Empowering a mesophilic inoculum for thermophilic nitrification: growth mode and temperature pattern as critical proliferation factors for archaeal ammonia oxidizers

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Abstract

Cost-efficient biological treatment of warm nitrogenous wastewaters requires the development of thermophilic nitrogen removal processes. Only one thermophilic nitrifying bioreactor was described so far, achieving 200 mg N L^{-1} d^{-1} after more than 300 days of enrichment from compost samples. From the practical point of view in which existing plants would be upgraded, however, a more time-efficient development strategy based on mesophilic nitrifying sludge is preferred. This study evaluated the adaptive capacities of mesophilic nitrifying sludge for two linear temperature increase patterns (non-oscillating vs. oscillating), two different slopes (0.25 vs. 0.08 °C d^{-1}) and two different reactor types (floc vs. biofilm growth). The oscillating temperature pattern (0.25 °C d^{-1}) and the moving bed biofilm reactor (0.08 °C d^{-1}) could not reach nitrification at temperatures higher than 46°C. However, nitrification rates up to 800 mg N L^{-1} d^{-1} and 150 mg N g^{-1} volatile suspended solids d^{-1} were achieved at a temperature as high as 49°C by imposing the slowest linear temperature increase to floccular sludge. Microbial community analysis revealed that this successful transition was related with a shift in ammonium oxidizing archaea dominating ammonia oxidizing bacteria, while for nitrite oxidation *Nitrospira* spp. was constantly more abundant than *Nitrobacter* spp.. This observation was accompanied with an increase in observed sludge yield and a shift in maximal optimum temperature, determined with ex-situ temperature sensitivity measurements, predicting an upcoming reactor failure at higher temperature. Overall, this study achieved nitrification at 49°C within 150 days by gradual adaptation of mesophilic sludge, and showed that ex-situ temperature sensitivity screening can be used to monitor and steer the transition process.

1. Introduction

The extensive production of inorganic nitrogen fertilizers is crucial to sustain food production for the increasing global population and living standard (Erisman et al. 2008). This, however, resulted in the accumulation of reactive nitrogen species in many natural ecosystems, causing a worldwide environmental problem (Galloway et al. 2014). Ammonia nitrogen is a major wastewater component inducing eutrophication and fish mortality when released in water bodies without prior treatment (Camargo and Alonso 2006). Nitrification, the microbial oxidation of ammonium to nitrate, plays a key role in the initial transformation of reactive nitrogen in wastewater treatment. Aerobic ammonium-oxidizing bacteria (AOB) and archaea (AOA) catalyze the first, rate-limiting step, i.e. oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) (also known as nitritation), while the successive oxidation to nitrate (NO_3^-) (also referred to as nitratation), is usually carried out by aerobic nitrite-oxidizing bacteria (NOB). Nitrification is conventionally followed by the reductive denitrification process to achieve complete nitrogen removal, although short-cut nitrogen removal processes, such as partial nitritation/anammox (PN/A), are gaining importance over the last years (Lackner et al. 2014, Vlaeminck et al. 2012).

Although nitrification is an established biological process to treat ammoniacal wastewater, applications above 40°C still represent a significant challenge. The development of thermophilic nitrification could enable the treatment of warm wastewaters, such as hot industrial wastewater and thermophilic anaerobic digester supernatant, without the need of additional cooling and thus lower both the capital as operational costs. Experiences with carbon treatment, moreover, suggest that a more stable process with higher specific rates (smaller bioreactors), a lower sludge production and a lower level of contamination could be achieved at thermophilic conditions (Lapara and Alleman 1999). Thermophilic nitrogen

removal would, thus, not only be a sustainable and an economically favorable solution for the treatment of warm wastewaters, but also for wastewaters on sites with excess available heat.

Two fundamentally different strategies can be used to achieve thermophilic nitrification for wastewater treatment, including a strategy based on a thermophilic nitrifying inoculum or based on a mesophilic community which has been adapted to higher temperatures. As both thermophilic AOA (e.g. "*Candidatus* Nitrosocaldus yellowstonii", "*Candidatus* Nitrososphaera gargensis") and NOB (e.g. *Nitrospira calida*) have been separately enriched from terrestrial hot springs, respective environmental samples may serve as inoculum to enrich a thermophilic nitrifying community (de la Torre et al. 2008, Hatzenpichler et al. 2008, Lebedeva et al. 2011). Indeed, Courtens et al. (*Under review*) recently showed the enrichment of autotrophic thermophilic nitrifiers from compost samples and the successful operation of a thermophilic nitrifying bioreactor at 50°C with biotechnological potential. However, the low growth rate and/or low relative abundance of those thermophilic autotrophs in environmental samples may result in very long and laborious enrichment processes, which may impede the upgrade of existing wastewater treatment plants. From a practical point of view the second strategy, in which existing mesophilic nitrifying communities are adapted to elevated temperatures, may thus be more appropriate. Shore et al. (2012) achieved complete nitrification at 40°C applying a stepwise temperature increase from 30 to 40°C (10°C d^{-1}) to a moving bed biofilm reactor (MBBR). In a parallel MBBR the temperature was increased from 30 to 45°C (15°C d^{-1}), however, losing all nitrifying activity. Slightly higher nitrification temperatures (42.5°C) were reached by Courtens et al. (2014a) who imposed smaller temperature differences (2.5°C d^{-1}) from 40°C on. It was furthermore demonstrated that salt amendment can be used as a tool to reach more efficient temperature transitions. However, from those studies it is clear that no 'real' thermophilic (>45°C) nitrification can be achieved through a stepwise temperature increase pattern (> 2.5°C d^{-1})**,** although short-term activity

96 measurements of mesophilic sludge (34°C) showed nitrifying potential up to 50°C (Lopez-
97 Vazquez et al. 2014).

98 Therefore, in this study, the adaptive capacities of mesophilic nitrifying sludge to gradual
99 temperature increase patterns were explored. In a first reactor experiment, a non-oscillating
100 linear temperature increase (0.25°C d^{-1}) was compared with an oscillating increase (amplitude
101 2°C) with the same final slope. Pre-exposure to a certain stress can in some cases result in an
102 increased resilience towards this stress as shown for copper stress in denitrifiers (Li et al.
103 2014, Philippot et al. 2008). In a second experiment, a linear temperature increase with a
104 lower slope (0.08°C d^{-1}) was investigated, in which a floccular growth system (SBR) was
105 compared with a biofilm based system (MBBR). Biomass retention of the slow growing
106 thermophilic autotrophs is essential, and could eventually be favored through a biofilm based
107 reactor system. Finally, the nitrifying community was closely monitored by batch activity
108 tests and molecular analyses during the linear temperature increase to elucidate the adaptation
109 process or shifts in the microbial community.

2. Materials and methods

2.1. Reactor set-up and operation

An overview of the two reactor experiments and associated reactor parameters is presented in **Table 1**. In the first experiment with two identical lab-scale sequential batch reactors (SBR), a linear temperature increase (0.25°C d^{-1}) with (SBR_1) and without (SBR_2) an oscillation (amplitude 2°C, frequency 0.088 d^{-1}) were compared. In the second reactor experiment, a lower linear temperature increase (0.08°C d^{-1}) was applied, and a SBR (SBR_3) was compared with a MBBR. The majority of the process and feeding parameters were the same in all reactors to investigate the effect of temperature pattern and/or sludge aggregation (flocs versus biofilm) (**Table 1**).

The reactor vessels (working volume 2 L, diameter 12 cm) were jacketed, allowing temperature control with a circulating thermostatic water bath, and equipped with a stirring device. The reactor pH was controlled between pH 6.5 and 7.5 by dosage of 0.1 M NaOH/HCl, and continuous aeration was provided by air pumps through a diffuser stone. The synthetic medium consisted of $(NH_4)_2SO_4$ (10-800 mg N L^{-1}), 11-12 g $NaHCO_3$ g^{-1} N, KH_2PO_4 (10 mg P L^{-1}) and 0.1 mL L^{-1} trace element solution dissolved in tap water (Kuai and Verstraete 1998). The nitrogen loading was adjusted through the ammonium concentration in the influent. The 6- and 4-h cycle of the SBR consisted of a 330 and 210-min aerobic reaction period including three 25-min feeding periods, a 15-min settling period, a 5-min decanting period and a 10-min idle period.

The carrier material of the MBBR consisted of polyvinyl alcohol (PVA)-gel beads (Kuraray, Japan) at a volumetric filling ratio of 15%. All reactors were inoculated with the same commercial nitrifying inoculum (Avecom NV, Ghent, Belgium) at an initial biomass concentration of 2.4 ± 0.1 g volatile suspended solids (VSS) L^{-1}. To ensure sufficient biomass

134 growth on the carriers of the MBBR, a stabilization period (79 days) was included in the
135 second experiment. The MBBR was initially operated in the same sequencing batch
136 feeding/withdrawal mode during the stabilization period to ensure enough suspended biomass
137 for biomass growth on the carriers. Once growth was observed on the carriers, initially, half
138 of the suspended biomass was wasted (day 23 of the stabilization period). Further on, the
139 residual suspended biomass was gradually wasted at about 45 mg VSS d^{-1} until day 79 when
140 the settling period was excluded.

141 **2.2. Ex-situ nitrification activity tests**

142 In parallel with the second reactor experiment, batch activity tests were performed with the
143 SBR_3 sludge and MBBR carriers to monitor the progress of the optimal temperature for both
144 ammonia and nitrite oxidation. Temperature sensitivity measurements were performed at
145 reactor temperatures of 38°C, 40°C, 42°C, 44°C, 46°C and 48°C, in which the specific
146 ammonia and nitrite oxidizing activities were measured at the respective reactor temperature
147 ±2°C. For the SBR_3 sludge, 96-well plates with a working volume of 250 µL were used, while
148 the MBBR carriers were transferred in 24-well plates with a working volume of 1.5-2.5 mL.
149 Plates were incubated in a MB100-4A Thermoshaker (Hangzhou Allsheng Instruments,
150 China) at the specific temperature, in which oxygen was provided through intensive shaking
151 at 600 rpm. The buffer solution (pH 7) contained final concentrations of 2 g P L^{-1}
152 (KH_2PO_4/K_2HPO4), 1 g $NaHCO_3$ L^{-1} and 60 mg N L^{-1} ($(NH_4)_2SO_4$ or $NaNO_2$). The sensitivity
153 of ammonia and nitrite oxidation for free ammonia (FA) was also evaluated by determining
154 the specific activity at different ammonium concentrations (25-200 mg N L^{-1}). All treatments
155 were performed in sextuple, and liquid samples (2 µL) were taken over time for NH_4^+ and
156 NO_2^- analysis. These high-throughput activity measurements were highly optimized for each
157 sludge type prior to the actual tests, demonstrating no oxygen limitation as increased shaking
158 speed (250-600 rpm) did not increase nitrogen oxidation rates A validation experiment was

159 performed in which the obtained rates were not significantly different with rates obtained in
160 conventional 250 mL Erlenmeyer aerobic batch tests without oxygen limitation as monitored
161 with an online DO measurement (Windey et al. 2005).

162 **2.3. Sludge production and settleability**

163 Sludge production was evaluated through the observed sludge yield (Y), calculated using
164 cumulative terms, as described previously (Courtens et al. 2014b). Biomass settleability of the
165 floccular sludge was measured through the determination of the sludge volume index (SVI) in
166 a 1 L Imhoff cone, with the sludge height variation monitored for 5 min instead of 30 min to
167 prevent extensive cooling of the sludge.

168 **2.4. Molecular analyses**

169 Biomass samples (± 2 g) of the inoculum and the reactors (SBR_3 and MBBR) were collected
170 over time, and total DNA was extracted using the Fast-Prep24 instrument (MP-BIO,
171 Germany) as described previously (Vilchez-Vargas et al. 2013). DNA quality and quantity
172 were analysed electrophoretically on 1% (w/v) agarose gel and spectrophotometrically by
173 determination of the absorbance ratios at 260 nm and 280 nm and the absorbance at 260nm,
174 using a NanoDrop ND-1000 instrument (Thermo Scientific), respectively. Abundance of the
175 16S ribosomal RNA (rRNA) genes of *Nitrospira* spp. and *Nitrobacter* spp. and the functional
176 gene encoding the A subunit of the bacterial and archaeal monooxygenase (*amoA*) was
177 monitored using quantitative PCR (qPCR) assays on an ABI StepOnePlus real-time PCR
178 instrument (Life Technologies, Carlsbad, CA, USA). Whereas *amoA* abundance is a good
179 proxy for monitoring AOB and AOA abundance, the genera *Nitrospira* and *Nitrobacter*
180 represent major NOB. Reactions were performed in a total volume of 20 µl consisting of 10 µl
181 of iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 1.0 µl
182 DNA template (diluted), 0.3 (*Nitrobacter*), 0.5 (*amoA*) or 0.6 µl (*Nitrospira*) of each forward

and reverse primer, adjusted to a final volume of 20 µl with H_2O (Table S1). Amplifications were run as follows: initial denaturation for 2 min at 95 °C followed by 40 cycles of 15 s denaturation at 94 °C, 30 s annealing at the temperature mentioned in Table S1 (supplemental information) and 30 s elongation at 60 °C. Each sample extract was amplified in triplicate and target quantification was performed using a standard curve. Standard curves (range: 1.0E+02 - 1.0E+07 copies μl^{-1}) were generated using six ten-fold dilutions of target DNA from Fosmid54D9 (Treusch et al. 2005), *Nitrosomonas europaea* DSM 28437, *Nitrobacter winogradskyi* DSM 10237 and *Nitrospira moscoviensis* DSM 10035. Additional positive/negative controls and a melting curve analysis were performed in all analyses to verify target specific amplification, the absence of contaminants, and to confirm product specificity, respectively.

The overall community structure was analyzed using paired-end high-throughput sequencing (MiSeq Illumina platform) of amplified V5-V6 regions of the 16S rRNA gene, using the universal primers 807F and 1050R (Bohorquez et al. 2012). Amplification, library preparations, sequencing and bioinformatic processing of sequences was done according to Camarinha-Silva et al. (2014) with some modifications. Prior to the addition of barcodes and Illumina adapters the template was enriched by 20 PCR cycles using primers 807F and 1050R. Raw sequences were assembled (Cole et al. 2014) and subsequently aligned using MOTHUR (gotoh algorithm with the SILVA reference database) prior to preclustering allowing two mismatches (Schloss et al. 2009). Next, sequences were clustered at a sequence similarity cut-off value of 99% to define species-level operational taxonomic units (OTUs). Only OTUs (phylotypes,Phy) exhibiting an average abundance of at least 0.001% of the total communities and a sequence length >200bp were considered for further analysis. Phylogenetic analyses were performed with MEGA5 (Tamura et al. 2011) using the neighbor-joining method with Jukes-Cantor correction and pairwise deletion of gaps/missing data. A

total of 1000 bootstrap replications were performed to test for branch robustness. A heat map was generated using gplots and RColorBrewer packages.

2.5. Chemical analyses

Ammonium (Nessler method), total suspended solids (TSS) and volatile suspended solids (VSS) were measured according to standard methods (Greenberg et al. 1992). The biomass concentration in the MBBR was determined through extraction of the biomass from the PVA carriers and subsequent protein measurement. The protein content was then translated to a VSS concentration using the average protein content of the MBBR sludge, 0.31 g protein g^{-1} $VSS_{MBBR\ sludge}$ as determined. The carriers were cut in fine pieces and incubated in 1 M NaOH for 2 hours at 46°C with regular mixing for biomass extraction. To determine the protein concentration in the extract, the method developed by Lowry was used with bovine serum albumin (BSA) as the standard (Lowry et al. 1951). Nitrite and nitrate were determined on a 930 Compact Ion Chromatograph (Metrohm, Switzerland), equipped with a conductivity detector. Dissolved oxygen (DO) and pH were measured with an HQ30d DO meter (Hach Lange, Germany) and a Dulcotest pH-electrode PHEP 112 SE (Prominent, Germany), respectively. In the batch activity tests, the liquid samples for ammonium and nitrite determination were always immediately analyzed spectrophotometrically with the Berthelot and Montgomery reaction, including a triplicate standard curve for each analysis run. Measurements were obtained using a Tecan infinite plate reader (Tecan, Switzerland), and biomass was quantified through protein concentrations.

3. Results

3.1. Oscillating versus non-oscillating linear temperature increase

The adaptive capacities of mesophilic nitrifying sludge were first evaluated for two different gradual temperature increase patterns. An oscillating temperature increase with an amplitude of 2°C and a frequency of 0.088 d^{-1} was compared with a non-oscillating increase with the same linear slope (0.25°C d^{-1}) as shown in **Figure 1**. Prior to any temperature increase, the reactors were started up identically at 37°C reaching ammonium removal rates of 180 ± 14 mg N L^{-1} d^{-1} or 136 ± 10 mg N g VSS^{-1} d^{-1} after one week of stabilization. Nitrite accumulation was negligible in both reactors and nitrate production accounted for 95% of the ammonium removal. Up to 40°C, no changes in volumetric ammonium removal rates were observed in both reactors. Further temperature increase above 40°C, however, negatively affected the nitrifying activity in both reactors, with a more pronounced effect in the oscillating reactor. At 42°C, only 15% of the initial volumetric nitrifying activity remained in the oscillating reactor (26 ± 5 mg N L^{-1} d^{-1}) while 50% remained in the non-oscillating one (90 ± 3 mg N L^{-1} d^{-1}) (**Figure 1**). Although the non-oscillating reactor seemed to better resist the temperature increase, the decreasing trend pursued in both reactors finally resulting in an entire loss of activity at 45°C in both reactors, suggesting that the imposed slope of 0.25°C d^{-1} was too high.

3.2. Floccular versus biofilm based reactor system

3.2.1. Reactor performance

In the second reactor experiment, a linear temperature increase with a lower slope was investigated (0.08-0.16°C d^{-1}), in which a SBR (SBR_3) was compared with a MBBR (**Table 1**). A 79-day stabilization period at 38°C allowed sufficient acclimatization of the inoculum and, more specifically, biomass growth on the PVA gel carriers of the MBBR. The suspended

biomass in the MBBR was gradually wasted during this period, while clear attached growth was observed on the PVA carriers (**Figure S1**). At the start of the actual experiment the settling was excluded to waste all the suspended sludge. This resulted in roughly a doubling of the attached growth (**Figure 2C**) and a further increase of the ammonium removal rate up to 580 ± 44 mg N L^{-1} d^{-1} (**Figure 2B**). As the SBR_3 sludge content also sharply increased from about 3.3 g VSS L^{-1} (day 6) to 4.5 g VSS L^{-1} (day 16), eventually endangering settling behavior, about one third of the SBR_3 sludge was wasted before the start of the temperature increase. Concurrently, the loading was lowered by one third to prevent overloading, reaching comparable volumetric nitrification rates in both reactors (**Figure 2B**). From day 20 on, temperature was gradually increased in both reactors at a slope of 0.16°C d^{-1} (**Figure 2A**). In accordance with the first reactor experiment, from 38°C to 40°C, no negative effect on the nitrification performance was observed. On the contrary, volumetric rates slightly increased (**Figure 2B)**. As temperatures above 40°C initiated reactor failing in the first experiment (**Figure 1)**, from 40°C on, the imposed slope was halved to 0.08°C d^{-1} (day 32). At that moment, a technical failure of the pH controller led to acidification (pH 5) in the MBBR resulting in a 18% decrease of nitrification performance. The MBBR recovered, even though temperature further increased. Stable ammonium removal rates of 563 ± 52 mg N L^{-1} d^{-1} (SBR_3) and 358 ± 40 mg N L^{-1} d^{-1} (MBBR) were observed in both reactors until 45°C. From 45.5°C on, however, ammonium removal rates gradually decreased in the MBBR from 358 ± 40 to 23 ± 8 mg N L^{-1} d^{-1} at 46.5°C (**Figure 2B)**. As more than 90% of the activity was lost, temperature increase was ceased in the MBBR. In contrast, volumetric rates increased in the SBR_3 up to 776 ± 62 mg N L^{-1} d^{-1} at a temperature as high as 49°C, corresponding with a specific ammonium removal rate of 155 ± 24 mg N g VSS^{-1} d^{-1} (**Figure 2C).** Nitrite accumulation was observed from temperatures higher than 49°C up to 200 mg N L^{-1}. As batch activity tests with SBR_3 sludge showed that nitrite concentrations up to 500 mg N L^{-1} did not

have a significant effect on the ammonium oxidizing activity ($p<0.05$, **Figure S2**), the loading rate was not adjusted. At 49.5°C, a malfunctioning of the pH controller pump now also acidified the SBR_3 (pH 3-4), resulting in a decrease of ammonium removal activity to 30 mg N L^{-1} d^{-1}. The temperature in the SBR_3 was decreased to 48.5°C to allow for recovery of the SBR_3. Ammonium oxidation rates increased again reaching >300 mg N L^{-1} d^{-1} after 50 days, while nitrite oxidation could not be recovered (**Figure S3**). Overall, the highest temperature where complete and stable nitrification was observed was 45.5°C and 49°C in the MBBR and SBR_3, respectively.

3.2.2. Community adaptation

The adaptive capacity of the SBR_3 and MBBR sludge towards the imposed temperature increase was closely monitored with parallel batch activity tests. Every 2°C along the temperature increase, specific ammonium and nitrite oxidizing activities of both sludge types were measured at the respective reactor temperature and at plus and minus 2°C. The results of these batch activity tests are presented in **Figure 3**. Similar observations were made for both reactors up to 42°C. Although the differences were small, it appeared that between 38 and 42°C, the temperature with the highest ammonium oxidizing activity was 40°C. Although the ammonium oxidation optimum in the SBR_3 gradually shifted from 40°C towards 46-48°C (**Figure 3A)**, the MBBR optimum did not get higher than 42°C (**Figure 3B)**. Moreover, at a reactor temperature of 44°C, no ammonium oxidation activity could be measured in the MBBR sludge at 46°C, clearly predicting the MBBR crash at 46°C (**Figure 3B)**. Despite the loss of ammonium oxidation at 46°C, the batch activity test indicates that the MBBR's nitrite oxidizers were still active up to 48°C (**Figure 3D)**. The nitrite oxidizers in the SBR_3 seemed to be adapted once the reactor reached 48°C, but a significant inhibition was observed at 50°C (**Figure 3C)**. Indeed, temperatures higher than 49°C led to nitrite accumulation in the SBR_3 (**Figure 2B**).

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3.2.3. Free ammonia sensitivity

Sensitivity of the nitrifying sludge towards elevated free ammonia (FA) was evaluated along the temperature increase. No significant inhibition of ammonium oxidation could be observed in both reactors by FA up to 6 mg N L^{-1}, in contrast, ammonium oxidation was stimulated by elevated FA (**Figure S4 A and B**).

The SBR_3's nitrite oxidizers were only slightly or not inhibited by FA up to 6 mg N L^{-1} at the lower operating temperatures (38-42°C), but were strongly inhibited at 46-48°C with a 50% (IC_{50}) and 100% (IC_{100}) inhibitory concentration of 0.67 ± 0.01 and 1.42 ± 0.08 mg NH_3-N L^{-1}, respectively (**Figure S4 C)**. The opposite trend was observed in the MBBR. Nitrite oxidation was clearly inhibited at 38-40°C, with an IC_{50} of 0.48 ± 0.07 mg NH_3-N L^{-1}, while the inhibition by FA disappeared at elevated temperatures (44-46°C) (**Figure S4 D**), possibly due to an increased diffusion limitation as the biomass concentration in the MBBR, and thus thickness of the biomass, strongly increased over time/temperature.

3.2.4. Sludge production and settleability

The increasing temperature initially induced a sharp decrease in sludge production in the SBR_3. The observed sludge yield halved from 0.074 to 0.035 g VSS g^{-1} N from 38°C to 42°C, whereas it increased again from 44°C to a yield of 0.067 ± 0.005 g VSS g^{-1} N up to 48°C (**Figure S5**). In contrast, sludge production in the MBBR was equal to 0.11 g VSS g^{-1} N until 42°C, whereupon it decreased and finally became negative at 46°C as a result of biomass die off (**Figure 2C**). Settling behavior of the SBR_3 sludge was stable up to 44°C, with a SVI_5 of 241 ± 38 mL g^{-1}, and improved at 46-48°C with a SVI_5 of 154 ± 2 mL g^{-1} (**Figure S5**). The sludge residence time (SRT) in the SBR_3 was 92 ± 7 days, while the SRT of the MBBR was considered infinite as nearly no suspended sludge could be measured in the effluent.

3.2.5. Functional community analysis

The abundance of selected key groups of nitrifying microorganisms was assessed along the temperature increase by means of qPCR. The reactors were inoculated with a subsample of the same inoculum, comprising a relatively well-balanced amount of AOB versus AOA ($2.1x10^9$ versus $3.5x10^8$ *amoA* gene copies g^{-1} VSS) and of the nitrite oxidizers *Nitrospira* spp. versus *Nitrobacter* spp. ($6.7x10^9$ versus $4.8x10^{10}$ 16S gene copies g^{-1} VSS). The AOB dominance was preserved in both reactors after the stabilization period reaching an AOB/AOA ratio of 279 and 7091 in the SBR_3 and MBBR, respectively. The bacterial *amoA* gene abundance kept stable up to 45°C at around 10^{10} copies g^{-1} VSS in both reactors, and then gradually decreased (**Figure 2D**). Clear differences in AOA abundances were, however, observed between the different reactors. The MBBR biomass retained significantly less AOA compared with the SBR_3 sludge after the stabilization period (**Figure 2D**, day 16). Moreover, a steep increase in AOA abundance by 3 orders of magnitude was observed in the SBR_3 at about 44°C, rising from $1.0x10^7$ to $2.9x10^{10}$ copies g^{-1} VSS, while the AOA abundance in the MBBR only slightly increased by two orders of magnitude to $8.6x10^8$ copies g^{-1} VSS at 46°C. This shows a clear shift in dominant ammonia oxidizers in the SBR_3 from AOB to AOA from 45°C on, while this shift never completely occurred in the MBBR. For nitrite oxidation, *Nitrospira* spp. were dominant over *Nitrobacter* spp. in both reactors over the whole experiment (**Figure 2D**). The observed trends in key nitrifier abundances were confirmed by community structure analysis through sequencing of the V5V6 region of the 16S rRNA gene (**Figure S6**). The most dominant AOB retrieved in the MBBR (Phy1) appeared to belong to the *Nitrosomonas europeae* species (**Figure S7)** and the most dominant AOA in the SBR_3 (Phy8) as well as the inoculum (Phy56) belonged to the *Nitrososphaera* genus (**Figure 4**). Interestingly, this AOA (Phy8) only showed a 95% similarity with the AOA initially detected

353 in the inoculum (Phy56). The closest related known NOB of the dominant *Nitrospira* in both
354 reactors is *Nitrospira japonica J1* with 91% similarity (**Figure 5**).

355

4. Discussion

4.1. Overall performance

The adaptive capacities of mesophilic nitrifying sludge over different linear temperature increase patterns and different sludge growth modes were explored in this study of which the main results are summarized in **Table 1**. A non-oscillating temperature pattern (SBR_2) appeared to be more effective than an oscillating pattern (SBR_1) for the tested slope of 0.25°C d^{-1} as both the volumetric as specific rates were 2-3 times higher. In general, the 'low-slope' reactors (SBR_3 and MBBR) reached 3 to 30 times higher volumetric rates than the 'high-slope' reactors (SBR_1 and SBR_2), at significantly higher temperatures. Finally, the biofilm based system (MBBR) showed 2.5 times lower rates than the parallel floccular growth system (SBR_3). Overall, within the range of the tested parameters/combinations in this study, the highest temperature with moreover the highest volumetric and specific nitrification rates were achieved through the transition of mesophilic nitrifying sludge by a slow, non-oscillating linear temperature increase (SBR_3).

The successful transition of the SBR_3 towards thermophilic temperatures was, remarkably, accompanied with a change in observed sludge production (**Figure S5**). The decreasing trend sharply reversed at 44°C finally resulting in comparable observed sludge yields at 38°C and 48°C (0.0687 ± 0.005 g VSS g^{-1} N). It is possible that the temperature window 38-42°C can be considered as sub-optimal for both mesophiles and thermophiles, leading to increased decay rates and/or decreased growth rates, and hence a significantly lower net biomass production. Overall, the observed sludge yields were lower than reported values for combined AOB and NOB sludge yield of 0.19-0.21 g VSS g^{-1} N at mesophilic temperatures (Barnes and Bliss 1983, Henze et al. 2008). Although shifts in net biomass production, i.e. the observed yield, coincided with the AOA vs. AOB dominance shift in this study, future

research should map the underlying biokinetic parameters. Besides growth and decay rate, also the oxygen and nitrogen affinity constants of the respective nitrifiers at different temperatures deserve attention to allow accurate modeling of nitrification at any elevated temperature.

In parallel, a clear shift in optimum temperature was observed with the ex-situ activity measurements. These small, fast, high-throughput activity tests, based on simple spectrophotometrical measurements, could predict the loss of ammonium and nitrite oxidation in the MBBR and SBR_3 (**Figure 3**), respectively. One could thus lower the slope of the imposed temperature slope when the optimum does not seem to evolve with the current temperature and so, steer the temperature increase strategy to achieve thermophilic nitrification.

4.2. Temperature increase pattern

Pre-exposure to a certain stress can result in an increased resilience to a secondary exposure (Philippot et al. 2008, Ryall et al. 2012). In the framework of this study, a pre-exposure to an elevated temperature can e.g. induce the production of heat-shock proteins (HSP) that could possibly protect the biomass during a secondary temperature increase and so, improve the adaptive capabilities. This study however showed that the tested oscillating temperature pattern did not improve the adaptive capabilities of mesophilic nitrifying sludge towards higher temperatures (**Figure 1**). The tested amplitude of 2°C was possibly too high to observe beneficial effects and thus, smaller oscillating could eventually give better results.

The linear character of the imposed temperature pattern in this study was clearly more successful than stepwise temperature increases reaching only maximum nitrification temperatures of 40 and 42.5°C (Courtens et al. 2014a, Shore et al. 2012). This is in line with observations at a lower temperature range (10-20°C) in which the negative effect of a sudden

temperature decrease on nitrification was much stronger than a gradual temperature decrease (Hwang and Oleszkiewicz 2007). Although nitrifiers are known to cope with relatively high seasonal temperature changes in wastewater treatment plants in moderate climates (e.g. 10°C-30°C at DC Water, Washington, USA), this study showed that a relatively low slope in temperature increase was essential to allow the transition of nitrifiers to temperatures higher than 38°C-42°C.

4.3. Sludge growth mode

A floccular growth system (SBR_3) was compared with a biofilm based system (MBBR), as biomass retention of the slow growing autotrophs is essential during the transition process, and could eventually be favored through a biofilm based reactor system. Experiences with thermophilic carbon treatment showed that thermophilic aerobic processes suffer from poor sludge settling properties (Suvilampi and Rintala 2003), thus, operation of settling based system such as a SBR may be threatened. Remarkably, settling behavior of the SBR_3 sludge in this study did not deteriorate (**Figure S5**), resulting in only minor differences in sludge retention time between both reactors.

The MBBR was initially hypothesized to better cope with the temperature transition, as biofilms show increased resistance to many types of environmental challenges (Gilbert et al. 2002). Recently, Gilbert et al. (2015) showed that nitrate production in a partial nitritation/anammox MBBR was more resilient against a gradual temperature reduction (20°C to 10°C, 0.07 °C d^{-1}), compared with a SBR, though ammonium oxidation declined similarly in both reactor types. Several observations, such as the increased resistance of biofilms towards antibiotics, are mainly explained by the restricted diffusion (Mah and O'Toole 2001). Recently, other factors, such as slow growth rate, high culture density and heterogeneity, were shown to influence the general stress response in biofilms (Mah and O'Toole 2001, Ryall et

al. 2012), and could eventually favor the adaptive capacities of nitrifiers towards elevated temperatures. This study is however in contrast with this hypothesis, as the ammonium oxidation MBBR failed around 46°C, while it could still be maintained until 49°C in the SBR_3 (**Figure 2B**). The successful transition in the SBR_3 seemed to be related to the observed shift of AOB to AOA dominance that was not completely achieved in the MBBR (**Figure 2D**). This is in accordance with literature, where most described thermophilic ammonium oxidizers are archaeal (de la Torre et al. 2008, Hatzenpichler et al. 2008, Lebedeva et al. 2013). The slower growth rate of the nitrifiers in the MBBR, initially supposed to favor the general stress response on a short term (Ryall et al. 2012), probably delayed the essential selection process on a long term. Indeed, an increase in AOA abundance in the biofilm was also observed, though one month later than in the SBR_3 (**Figure 2D)**. Furthermore, although both reactors were inoculated with the same AOA/AOB ratio, the relative decrease in AOA during the stabilization period was more pronounced in the biofilm than in the flocs resulting in an initially lower AOA abundance in the biofilm. The late start of the increasing trend of AOA in the MBBR suggests that the essential shift could eventually also have been achieved with an even lower slope of temperature increase. Potentially a stronger selection for fast growing micro-organisms was made in the MBBR versus the SBR giving more advantage to the faster growing AOB. Another possible reason for the differential stimulation of AOA vs. AOB in both systems might be the levels and dynamics of DO and NH_4^+ concentrations, as key substrates for ammonia oxidation and potentially niche differentiation. Figure S8 displays the typical concentration profiles, highlighting more frequent fluctuations of the bulk concentrations of both substrates along with lower concentrations of NH_4^+ in the SBR compared to the MBBR, possibly favoring AOA stimulation. Future research should focus on the actual substrate availability for the ammonia oxidizers within the flocs versus biofilm.

Besides the actual abundance of AOA in the biofilm, different, less thermotolerant, AOA species could have been enriched in the biofilm, compared with the floccular sludge.

Observations regarding nitrite oxidation were in line with literature, stating that *Nitrospira* is the most dominant nitrite oxidizer up to 60°C (Edwards et al. 2013, Lebedeva et al. 2011, Marks et al. 2012). In this study, no shifts were observed, and *Nitrospira* was dominant in both reactors over the entire experiment (**Figure 2D**). However, remarkable differences in free ammonia sensitivity were observed between reactors and over time (**Figure S4**), suggesting that a possible selection on strain level occurred during the transition. Overall, nitrite oxidizers were much more sensitive compared with the ammonium oxidizers, finally resulting in the development of a partial nitritation reactor at 48.5°C, opening opportunities for short-cut nitrogen removal processes.

4.4. Practical implications

The results of this study suggest that existing mesophilic nitrifying wastewater plants can be upgraded to thermophilic systems through a slow, non-oscillating linear temperature increase. Excluding the stabilization period, which is non-relevant for existing plants, this could be achieved in about 140 days. Close monitoring of the transition by high-throughput activity tests as described in this study, could moreover allow an even faster transition period. It should be emphasized that, beside the temperature increase pattern, the presence of AOA in the mesophilic sludge appeared to be essential for a successful transition. The fact that AOA appear to be distributed in wastewater treatment plants worldwide, even in equal or higher abundance than AOB (Limpiyakorn et al. 2013), opens thus opportunities for thermophilic nitrogen removal.

5. Conclusions

- The oscillating temperature pattern with an amplitude of 2°C and a slope of 0.25°C d^{-1} achieved a low nitrification rate of 26 ± 5 mg N L^{-1} d^{-1} at 42°C and lost all activity at 45°C.

- The moving bed biofilm reactor subjected to a slope of 0.08-0.16°C d^{-1} was able to oxidize ammonium up to 46°C, though, at a low volumetric rate of 32 ± 7 mg N L^{-1} d^{-1}.

- Nitrification rates of up to 800 mg N L^{-1} d^{-1} and 170 mg N g VSS^{-1} d^{-1} were achieved at 49°C through gradual adaptation (0.08 °C d^{-1}) of mesophilic nitrifying sludge in suspension (SBR_3).

- The successful transition from mesophilic to thermophilic ammonia oxidation in SBR_3 was linked to a dominance shift of archaeal above bacterial ammonia oxidizers.

- Ex-situ batch activity measurements can serve as a good tool to monitor the process response to transition, predicting reactor failures, thus enabling steering of the temperature increase pattern.

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614

615 **Table and Figure captions**

616 **Table 1.** Overview of reactor parameters, temperature increase patterns, volumetric and
617 biomass specific rates achieved at the highest temperature where complete nitrification was
618 observed in the two different reactor experiments. Averages calculated over at least 3
619 hydraulic retention times (± 3 operation days). n.a.: not applicable, SBR: sequencing batch
620 reactor, MBBR: moving bed biofilm reactor, VER: volumetric exchange ratio, HRT:
621 hydraulic retention time.

622

623 **Figure 1.** Temperature increase pattern **(A)** and nitrifying reactor performance **(B)** of two
624 sequential batch reactors comparing a linear with an oscillating temperature increase (0.25°C
625 d^{-1}).

626 **Figure 2.** Operation and performance characteristics of SBR_3 (left) and MBBR (right). **(A)**
627 Temperature increase patterns. **(B)** Volumetric ammonium removal and nitrite/nitrate
628 production rates. **(C)** Specific rates (left axis) and sludge content (right axis). **(D)** Abundance
629 of nitrifiers as determined by qPCR.

630 **Figure 3**. Relative temperature activity curves for ammonium **(A,B)** and nitrite **(C,D)**
631 oxidation of the SBR_3 **(A,C)** and MBBR **(B,D)** sludge. Each color curve represents a batch
632 test performed at a certain reactor temperature, of which the temperature is indicated with a
633 symbol. Per batch test, the temperature where the highest activity was measured was indicated
634 as the 'optimum temperature' and assigned as 100%. All experiments were performed in
635 sextuple, and statistically significant optima (student's t-test, $p<0.05$) are indicated with an
636 asterisk.

637 **Figure 4.** Phylogenetic relationships between the most dominant archaeal 16S rRNA gene
638 sequences in the SBR_3 reactor (Phy8) and the inoculum used (Phy56) and all described AOA

639 cultures or isolates, as well as relevant environmental clone sequences. Phy7 refers to the
640 archaeal 16S rRNA gene sequence detected in the thermophilic nitrifying reactor enriched
641 from compost by Courtens et al. (*Under review*).

642 **Figure 5.** Phylogenetic relationships between the most dominant *Nitrospira* 16S rRNA gene
643 sequence in the SBR_3 and MBBR (Phy3) and all described *Nitrospira* cultures or isolates, as
644 well as relevant environmental clone sequences. Phy1 refers to the *Nitrospira* 16S rRNA gene
645 sequence detected in the thermophilic nitrifying reactor enriched from compost by Courtens et
646 al. (*Under review*).

Table 1. Overview of reactor parameters, temperature increase patterns, volumetric and biomass specific rates achieved at the highest temperature where complete nitrification was observed in the two different reactor experiments. Averages calculated over at least 3 hydraulic retention times (± 3 operation days). n.a.: not applicable, SBR: sequencing batch reactor, MBBR: moving bed biofilm reactor, VER: volumetric exchange ratio, HRT: hydraulic retention time.

	Experiment 1		**Experiment 2**	
Reactor(type)	SBR_1	SBR_2	SBR_3	MBBR
Linear temperature increase	Oscillating	Steady	Steady	
Linear slope (°C d^{-1})	0.25		<40°C: 0.16 >40°C: 0.08	
Oscillating amplitude (°C)	2	n.a.	n.a.	
Oscillating frequency (d^{-1})	0.088	n.a.	n.a.	
Experimental periods				
Stabilization (d)	7		79	
Temperature increase (d)	50		150	
VER (%)	25		20	
Cycle duration (h)	6		4	
Flowrate (L)	2.1 ± 0.2		2.1 ± 0.3	
HRT (d)	1.0 ± 0.2		1.0 ± 0.2	
Highest temperature (°C)	42	42	49	45.5
Ammonium conversion rates*				
Volumetric (mg N L^{-1} d^{-1})	26 ± 5	90 ± 3	794 ± 57	309 ± 30
Specific (mg N g^{-1} VSS d^{-1})	72**	139 ± 18	151 ± 7	67**

* In all cases, nitrite accumulation was negligible and nitrate formation > 90% of ammonium removal

** Only one biomass measurement available for the specific period

Figure 1

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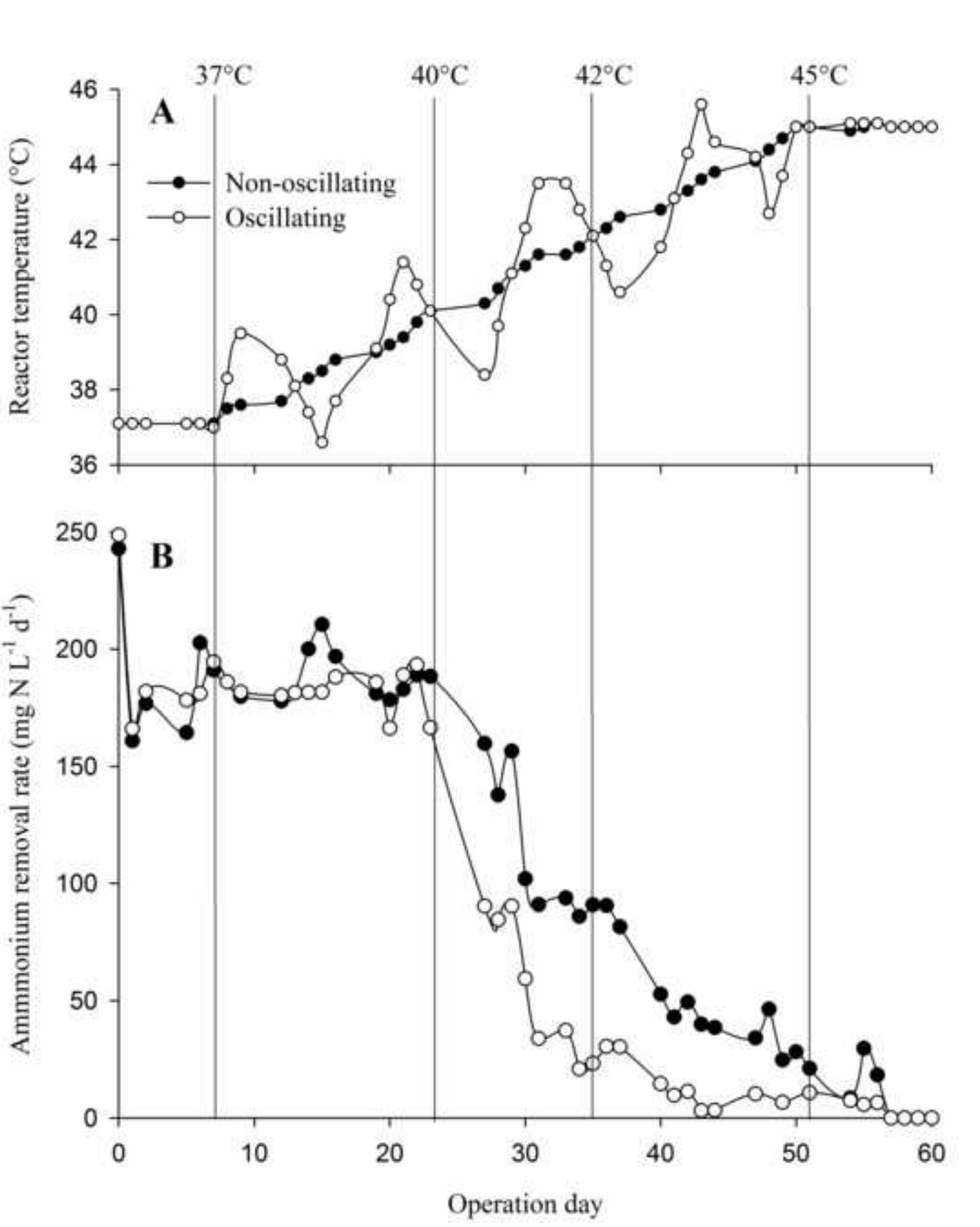

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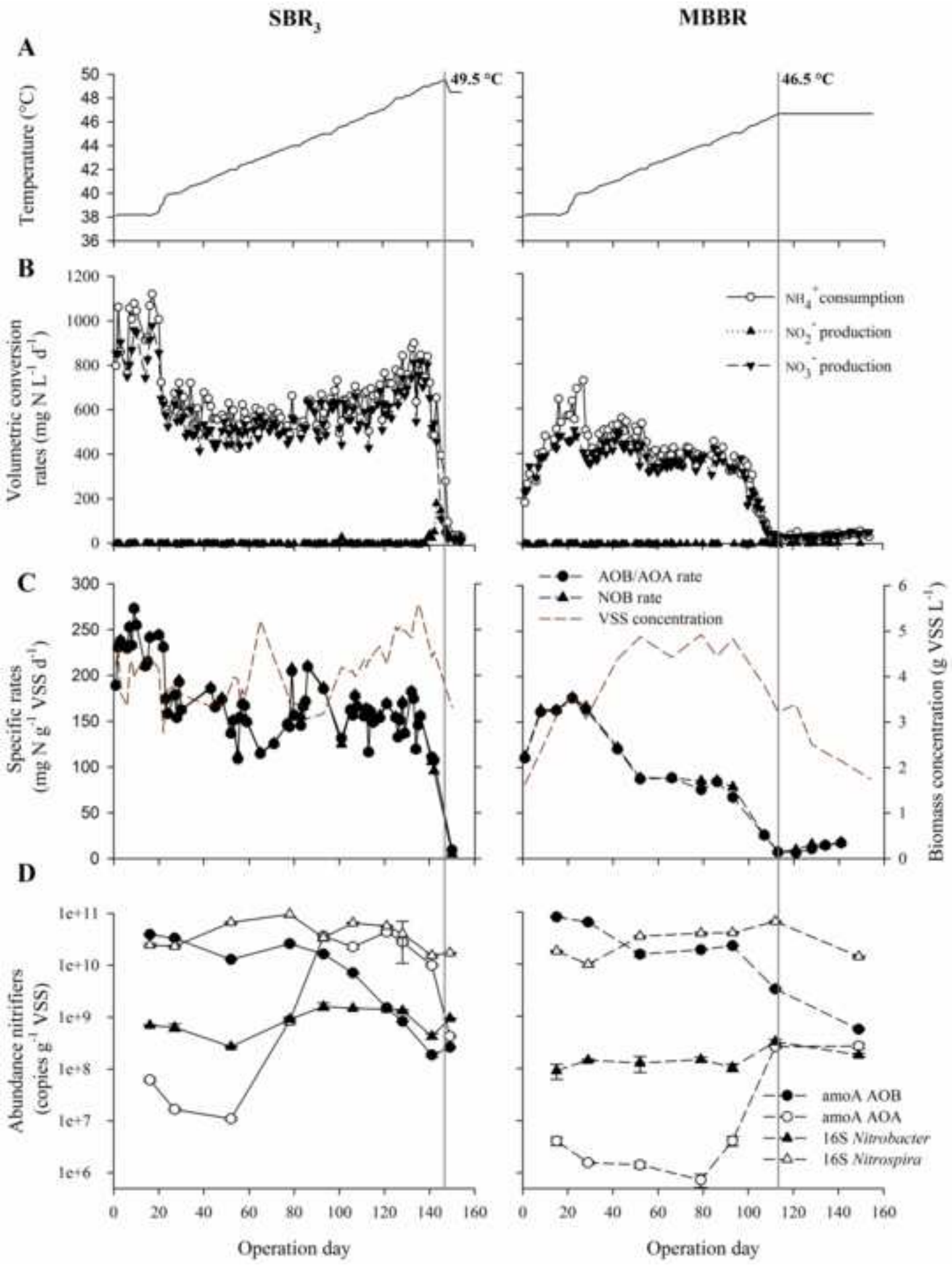

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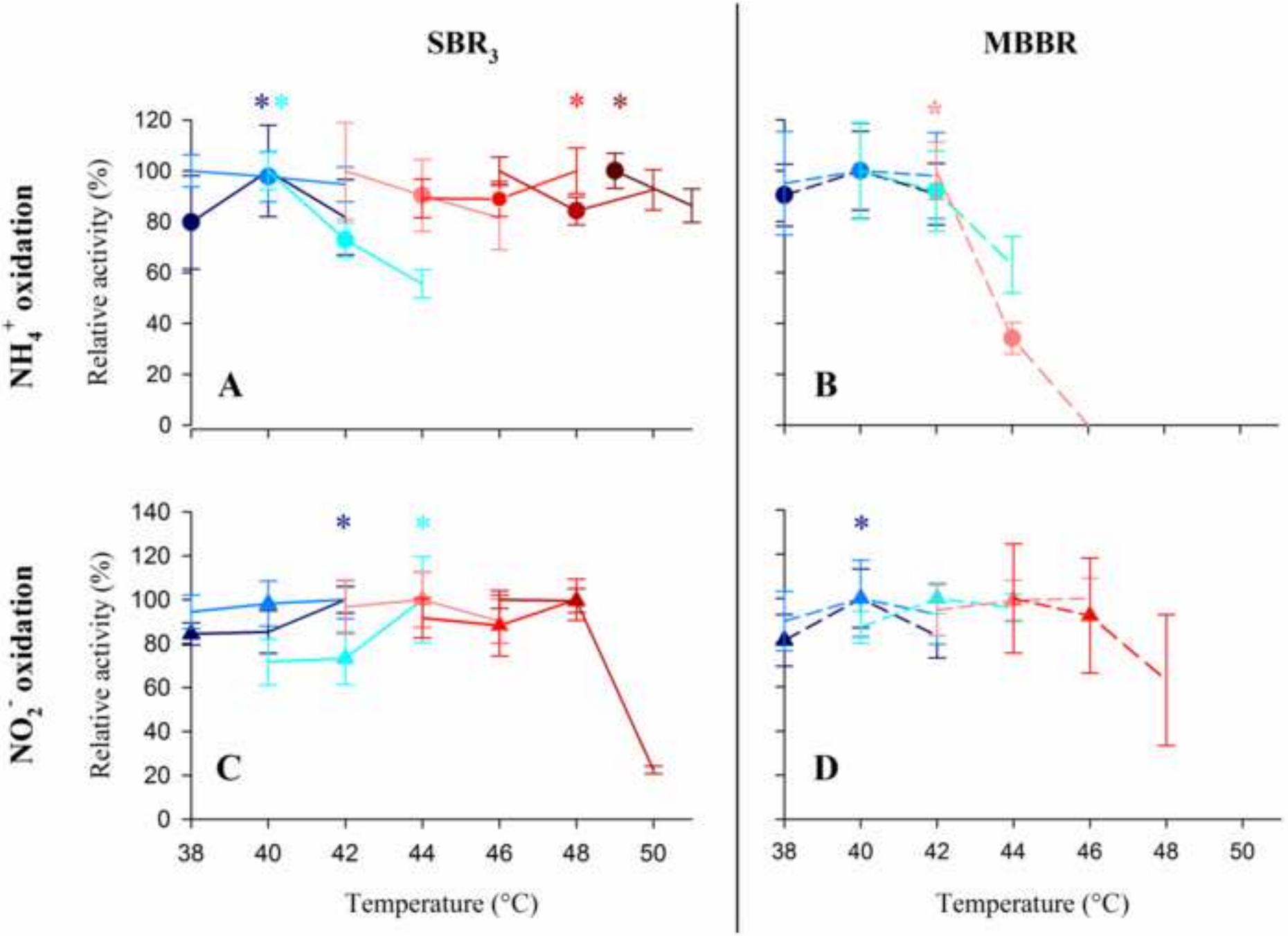

Figure 4

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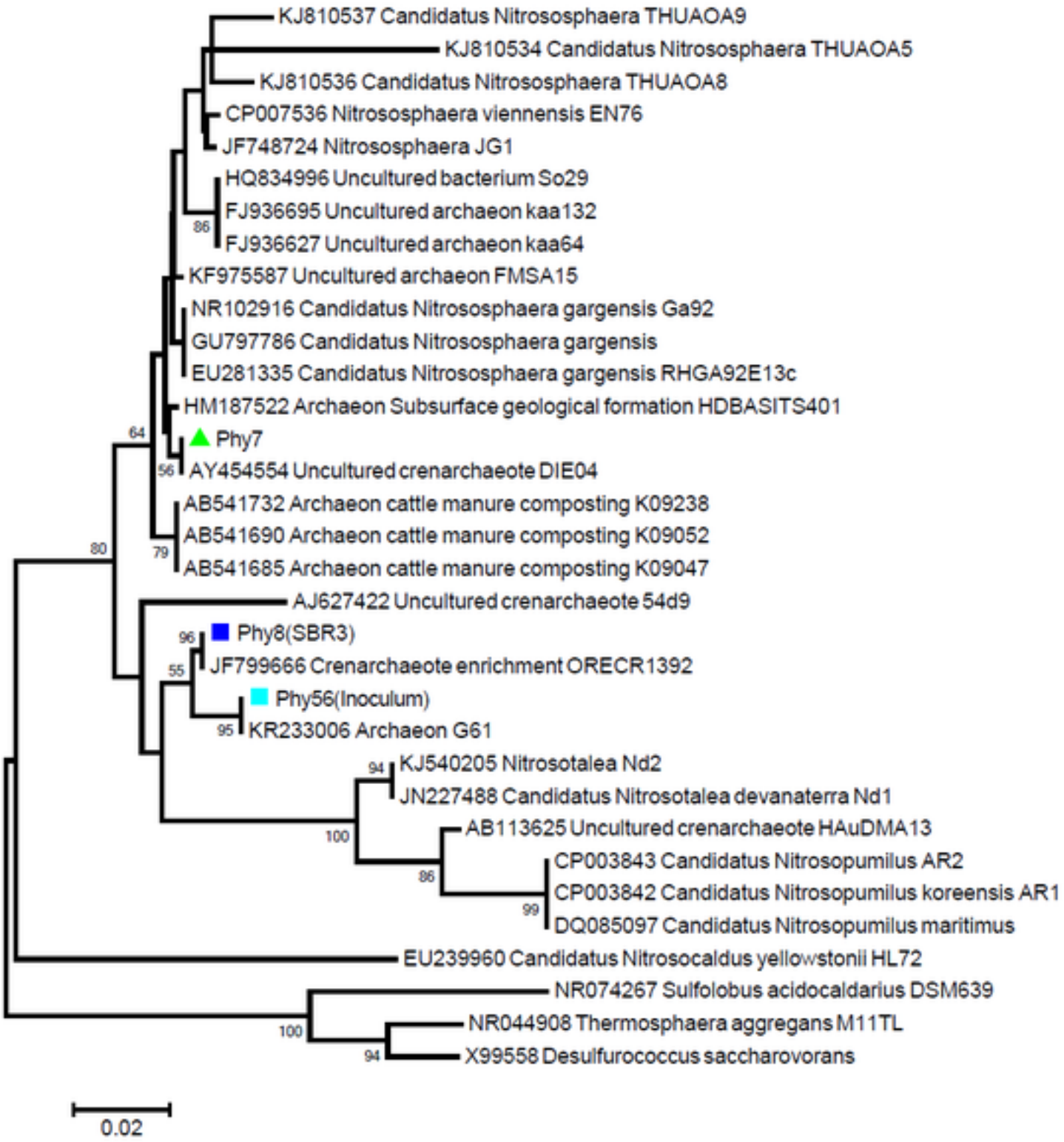

Figure 5

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