative microbial genera were also found for AerAOB (*Nitrosomonas*) and NOB (*Nitrospira* and Candidatus *Nitrotoga*). Candidatus Brocadia (belongs to Planctomycetes) dominated the microbial community in both floccular and granular sludge over the 180 days preservation with a relative abundance of 15% and 32%, respectively. For floccular sludge, the relative abundance of Candidatus *Brocadia* increased (up to 20 – 30%), whereas it was stable for granular sludge (25 – 31%). The characteristics of floccular and granular (different particle size distribution) probably determined the difference. Ma and Wang (2018) also reported a stable relative abundance of AnAOB in granular sludge (dominated by Candidatus *Kuenenia*). The relative abundance of *Nitrosomonas*, the only identified AerAOB, was stable around 2 – 6% and 1 – 2% in the floccular and granular sludge, respectively. The lower relative abundance in the granular sludge corresponded with the lower AerAOB activity capacity (Section – 3.1.2). A low relative abundance of representative NOB genera (*Nitrospira* < 0.6% and Candidatus *Nitrotoga* < 0.1%) was found in both sludge types. *Denitratisoma*, another nitrogen removal related genus following denitrifying route, was abundant in all different storage conditions (7 – 14% and 3 – 6% in floccular and granular sludge, respectively), which could convert NO_2^--N or NO_3^--N (redox in the present study) to N_2 (Vlaeminck et al., 2007). It was likely one of the reasons for the reduction of redox. Moreover, according to Kartal et al. (2007), AnAOB could reduce NO_3^--N or NO_2^--N to NH_4^+-N through dissimilatory reduction. That is probably another possible route for redox removal, but that was not proved in this study.

3.4 Properties of sludges during preservation

3.4.1 EC levels increased and negatively corrected with AnAOB activity

The EC value (Figure. S3) increased in all storage conditions due to biomass decay (releasing ions, e.g., PO_4^{3-}, NH_4^+, etc.), pH control (HCl or NaOH addition), and chemical addition (NH_4Cl, $NaNO_2$, or $NaNO_3$). Under anaerobic conditions, it only slightly increased over 180 days since no chemicals were added. In contrast, the EC value of the sludge stored in anoxic conditions rapidly increased, especially for 'NO_2^-+NH_4^+ (20℃)' probably due to the redox buffer and acid addition. There are two possible reasons to consume redox, i) the existence of denitrifiers (Section – 3.3), and ii) the occurrence of dissimilatory reduction (Kartal et al., 2007). That could also explain the lower EC levels of the sludge stored at 4°C than 20℃. The levels in anoxic conditions were close to or even higher than the half inhibition value (IC_{50}, 14.6 mS cm^{-1} (Lin et al., 2020)) after 180 days of storage. Besides, salinity suppression might be more serious on starved biomass than fresh ones. A negative correlation between EC values and AnAOB activity (R^2 = 0.8513, $p < 0.05$ for floccular sludge and R^2 = 0.8863, $p < 0.05$ for granular sludge) was found (Figure. 5A/B).

3.4.2 Heme c decreased

Heme c was positively decreased during the sludge preservation (Figure. S4), but there was no significant correlation between AnAOB activity and the heme c in both floccular (R^2 = 0.8286, $p > 0.1$) and granular (R^2 = 0.4703, $p > 0.1$) sludge. Even though the positive correlation has been proven during reactor operation and enrichment (Tang et al., 2010), this rule did not apply in the biomass storage process. Ma and Wang (2018) reported

the heme c remained stable during anammox starvation, although the AnAOB activity gradually decreased. That difference might cause by the different sludge types (anammox versus PN/A sludge) and dominant genera (Candidatus *Kuenenia* versus Candidatus *Brocadia*).

3.4.3 EPS decreased

EPS are metabolic products in bacteria and can represent the cell viability to a content (Wang et al., 2013). Regardless of the storage strategy, the EPS content in floccular and granular sludge gradually decreased (Figure-S5). That was in line with Zhang and Bishop (2003), who demonstrated that the EPS could be secreted from cells to serve as the energy source for the functional bacteria when suffering from starvation. Gao et al. (2012) also found a significant reduction of EPS in aerobic granular after storage. According to Figure – S5, the low temperature probably reduced the EPS hydrolysis rate, whereas the redox buffer addition did not affect that.

3.5 Anaerobic conditions at 20℃ were cost-effective sludge storage strategy

An OPEX analysis (Figure. 6) demonstrates that the sludge stored under 'no N spike (20℃)' has the lowest operational expense. Only 104 € ton VSS^{-1} and 22 € ton VSS^{-1} are needed when storing floccular and granular sludge over 180 days, respectively, which is considerably below the storage costs in the continuous presence of redox buffers (at least around 340 € ton VSS^{-1}). For 'no N spike (20°C)', pH control (base addition) is the main expense. Except for this, there are no additional costs (e.g., reagents and cooling, etc.). As the storage condition which could maintain the highest anammox activity, 'no N spike (4℃)' has the lowest cost in pH control, whereas the cooling price is extremely

high (752 € ton VSS^{-1}). This leads to the total cost of 'no N spike (4℃)' being more than 20 times higher than 'no N spike (20℃)'. Considering the most residual anammox activity after storage, 'no N spike (4℃)' is suitable for storing a small amount of sludge in the lab.

The cost of pH control (acid addition) during the sludge storage is higher when applying anoxic conditions than for anaerobic conditions. This may be due to the occurrence of denitrification or denitritation, gradually consuming H^+ and increasing pH. In addition, the redox buffer addition is another major expense. Even though 'NO_3^-+NH_4^+ (20℃)' cost less in pH control than 'NO_3^- (20℃)', it could consume more redox buffer probably due to the occurrence of both anammox and denitrification. Both strategies need a much lower cost than 'NO_2^-+NH_4^+ (20℃)', but they are still at least 5 times higher than that of 'no N spike (20℃)'.

The results of the OPEX per specific AnAOB activity (cost-effective analysis) reveal that only 2.3 and 0.6 € d kg NH_4^+-N^{-1} in 'no N spike (20℃)' from the stored floccular and granular sludge are needed. That is more than 15 times lower than anoxic storage strategies. Except for the 'no N spike (20℃)', 'no N spike (4℃)' is the best option even though it needs extremely high cooling costs. That proves the anaerobic condition is more cost-effective for PN/A sludge storage than anoxic ones, especially for the large scale.

3.6 Research application and prospect

The findings on the anammox-related parameters (e.g., AnAOB activity) are generical and can be extrapolated to any other sludge. While biomass decay is specific to the sludge tested (e.g., composition and proportion of functional microbes) and should not

be extrapolated directly. However, the dominant genus of the sludges in the present is Candidatus *Brocadia*, which is a typically anammox genus that present in many studied sludges (Ali et al., 2014; Oshiki et al., 2011). For another common AnAOB genus, Candidatus *Kuenenia*, similar findings to the present research were also found by other researchers (Ma and Wang, 2018; Ma et al., 2017). Thus, the conclusions proposed in the present research are expected most likely also applicable for other PN/A sludges with different dominant genera. Additionally, for the effect of proportions of functional microbes on the biomass decay, convergence is also expected between different PN/A sludge since most of the microbes in PN/A sludge are heterotrophs (85-90% in the mainstream versus 67-84% in this study) (Henze et al., 2000; Lotti et al., 2015; Strous et al., 1998).

According to the results of this study, the 4℃ without N spike ('no N spike (4℃)') storage strategy was the most effective in maintaining the highest biomass activity capacity after long-term storage. This strategy is recommended for preserving small amounts of sludge in the laboratory, but it is too costly for large-scale applications. For the winter bioaugmentation concept in the STPs, the 20°C without N spike ('no N spike (20℃)') strategy deserves to be chosen, since it is the most cost-effective storage strategy.

Before the full-scale application, there are still several aspects that need to be studied. Firstly, whether the activity of stored sludge can be reactivated quickly at low temperature (<15℃) is essential for the winter bioaugmentation application (stored sludge directly bioaugmented into the mainstream reactor). Secondly, the applicability of the conservation strategy on other AnAOB species (e.g., Candidatus *Kuenenia* and

Candidatus *Jettenia*, etc.) will be tested since the appropriate biomass preservation strategy is usually species-dependent (Ali and Okabe, 2015). Both floccular and granular sludge in this study were dominated by Candidatus *Brocadia*, which is the limitation of this research. Additionally, the Candidatus *Brocadia* has a higher growth rate, but low substrates affinity (r-strategist) compared to other AnAOB genera (Oshiki et al., 2011), which may be beneficial for 'no N spike' in the present research. Thirdly, whether the 'NO_2^-+NH_4^+' is suitable for biomass preservation in the presence of other elements that the metabolic activity requires. Finally, a pilot-scale system should also be established to verify the stability of the concept. Using the preservation strategy in large-scale sludge is still a challenge.

4. Conclusions

The anaerobic conditions resulted in the higher preservation of the AnAOB activity capacity (37 – 55%) than anoxic conditions during storage for 180 days. Despite the higher activity capacity retention at 4°C, 'no N spike (20℃)' is recommended to preserve sludge on a large scale, since it has the lowest value in OPEX per specific AnAOB activity (i.e., OPEX/anammox activity on Day-180, 2.3 and 0.6 € d kg NH_4^+-N^{-1} for floccular and granular sludge, respectively) making it the most cost-effective alternative. Successful biomass reactivation (20℃) and the remained predominant genus (Candidatus *Brocadia*) furthermore strengthen the potential of this approach. An exponential negative correlation between the electrical conductivity and the AnAOB activity was also found.

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