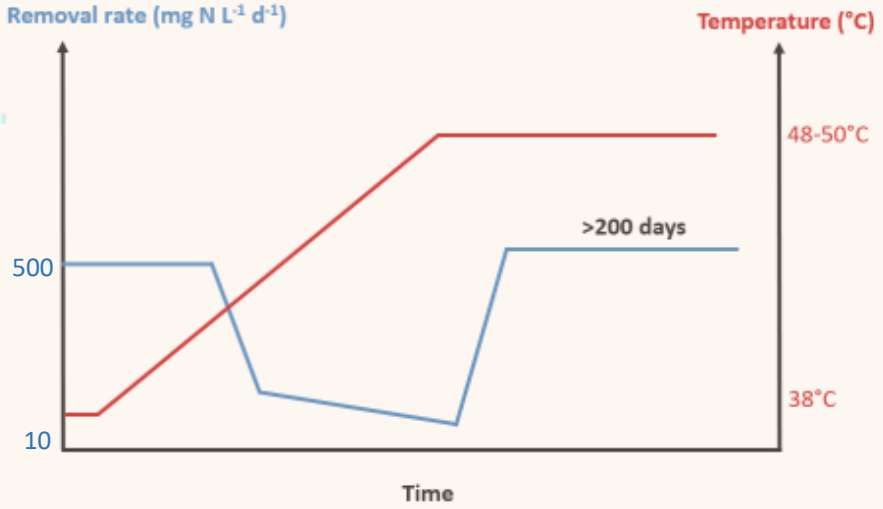
Adaptation and characterization of thermophilic anammox in bioreactors

Thermophilic N removal through ANAMMOX



Temperature adaptation & microbial community analysis in biofilm-based reactor

Characterizing kinetics in suspended biomass reactor

Economic assessment

Adaptation and characterization of thermophilic anammox in bioreactors

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Abstract

Anammox, the oxidation of ammonium with nitrite, is a key microbial process in the nitrogen cycle. Under mesophilic conditions (below 40°C), it is widely implemented to remove nitrogen from wastewaters lacking organic carbon. Despite evidence of the presence of anammox bacteria in high-temperature environments, reports on the cultivation of thermophilic anammox bacteria are limited to a short-term experiment of 2 weeks. This study showcases the adaptation of a mesophilic inoculum to thermophilic conditions, and its characterization. First, an attached growth technology was chosen to obtain the process. In an anoxic fixed-bed biofilm bioreactor (FBBR), a slow linear temperature increase from 38 to over 48°C (0.05-0.07°C d^{-1}) was imposed to the community over 220 days, after which the reactor was operated at 48°C for over 200 days. Maximum total nitrogen removal rates reached up to 0.62 g N L^{-1} d^{-1}. Given this promising performance, a suspended growth system was tested. The obtained enrichment culture served as inoculum for membrane bioreactors (MBR) operated at 50°C, reaching a maximum total nitrogen removal rate of 1.7 g N L^{-1} d^{-1} after 35 days. The biomass in the MBR had a maximum specific anammox activity of 1.1 ± 0.1 g NH_4^+-N g^{-1} VSS d^{-1}, and the growth rate was estimated at 0.075-0.19 d^{-1}. The thermophilic cultures displayed nitrogen stoichiometry ratios typical for mesophilic anammox: 0.93-1.42 g $N\text{-}NO_2^-{}_{removed}$ g^{-1} $N\text{-}NH_4^+{}_{removed}$ and 0.16-0.35 g $N\text{-}NO_3^-{}_{produced}$ g^{-1} $N\text{-}NH_4^+{}_{removed}$. Amplicon and Sanger sequencing of the 16S rRNA genes revealed a disappearance of the original "*Ca.* Brocadia" and "*Ca.* Jettenia" taxa, yielding *Planctomycetes* members with only 94-95% similarity to "*Ca.* Brocadia anammoxidans" and "*Ca.* B. caroliniensis", accounting for 45% of the bacterial FBBR community. The long-term operation of thermophilic anammox reactors and snapshot views on the nitrogen stoichiometry, kinetics and microbial community open up the development path of thermophilic partial nitritation/anammox. A first economic assessment highlighted that

40 treatment of sludge reject water from thermophilic anaerobic digestion of sewage sludge may
41 become attractive.
42

Keywords: AnAOB; biological nitrogen removal; nitrification; nutrient removal and recovery; sludge reject water; packed-bed biofilm reactor

1. Introduction

Anammox, the anoxic oxidation of ammonium with nitrite, is a microbial process autotrophically producing nitrogen gas, catalyzed by members of the Planctomycetes phylum. Interest in anammox has grown substantially over the past decades in both research and application for wastewater treatment. From its first prediction based on thermodynamics (Broda, 1977), two decades passed before the first enrichment culture was characterized (Strous et al., 1999a) and anammox was proposed as a cost-efficient alternative to denitrification for the treatment of wastewaters lacking organic carbon (Jetten et al., 1997). By 2014, more than 100 full-scale installations had been built, treating high-strength nitrogenous wastewater with ratios of biologically degradable chemical oxygen demand to nitrogen (bCOD/N) <3, under mesophilic conditions (Lackner et al., 2014). Current advances towards applying anammox to the treatment of cold wastewaters, i.e. below 15°C, could even enable energy-autarkic treatment of municipal wastewater (Agrawal et al., 2018).

Thermophilic conditions have been widely explored for carbon removal wastewater treatment processes, revealing advantages over mesophilic treatment such as lower sludge production, higher conversion rates and a more effective inactivation of pathogens (Lapara and Alleman, 1999, Layden et al., 2007, van Lier, 1996)~~., e~~ Efforts to develop thermophilic technologies for N removal, however, are scarce and recent. Thermophilic nitrogen removal could nonetheless offer a viable alternative to current mesophilic practices in industries generating warm nitrogenous wastewater, avoiding the need for cooling prior to mesophilic treatment. The biotechnological possibility of thermophilic nitrification (nitritation and nitratation), and denitrification as well as denitritation have been recently demonstrated at lab-scale, revealing

the benefit of lower heterotrophic sludge production (Courtens et al., 2016a, Courtens et al., 2016b, Courtens et al., 2014, Vandekerckhove et al., 2019a, Vandekerckhove et al., 2018, Vandekerckhove et al., 2019b). For carbon-rich wastewater streams (bCOD/N>4), thermophilic nitrification/denitrification would enable high-temperature treatment. Carbon-lean streams such as thermophilic digestates, however, are suitable candidates for shortcut nitrogen removal processes. Nitritation/denitritation is one possibility, but when bCOD/N drops below 2-3, only partial nitritation/anammox (PN/A) would enable nitrogen removal without exogenous carbon dosage (Vlaeminck et al., 2012).

The application of anammox bacteria to treat wastewater under thermophilic conditions has hardly been explored. The maximum growth temperature seems to be dependent on the species, i.e., 37°C for "*Candidatus* Kuenenia stuttgartiensis" (Egli et al., 2001), 42.5°C for "*Candidatus* Jettenia caeni" (Ali et al., 2015), 43°C for "*Candidatus* Brocadia anammoxidans" (Strous et al., 1999b) and 45°C for "*Candidatus* Brocadia sinica" (Oshiki et al., 2011). Nevertheless, the evidence of the presence of anammox bacteria in high-temperature environments suggests that some uncultured strains may have higher temperature growth limits. Byrne et al. (2009) found molecular evidence of the presence of anammox bacteria in hydrothermal vents in the mid-Atlantic Ocean. The anammox activity was confirmed in incubations with samples from chimneys, performed at 60°C and 85°C. Jaeschke et al. (2009) detected traces of ladderane lipids (a biomarker for anammox bacteria) and found 16S rRNA genes closely related to "*Candidatus* Kuenenia stuttgartiensis" in sediment and mat samples from a Californian hot spring at 52°C. Additionally, molecular evidence of anammox bacteria has been found in a geothermal spring in Japan (50-62°C) (Hirayama et al., 2005) and in oil reservoirs in China (55-75°C) (Li et al., 2010). Recently, anammox activity at 50°C was reported for 2 weeks in an upflow anaerobic sludge-blanket (UASB) reactor, with a total nitrogen removal rate of 0.53 ± 0.23 g N L^{-1} d^{-1}. This was

achieved after implementing a temperature increase of 2.5°C every two weeks (corresponding to 0.18°C d^{-1}) (Zhang et al., 2018).

Although anammox activity has been shown at 50°C, long-term cultivation and characterization of the bacterial stoichiometry, kinetics and community remain absent. This study reports on the first long-term operation (>200 days) of a thermophilic anammox bioreactor (48°C), obtained by imposing a linear temperature increase (with 0.05 ± 0.01°C d^{-1}) to a mesophilic anammox inoculum. A first reactor type was biofilm based to achieve a sufficiently high biomass retention, implemented as a fixed-bed biofilm reactor (FBBR). In this bioreactor we evaluated the changes in microbial community and stoichiometry. A second reactor type was based on suspended sludge, a membrane bioreactor (MBR), using microfiltration to retain the biomass. With the FBBR enrichment as inoculum, the MBR were operated at 50°C, and the specific anammox activity and the first thermophilic anammox growth rate were determined. Finally, an economic assessment was performed of the treatment of sludge reject water from thermophilic anaerobic digestion of sewage sludge.

2. Materials and methods

2.1. Adaptation and characterization in a fixed-bed biofilm reactor (FBBR) at 48°C

An anoxic upflow bioreactor (3L) was used in this experiment. The reactor was composed of a cylindrical section of 5.5 cm diameter and 60 cm height, and a spherical settling section at the top of the column, with a diameter of 15 cm. The reactor was fed from the bottom, and the effluent discharge occurred through an overflow at the top of the reactor. The column was packed with carrier material (Kaldness K1 and ceramic rings), was inoculated with granules from a mesophilic (±37°C) full-scale bioreactor treating anaerobically digested potato wastewater and sludge reject water through PN/A (Olburgen, The Netherlands), provided by Paques BV (The Netherlands). The column reactor was placed in a temperature controlled room (34°C) to prevent temperature fluctuation in the environment and was heated >40°C by a hose coiled around the reactor connected to water circulating thermostatic bath (Julabo MA-4). The temperature of the thermostatic bath (with a sensitivity of 0.01°C) was raised with 0.05 ± 0.01°C d^{-1}, starting from 37.5°C until over 48°C (measured daily inside the reactor with a sensitivity of 0.1°C). The temperature was measured at the top of the column reactor. The reactor was continuously fed with a synthetic, autotrophic medium that consisted of NH_4HCO_3 and $NaNO_2$, $CaCl_2$ (0.1 g L^{-1}), $MgSO_4.7H_2O$ (0.2 g L^{-1}), NaH_2PO_4 (0.015 g P L^{-1}), yeast extract (4.5 mg L^{-1}) and 1 mL L^{-1} medium of trace element solution A (9.15 g L^{-1} $FeSO_4.7H_2O$ and 9.62 g L^{-1} $Na_2EDTA.2H_2O$) and trace element solution B (Third et al., 2001). The medium was not flushed with N_2 gas and had the temperature of the climate room (34**°C)**. Ammonium and nitrite were dosed at a nitrite-limiting ratio of 0.8-0.9g NO_2^--N g^{-1} NH_4^+-N, avoiding the accumulation of nitrite. Water samples were collected regularly, filtered over 0.2 µm pore-size syringe filters and stored at 4°C prior to analysis of NH_4^+, NO_2^- and NO_3^-. The pH was controlled at 7.6 *via* the addition of 0.2 M HCl.

In-situ maximum anammox activity ($VRR_{Ntot,max}$) tests were performed regularly. The $VRR_{Ntot,max}$ was calculated as the volumetric rate of total nitrogen removal over the test period (mg NH_4^+-N and NO_2^--N L^{-1} d^{-1}). During these tests, the continuous feeding was interrupted and substrates $NaNO_2$ and NH_4HCO_3 were spiked at 33 ± 15 mg NO_2^-- N L^{-1} and a NO_2^--N/NH_4^+-N ratio of 1. Liquid samples were taken regularly during 1-8 hours, depending on the N consumption rate, and stored at 4°C prior to analysis of NH_4^+, NO_2^- and NO_3^-.

2.2. Cultivation and characterization in membrane bioreactors (MBR) at 50°C

Two membrane bioreactors (MBR, 1L each reactor) were started up on days on days 354 and 430. The bioreactors were inoculated with biomass from the FBBR. The reactors were fitted with a hollow fibre microfiltration membrane, with a pore size of 100 nm, that allowed for effluent discharge with complete biomass retention in the bioreactors. The fibers originated from a full-scale PALL Microza installation (Asahi Kasei Chemicals Corporation, Japan). The reactor vessel was jacketed and the temperature was controlled at 50°C by use of a circulating thermostatic water bath (Julabo MA-4). In both MBR, mixing was provided by a magnetic stirrer and the pH was controlled at 7.6**.** In MBR_1 the pH was controlled by dosing 0.2 M HCl. In MBR_2 the pH was not controlled. Instead the medium was amended with 2 g $NaHCO_3$ L^{-1} and the reactor was continuously flushed with N_2/CO_2 (90/10, v/v) at a flowrate of ca. 100 ml min^{-1} providing a constant pH of 7.6. A water-lock in the exhaust gas line provided enough overpressure to prevent air intrusion in the bioreactor. This was found to be more reliable than dosing HCl and prevents acid overdose in case of failure of the pH controller. The reactors were not run in parallel.

The synthetic medium for the MBR was the same as the FBBR's feed and was continuously fed to the systems. As for the FBBR, nitrite was the limiting nitrogen substrate. Ammonium and nitrite **were** supplemented to the medium at ratios between 0.8 and 1.1 g NO_2^--N g^{-1}

NH_4^+-N. Liquid samples handling, and i*n-situ* activity measurements in MBR_2 were performed as previously described for the FBBR. A total of three activity tests was performed on operation day 100.

2.3. Physical/chemical analyses of water and sludge

Ammonium (Nessler method), total suspended solids (TSS) and volatile suspended solids (VSS) were measured according to standard methods (APHA, 1992). Nitrite and nitrate were determined on a 761 Compact Ion Chromatograph (Metrohm, Switzerland) as explained in the supplementary material. The temperature inside the reactor was regularly measured with a digital thermometer. pH inside the reactor was regularly checked with a digital pH probe (Orbisint CPS11D).

2.4. Molecular analyses of the microbial communities

Samples were collected from the inoculum and from the FBBR biomass at 43 and 48°C (operation days 120 and 390) for analysis of the microbial community by amplicon sequencing. On the sample from the FBBR at 48°C, Sanger sequencing was performed as well. The samples were stored at -20°C prior to DNA extraction. DNA extraction was performed as explained in the supplementary material.

2.4.1. Amplicon sequencing and data processing

Illumina 16S rRNA gene amplicon libraries were generated and sequenced by BaseClear BV (Leiden, the Netherlands). The DNA extracts were sent to BaseClear B.V. (The Netherlands) for 16S rRNA gene amplicon sequencing on the Miseq platform for bacteria as explained in the supplementary material.

2.4.2. Sanger sequencing

Primers targeting the Planctomycetes community were used, namely Pla40f (CGGCRTGGATTAGGCATG), Pla46f (GGATTAGGCATGCAAGTC), 1378r

(CGGTGTGTACAAGGCCCGGGAACG) and 1492r (TACGGYTACCTTGTTACGACTT). The amplification products were purified as indicated in Supplementary Material and were sent to LGC Genomics (Germany) for Sanger Sequencing.

2.5. The economic prospect of thermophilic PN/A

Even though only snapshot views on the kinetics and stoichiometry of thermophilic nitritation and anammox are available, a first cost assessment was performed to evaluate which factors could render thermophilic PN/A economically interesting. The chosen source of wastewater was high-temperature (± 50°C) sludge reject water, originating from thermophilic anaerobic digestion of primary and secondary sewage sludge. Capital and operational expenditure (capex and opex) were compared for its mesophilic (30°C) and thermophilic (50°C) PN/A treatment. Typical influent and effluent characteristics from full-scale mesophilic treatment of sludge digestion reject water were assumed for both (**Table S.1**, Lackner et al. (2014)). The treatment plant consisted of a continuously fed single-sludge reactor tank, at a biomass concentration of 4 kg VSS m^{-3}. A lamella separator was installed for sludge retention, with a mixed liquor recycle stream back to the reactor tank whereas the produced biomass was wasted via a waste flow to a centrifuge. Even though in practice, sludge wasting from a sidestream PN/A occurs only on limited occasions and is usually sent back to the digester and mainstream treatment unit, this economic assessment incorporated the cost for wasting the produced sludge to see what the difference would be between mesophilic and thermophilic PN/A. In the mesophilic PN/A scenario, a cooling installation was added to the design to lower the temperature of the thermophilic sludge reject water. Applied kinetics are summarized in Supplementary material, Table S.2. Capex involved cooling installations (only for the mesophilic scenario), civil works, equipment, mechanical and electrical works, piping, working hours and profit/risk. Opex comprised cooling, sludge thickening (electricity consumption centrifuge), chemicals, aeration, pumping, mixing, sludge disposal, personnel,

analyses and maintenance. All details on data and assumptions linked to influent characteristics, kinetics, process design and operation and depreciation can be found in Supplementary material, section S.3. Process diagrams of the mesophilic and thermophilic scenario, along with several process parameters can also be found in Supplementary material, section S.3.3.(**Figure S.4 and S.5**).

3. Results & discussion

3.1. Adaptation and characterization in a fixed-bed biofilm reactor (FBBR) at 48°C

In a first phase, a biofilm-based reactor was chosen to achieve a sufficiently high biomass retention, implemented as a fixed-bed biofilm reactor (FBBR). The FBBR, inoculated with mesophilic PN/A granules, was subjected to a loading rate of 276 ± 18 mg N_{tot} L^{-1} d^{-1} at a temperature of 37.5°C. After 3 days of temperature increase to 38.1°C, low concentrations of nitrite accumulated (1.5 ± 0.6 mg N L^{-1}) (Figure S.1), after which the temperature increase was stopped and the loading rate was lowered to 200 mg N_{tot} L^{-1} d^{-1} in order to prevent substrate accumulation. The *in-situ* batch activity measurements showed a decreasing maximum total nitrogen removal rate ($VRR_{Ntot,max}$) for the first 26 days (**Figure 1**). On day 39, $VRR_{Ntot,max}$ did not decrease relative to the previous $VRR_{Ntot,max}$ determination, after which the daily temperature increase of 0.05 ± 0.01°C d^{-1} was resumed.

The ratios $NO_2^-{}_{removed}/NH_4^+{}_{removed}$ and $NO_3^-{}_{produced}/NH_4^+{}_{removed}$ are common indicators of the anammox nitrogen stoichiometry, with reported values of 1.15-1.32 and 0.16-0.26 g N g^{-1} N respectively (Lotti et al., 2014c, Strous et al., 1999b). The $NO_2^-{}_{removed}/NH_4^+{}_{removed}$ ratio in the FBR remained at 1.16 ± 0.09 g N g^{-1} N as long as the temperature was below 43°C. Additionally, the nitrate production was lower than expected from anammox stoichiometry. During the first 40 days, the $NO_3^-{}_{produced}/NH_4^+{}_{removed}$ ratio was 0.02 ± 0.02 g N g^{-1} N at a

229 temperature of 38.1°C. This points towards endogenous denitrification, i.e. decay products in
230 the reactor acting as electron donors for nitrate reduction, as some members of the inoculum
231 microbial community depending on oxygen and/or organics likely could not survive under
232 the newly imposed anoxic autotrophic conditions (Wang et al., 2015).

233 After this start-up phase, the performance of the FBBR was stable at temperatures up to
234 43°C, with limited nitrite accumulation and a $VRR_{Ntot,max}$ of 703 ± 212 mg N L^{-1} d^{-1} (**Figure 1**
235 **and Figure S.1**). From day 40 onwards, the $NO_{3\ produced}^{-}/NH_{4\ removed}^{+}$ ratio was higher at 0.12 ±
236 0.02 g N g^{-1} N, but still below the typically reported range for anammox. From 43°C
237 onwards, total nitrogen removal rate in the reactor (VRR_{Ntot}) (45 ± 13 mg N L^{-1} d^{-1}) and
238 relative nitrate production (0.14 ± 0.06 g N g^{-1} N) remained low, with a $NO_{2\ removed}^{-}/NH_{4\ removed}^{+}$
239 ratio of 1.17 ± 0.30 g N g^{-1} N. The linear temperature increase was
240 maintained until 48°C, after which the activity increased again, with a $VRR_{Ntot,max}$ of 529 ±
241 103 mg N L^{-1} d^{-1}. The increase in anammox activity was accompanied by an increase in
242 nitrate production to 0.20 ± 0.03 g N g^{-1} N, which is within the typically reported range,
243 possibly suggesting a lower contribution of denitrification. The period of low activity could be
244 the consequence of low initial numbers of the thermophilic anammox species, that took over 100 days
245 to provide a measurable activity from the beginning of the linear T increase. The $NO_{2\ removed}^{-}/NH_{4\ removed}^{+}$
246 ratio remained stable at 1.03 ± 0.11 g N g^{-1} N at thermophilic temperatures,
247 slightly below the literature values. Overall, stable operation for over 200 days was achieved
248 at 48°C.

249 Key to the successful selection for thermophilic anammox bacteria, or adaptation of
250 mesophilic anammox bacteria to thermophilic conditions, was possibly the slow temperature
251 increase allowing the microorganisms to adapt to changing conditions. Previous studies
252 investigated temperature shocks and found that, for example, overheating from 35 to 48°C for
253 one hour resulted in anammox bacteria damage and death in an irreversible process, with

recovery requiring two weeks of operation back at 35°C (Liu et al., 2015). Another study examined the effect of a stepwise change of temperature between 35 and 46°C on an anammox sequencing batch reactor and found that it caused lysis of anammox cells after which almost no activity was found back. The activity recovered over a period of about 20 days and subsequently increased again after restoring the initial temperature of 35°C (Isanta et al., 2015). It was also found that thermophilic anammox bacteria could not be selected at 55°C from industrial coke-oven wastewater sludges (Toh et al., 2002). As opposed to the temperature shocks in previous studies, this study imposed a slow temperature increase lasting over 200 days, which enabled the development of a thermophilic anammox community with sustained activity up to 48°C.

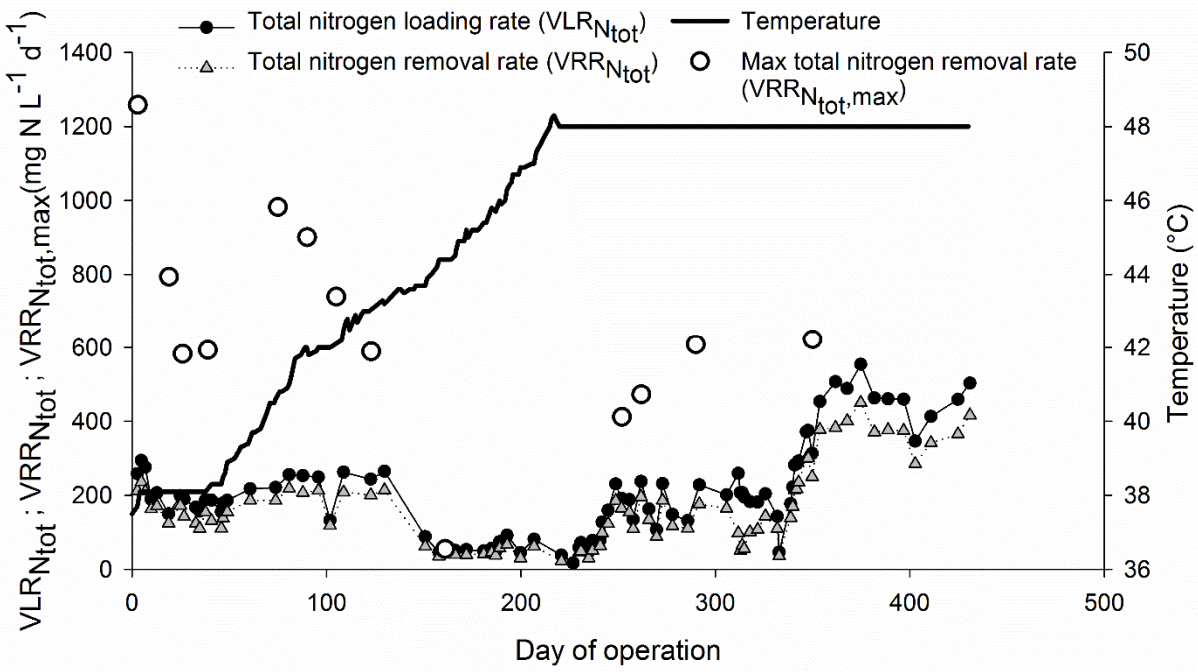


Figure 1: Reactor performance of the thermophilic anammox FBBR throughout the temperature increase and subsequent operation at constant high temperature of 48°C, together with the maximum *in-situ* total nitrogen removal ($VRR_{Ntot,max}$).

3.2. Stoichiometry and kinetics in membrane bioreactors (MBR) at 50°C

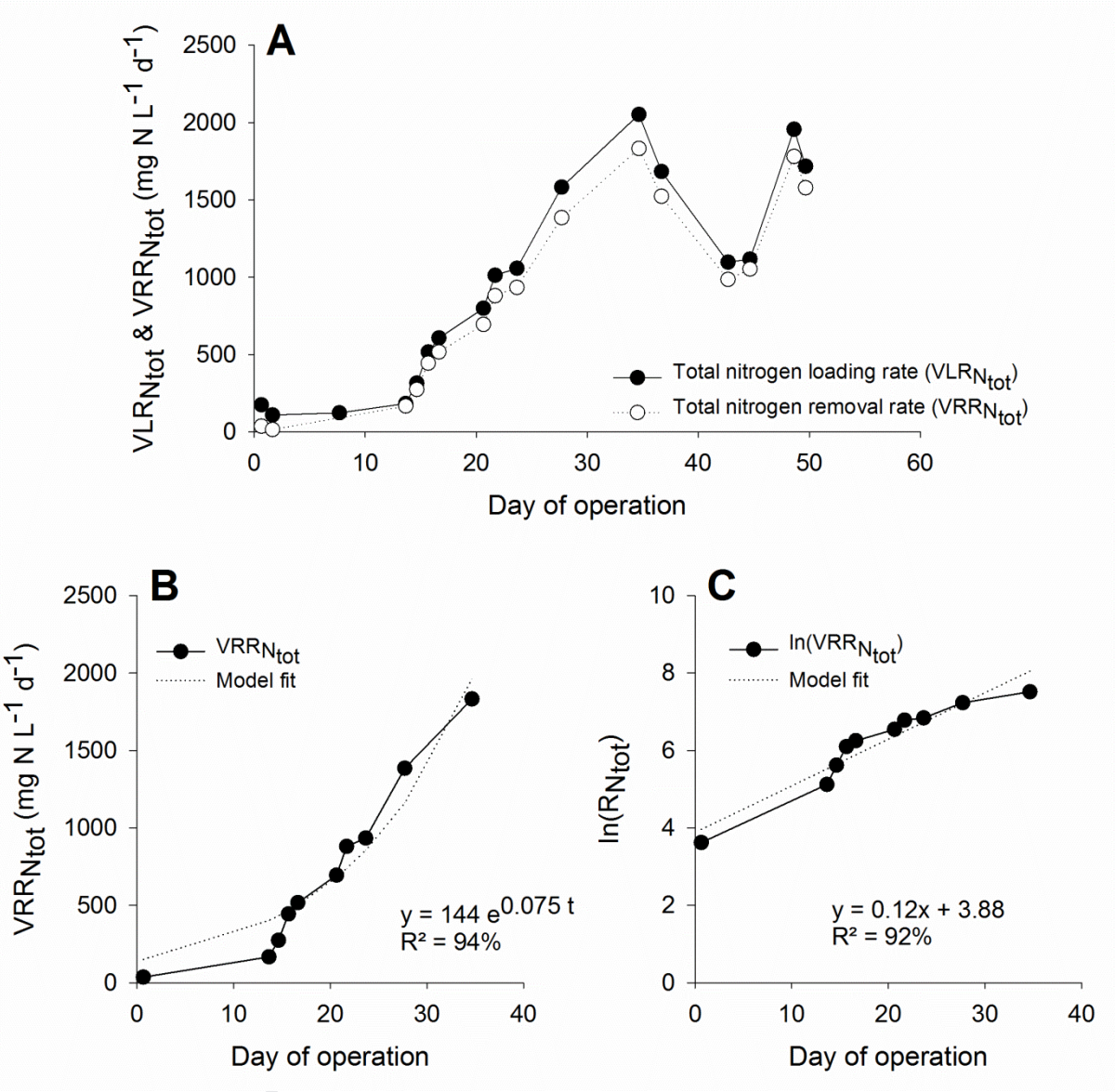
After obtaining anammox activity at 48°C in the FBBR, part of the culture was used to inoculate two membrane bioreactors operated at 50°C (MBR_1 and MBR_2). Cultivating anammox bacteria in these MBR enabled homogeneous sampling for the determination of biomass concentration, which was not feasible in the FBBR. This enabled the determination of several kinetic parameters, being the maximum biomass-specific anammox activity and the net growth rate (μ_{net}). Within 35 days, a high total nitrogen removal rate of 1.8 g N L^{-1} d^{-1} was achieved in MBR_1, with $NO_2^-{}_{removed}/NH_4^+{}_{removed}$ and $NO_3^-{}_{produced}/NH_4^+{}_{removed}$ of 1.14 ± 0.16 and 0.22 ± 0.05 g N g^{-1} N respectively, consistent with known mesophilic anammox stoichiometry (**Figure 2, A**). The obtained volumetric rates were about a factor 3 higher than a previous study achieving anammox activity at 50°C in a bioreactor using salt amendment for 2 weeks (0.53 ± 0.23 g N L^{-1} d^{-1}) (Zhang et al., 2018).

The increasing anammox activity during the first 35 days of MBR_1 operation was used to estimate the net growth rate (μ_{net}), the result of the actual growth rate and the decay rate. Over the activity increase, substrate levels were 28.7 ± 15.5 mg NH_4^+-N L^{-1} and 12.3 ± 17.9 mg NO_2^--N L^{-1}. However, the affinity indices of this culture were not known, so it is not sure that the anammox bacteria were growing at their maximum rate (μ_{max}). Nonetheless, μ_{net} is a valuable parameter, providing insight into the achievable thermophilic anammox growth rate.

Three different approaches were used for the estimation of μ_{net} at 50°C. First, an exponential model was fitted to the experimental data using the least squares method (**Figure 2, B**), yielding a μ_{net} of 0.075 d^{-1} (R^2=94%). Secondly, the total nitrogen removal rate was linearized by log-transformation and fitted by a linear equation using the least squares method (**Figure 2, C**) (van der Star et al., 2007), resulting in a μ_{net} of 0.12 ± 0.01 d^{-1} (R^2=92%). Thirdly, μ_{net} was calculated at every time interval (1) (Laureni et al., 2015), rendering an average value of 0.19 ± 0.18 d^{-1} (**Figure S.2**).

$$\mu_{net} = \frac{\ln\left(\frac{{}^{R}N_{tot,t_{n+1}}}{{}^{R}N_{tot,t_n}}\right)}{(t_{n+1}-t_n)} \quad (1)$$

Each method yielded a different μ_{net}, so caution should be used when using the estimated values. Nonetheless, the obtained μ_{net} range of 0.075-0.19 d^{-1} is after rescaling to 20°C (Arrhenius function with theta 1.1 (Seuntjens et al., 2018)) comparable to growth rates of anammox bacteria measured under mesophilic conditions (Table 1). When comparing reactors with high sludge retention time (SRT), the obtained μ_{net} in this study is relatively high compared to growth rates for mesophilic anammox bacteria. The high growth rates observed by Lotti et al. (2015) were obtained by imposing a low SRT to the bioreactor.



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304 **Figure 2:** (A) Reactor performance of MBR_1, with the imposed total nitrogen loading rate
305 ($VLR_{V,Ntot}$) and the total nitrogen removal rate ($VRR_{V,Ntot}$). The increasing anammox activity
306 was used to estimate the net anammox growth rate (μ_{net}) in MBR_1 based on (B) an
307 exponential fit to the experimentally determined VRR_{Ntot} and (C) a linear fit to the natural
308 logarithm of the experimentally determined VRR_{Ntot}, both using the least squares method.

309

310 **Table 1:** Overview of the anammox μ_{max} and doubling time at different temperatures and conditions, with their respective reactor SRT and
311 wastewater type. N/A: not determined. AnAOB: anammox bacteria (anaerobic ammonia oxidizing bacteria.

Temperature (°C)	SRT (d)	μ_{max} (d^{-1})	$\mu_{max,20°C}$[f] (d^{-1})	q_{max} (g NH_4^+-N g^{-1} VSS d^{-1})	AnAOB abundance (%)	q_{max} (g NH_4^+-N g^{-1} AnAOB-VSS d^{-1})	$q_{max,20°C}$[f] (g NH_4^+-N g^{-1} AnAOB-VSS d^{-1})	Doubling time (d)	Reference
10	60-140	0.005[a]	0.013	0.03	80	0.04	0.10	138.6	(Lotti et al., 2014b) [1]
12.5	>60	0.009[a]	0.018					77.0	(Laureni et al., 2015) [1]
15	60-140	0.009 [a]	0.014	0.10				77.0	(Lotti et al., 2014b) [1]
15	150	0.017[b]	0.027					40.8	(Lotti et al., 2014a) [2]
20	60-140	0.02 [a]	0.020	0.13	40	0.33	0.33	34.7	(Lotti et al., 2014b) [1]
29	>60	0.03[a]	0.013					23.9	(Laureni et al., 2015) [1]
30	3.0-4.1	0.24-0.33[c]	0.110	1.1-3.4	97	1.1-3.5	0.42-1.35	2.1-2.9	(Lotti et al., 2015) [2]
30	12	0.083[c]	0.032	2.01	98	2.05	0.78	8.4	(Lotti et al., 2014c)
32-33	N/A	0.06[d]	0.019	0.66[e]	74	0.89	0.28	10.7	(Strous et al., 1998) [2]
30-40	45-160	0.04-0.35[c]	0.047	0.25	55	0.45	0.11	2-18	(van der Star et al., 2007) [3]
35-40	N/A	0.098[d]	0.018					7.0	(Oshiki et al., 2011)[2]
38	12-16	0.062-0.084[b]	0.013					8.3-11.1	(van der Star et al., 2008) [2]
50	**N/A**	**0.075-0.19[a,b,g]**	**0.004-0.011**	1.1	**45[e]**	**2.4**	**0.14**	**3.6-9.2**	**This study** [2]

[a]: Calculated from the change in anammox activity over time
[b]: Based on a linear fit of the natural logarithm of increasing anammox rate
[c]: Calculated as 1/SRT
[d]: Based on maximum anammox activity (q_{max}) and biomass yield (Y)
[e]: Calculated from the maximum conversion capacity compared to steady state reactor operation
[f]: Normalized μ_{max} at 20°C using the Arrhenius equation with $\theta = 1.1$ (Seuntjens et al., 2018)
[g]: Not necessarily μ_{max} that was determined here (see section 3.2)
e: The AnAOB abundance of the FBBR was assumed in this calculation
[1]: Pretreated municipal wastewater
[2]: Synthetic medium
[3]: Centrifuged digestate

In MBR_2, operated for >100 days, the biomass was kept in suspension and the biomass-specific anammox activity could be determined. Anammox stoichiometry was observed in MBR_2, with $NO_2^-{}_{removed}/NH_4^+{}_{removed}$ and $NO_3^-{}_{produced}/NH_4^+{}_{removed}$ ratios of 1.10 ± 0.13 and 0.19 ± 0.03 g N g^{-1} N, respectively. The actual biomass-specific total nitrogen removal rates ($R_{X,Ntot}$) in the reactor 0.52 ± 0.26 g N g^{-1} VSS d^{-1} (**Figure S.3, B**). A maximum biomass-specific anammox activity of 1.1 ± 0.1 g NH_4^+-N g^{-1} VSS d^{-1} (1.8 ± 0.2 g N_{rem} g^{-1} VSS d^{-1}) was obtained from an *in-situ* activity measurement. These rates are higher than previously reported from reactors operated at SRT >45d under mesophilic conditions and are comparable to those obtained by Lotti et al. (2015) in a reactor operated at very short SRT (<3 d) (**Table 1**). However, when considering the anammox bacteria abundance and after rescaling to 20°C (Arrhenius function with theta 1.1 (Seuntjens et al., 2018)), this rate is in the lower range of reported values (**Table 1).** The biomass yield can be considered as the ratio of growth rate (0.075-0.19 d^{-1}) and the biomass specific anammox activity (1.1 g NH_4^+-N g^{-1} VSS d^{-1}). This results in an estimated biomass yield of 0.07 and 0.17 g VSS g^{-1} NH_4^+-N, which is comparable to the reported 0.12 g VSS g^{-1} NH_4^+-N (Strous et al., 1998). In this study, biomass was only extracted for occasional sampling, rendering an estimated SRT of ± 300 days. It remains to be investigated whether a shorter SRT control would render higher thermophilic activities as well.

3.3. A potentially new thermophilic anammox taxon

16S rRNA gene amplicon sequencing was used to characterize the bacterial community in the FBBR inoculum and in the reactor at 43 and 48°C (operation days 120 and 390) (**Figure 3, B and C**). The anammox bacteria in the inoculum were classified as "*Candidatus* Brocadia" and "*Candidatus* Jettenia" and accounted only for 1.5 and 1.3% of the microbial community, respectively. At 43°C, no "*Ca.* Jettenia" was detected, whereas "*Ca.* Brocadia" was present at a

relative bacterial abundance of 2.1% and was the most abundant anammox species. The loss of "*Ca. Jettenia*" could be attributed to its reported temperature range of activity, being slightly lower than reactor conditions (20-42.5°C) (Ali et al., 2015). The activity of "*Ca.* Brocadia", on the other hand, has been confirmed at a wider range of temperatures (25-45°C) (Oshiki et al., 2011). Thermophilic conditions (48°C) resulted in a bacterial community highly enriched in a "*Ca.* Brocadia"-related anammox species (46.4% of the total bacterial community). The reactor conditions (anoxic conditions and autotrophic medium) and the increasing temperature likely resulted in the selection and high abundance of this putative thermophilic anammox species. Previous research has also shown that a temperature shock on an anammox reactor resulted in an anammox enriched community after a recovery process, as opposed to a very diverse microbial community before the temperature shock (Isanta et al., 2015).

Parallel to 16S rRNA gene amplicon sequencing of the thermophilic community, Sanger sequencing was performed on the DNA sample of the FBBR at 48°C, using primers targeting the *Planctomycetes* community. Three out of four sequence pairs could be merged into contigs, all yielding a 96-97% similarity to an uncultured anammox bacterium from a geothermal spring in Japan (Hirayama et al., 2005). Two of the Sanger sequences (pla46f 1492r and pla40f 1492r) were used to construct a phylogenetic tree comparing the obtained Sanger sequences to all anammox bacteria reference sequences. The closest cultured relatives were "*Ca. Brocadia caroliniensis*" (94-95% similarity) and "*Ca. Brocadia anammoxidans*" (94-95% similarity) (**Figure 3A**). Considering the currently applied sequence similarity thresholds of 95% (for genus) and 98.7% (for species) (Rossi-Tamisier et al., 2015), we hypothesize that the community harboured a new anammox species. However, further research is necessary to establish its

phylogenetic novelty and to attribute the necessary (functional) genes to this *Planctomycete* to validate its association with the observed ammonium oxidation.

Both *Rhodocyclaceae* and RBG1 become abundant at 43°C, at the same time that the anammox activity, as observed through in-situ tests, collapses. The switch from a mesophilic temperature region to moderately thermophilic conditions caused a change in the whole microbial community in which mesophiles die or are washed out, while microorganisms that tolerate higher temperatures take over. The *Rhodocyclaecae* that became enriched at 43°C are affiliated to the genus *Denitratisoma*, that seem to have thrived by performing denitrification on the decay products of collapsing mesophiles. The RBG-1 are affiliated to the genus of *Zixibacteria* (Castelle et al., 2013). It has been found in anoxic sections in anoxic sediments, in the sulfate-methane transition zone (Baker et al., 2015), and has been suggested to scavenge reduced carbon products. It is capable of nitrate and nitric oxide reduction as well as of sulfate reduction (Momper et al., 2017). It seems plausible that it contributed to the denitrification of biomass decay products during this T transition period, just like *Rhodocyclaceae*.

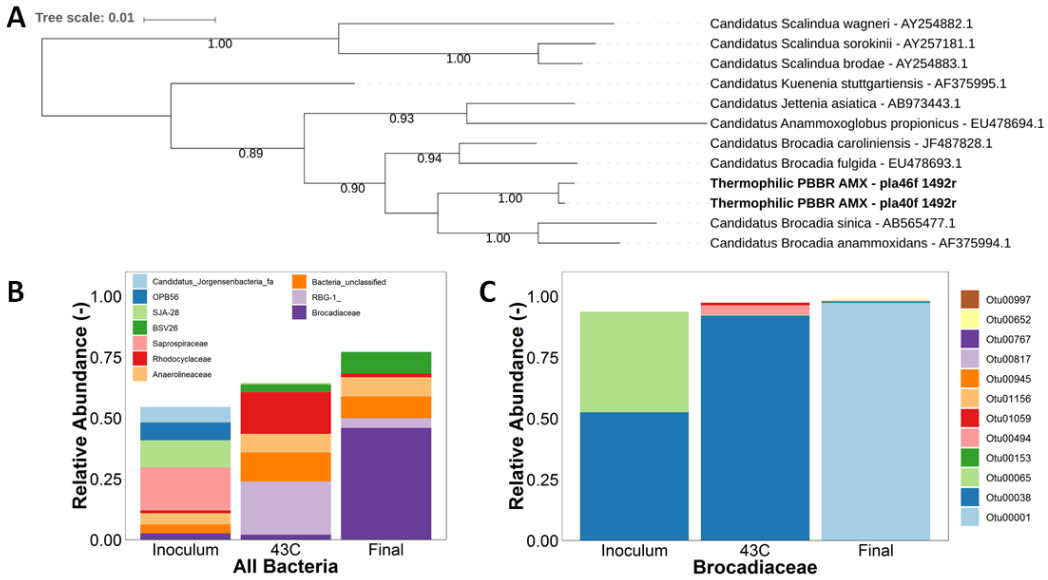

Figure 3: A: Phylogenetic tree displaying the evolutionary distance between the Sanger sequence of the 2 PCR products from the thermophilic FBBR (almost full 16S rRNA gene) and anammox bacteria reference sequences. The tree was built in MEGA7 using the maximum likelihood method with 500 bootstrap replications. The numbers at the nodes are the bootstrap values. B: Evolution of the relative abundance of anammox species in the FBBR during the transition from mesophilic to thermophilic temperatures. C: Shift in composition of the microbial community during the transition from mesophilic to thermophilic temperatures. In the inoculum, OTU38 and OTU65 were the two most abundant anammox species, classified as "*Candidatus* Brocadia" and "*Candidatus* Jettenia" respectively. At 43°C, OTU38 ("*Candidatus* Brocadia") was the most abundant anammox species and OTU65 ("*Candidatus* Jettenia") disappeared. At thermophilic conditions, a new anammox species replaced OTU38 and was classified as "*Candidatus* Brocadia".

3.4. Practical outlook

The presented proof of principle of thermophilic anammox biotechnology opens up a new route for thermophilic biological nitrogen removal, namely through PN/A. Thermophilic nitrification has been obtained in lab-scale bioreactors and its kinetics have been determined elaborately (Vandekerckhove et al., 2019a, Vandekerckhove et al., 2019b), setting the foundation for research on suppressing nitrite oxidation to obtain partial nitritation and integrating with thermophilic anammox in a single-sludge bioreactor. Thermophilic ammonia oxidation is

406 dominated by ammonia oxidizing archaea (AOA) (Vandekerckhove et al., 2019b). Only two
407 heterotrophic and one autotrophic thermophilic AOB has been isolated so far (Itoh et al., 2013,
408 Mevel and Prieur, 2000, Shimaya and Hashimoto, 2011). Kinetic comparison between
409 thermophilic AOA and mesophilic AOB revealed that thermophilic AOA had a higher biomass
410 yield, lower biomass-specific ammonia oxidation rate, lower growth rate and higher substrate
411 affinity (Vandekerckhove et al., 2019a, Vandekerckhove et al., 2019b). This first demonstration
412 of long-term cultivation of anammox bacteria under thermophilic conditions, with volumetric
413 activities and observed growth rates which are comparable to those reported under mesophilic
414 conditions (Table 1), suggests the feasibility of the application of anammox to warm
415 temperature.

416 The obtained maximum biomass-specific anammox activity in this study indicates that similar
417 rates might be possible in thermophilic compared to mesophilic sidestream anammox treatment
418 of, for example, reject water from thermophilic sludge digestion (De Vrieze et al., 2016).
419 However, more research is needed to evaluate the feasibility of thermophilic PN/A. First, the
420 economic prospect of thermophilic PN/A should be compared to current practices of cooling
421 thermophilic digestates prior to mesophilic PN/A. Secondly, more information on the anammox
422 biomass yield and decay rate is necessary to enable a better comparison to mesophilic kinetics.
423 Thirdly, the effect of organic carbon, pH, temperature fluctuations, free ammonia/free nitrous
424 acid, recurrent dissolved oxygen exposure and real wastewater matrices on the anammox activity
425 should be investigated as stepping stones towards thermophilic PN/A. Fourthly, considering the
426 high anammox activity compared to thermophilic AOA activity (1.1 vs. ca 0.2 g NH_4^+-N g^{-1} VSS
427 d^{-1}), the obtainable nitritation rate could be the limiting factor in a PN/A system and requires
428 further research. However, in mesophilic PN/A systems, nitritation is the limiting factor as well.

Finally, mesophilic reactor configurations implemented in practice include moving-bed biofilm reactors (MBBR), granular sludge processes and sequencing batch reactors (SBR) (Lackner et al., 2014). Considering the configurations tested in this study (FBBR and MBR), there is a need to evaluate more practically relevant reactors to facilitate the implementation.

3.5. The economic prospect of thermophilic PN/A

Even though limited data is available on the kinetics and stoichiometry of thermophilic nitritation and anammox, a first cost assessment was performed to evaluate which factors could render thermophilic PN/A economically interesting. The chosen source of wastewater was high-temperature (± 50°C) sludge reject water, originating from thermophilic anaerobic digestion of primary and secondary sewage sludge. Capital and operational expenditure (capex and opex) were compared for its mesophilic (30°C) and thermophilic (50°C) PN/A treatment.

Both scenarios showed a similar total treatment cost, with thermophilic PN/A cost only € 0.03 kg^{-1} N or 2.5% above the mesophilic one. Opex dominated the total treatment cost, amounting to 73 and 75% for the thermophilic and mesophilic scenario, respectively (**Table 2**). Capex and opex associated with influent cooling for the mesophilic scenario contributed only 5.1% to the total treatment cost, rendering limited savings attributed to avoided cooling for thermophilic treatment. The extra costs for thermophilic PN/A were mainly attributed to a higher reactor capex, due to the need for insulation, and an increased opex for sludge disposal. The extra cost for insulation is inevitable. The slightly higher sludge disposal cost **(9.5%)** was caused by the higher observed biomass production, mainly because thermophilic AOA have a higher maximum biomass yield (Y_{max}) and lower decay rate (k_d) than mesophilic AOB (Vandekerckhove et al., 2019a, Vandekerckhove et al., 2019b). However, knowledge on the Y_{max} and k_d of thermophilic

versus mesophilic ammonia-oxidizing organisms is still limited (Vandekerckhove et al., 2019a, Vandekerckhove et al., 2019b), which is also the case for anammox bacteria, rendering the need for a more thorough investigation to accurately predict the sludge production difference. Also, as sludge from a sidestream PN/A is usually sent back to the anaerobic digester and to the mainstream treatment unit, the actual impact on overall sludge disposal cost will be more limited. Currently, the expected thermophilic nitritation rate is below the rates observed in full-scale mesophilic PN/A systems. However, if the thermophilic nitritation rate could be increased to the mesophilic rate and thermophilic and mesophilic sludge production would be the same, the total treatment cost for thermophilic PN/A would be 2.3% cheaper than mesophilic PN/A. As current knowledge on thermophilic nitritation is still limited, further research could focus on obtaining higher rates, by investigating different reactor conditions or other microbial species with higher conversion rates.

Overall, the first economic estimation highlighted that thermophilic PN/A could be cost competitive to mesophilic PN/A. This means that warm wastewaters, for example reject water from thermophilic digestion, would not need cooling prior to mesophilic PN/A, but could be treated thermophilically in a cost-effective way. Thermophilic anaerobic digestion obtains higher conversion rates, higher biogas yield, higher reduction of pathogens and contributes to higher energy and nutrient recovery compared to mesophilic digestion (De Vrieze et al., 2016). Currently, the use of cooling equipment prior to mesophilic treatment is prone to failure. When the cooling does not work, a temperature shock would inhibit the PN/A process and result in treatment failure. Considering the importance of preventing treatment failure and preventing excessive amount of reactive nitrogen to be sent back to the mainstream treatment unit or to the environment, thermophilic PN/A would be a valuable alternative to the current practices. Also,

475 thermophilic conditions prevent the growth of mesophilic pathogens and enables a more efficient
476 hygienization of the wastewater (Lapara and Alleman, 1999). For example, legionella grows in
477 biofilms in wastewater treatment plants under mesophilic conditions (25-45°C), thermophilic
478 treatment would prevent growth of such pathogens (Caicedo et al., 2019).

479 Further research and development on thermophilic nitritation and anammox stoichiometry and
480 kinetics should enable a more accurate assessment.

481

Table 2: Estimation of capital and operational expenditure (capex and opex) for mesophilic and thermophilic partial nitritation/anammox (PN/A) on sludge digestion reject water from thermophilic anaerobic digestion of primary and secondary sewage sludge, with the most important differences underlined. Extensive details on data and assumptions linked to influent characteristics, kinetics, process design and operation and depreciation can be found in Supplementary material, section S.3.

	Mesophilic PN/A (30°C)			Thermophilic PN/A (50°C)		
	€	**€ kg^{-1} N$_{removed}$**	**%**	**€**	**€ kg^{-1} N$_{removed}$**	**%**
Capex						
Cooling installation	60 000	0.02	1.7	0	0.00	0.0
Civil works	188 214	0.05	4.2	348 057	0.10	8.2
Equipment	171 243	0.05	4.2	171 014	0.05	4.1
Mechanical works	27 500	0.01	0.8	27 500	0.01	0.8
Piping	62 250	0.02	1.7	62 250	0.02	1.6
Electrical works	80 000	0.02	1.7	80 000	0.02	1.6
Working hours	327 360	0.09	7.6	327 360	0.09	7.4
Profit / risk	147 302	0.04	3.4	172 205	0.05	4.1
Total capex	**1 063 869**	**0.30**	**25.2**	**1 188 387**	**0.33**	**27.0**
Opex						
Cooling		0.04	3.4		0.00	0.0
Centrifuge		0.01	0.8		0.01	0.8
Chemicals		0.09	7.6		0.09	7.4
Aeration		0.13	10.9		0.13	10.7
Pumping and mixing		0.03	2.5		0.03	2.5
Sludge disposal		0.21	17.6		0.23	18.9
Personnel		0.23	19.3		0.23	18.9
Analyses		0.03	2.5		0.03	2.5
Maintenance		0.11	9.2		0.12	9.8
Total opex		**0.89**	**74.8**		**0.89**	**73.0**
Total		**1.19**	**100.0**		**1.22**	**100.0**

4. Conclusions

Long-term thermophilic anammox reactor operation was demonstrated for the first time, both for ~~an~~ attached and suspended growth. In the FBBR, with maximum total nitrogen removal rates up to 0.62 g N L^{-1} d^{-1} (48°C), an adapted enrichment community was obtained by imposing a linear temperature increase (0.05-0.07°C d^{-1}) to a mesophilic PN/A inoculum. Kinetic characterization in subsequent MBR operation at 50°C revealed an estimated net growth rate between 0.075 and 0.19 d^{-1} and a maximum biomass-specific anammox activity of 1.1 ± 0.1 g NH_4^+-N g^{-1} VSS d^{-1}. When taking into account anammox bacteria abundance and rescaling to 20°C, however, these rates were relatively low compared to mesophilic anammox. In all reactors, the nitrogen stoichiometry known for mesophilic anammox was observed ($NO_2^-{}_{removed}/NH_4^+{}_{removed}$ 1.03-1.14 g N g^{-1} N; $NO_3^-{}_{produced}/NH_4^+{}_{removed}$ 0.20-0.22 g N g^{-1} N). Amplicon and Sanger sequencing of the 16S rRNA gene revealed *Planctomycetes* members with only 94-95% similarity to *Brocadia anammoxidans* and *B. caroliniensis*, accounting for 45% of the adapted thermophilic bacterial community in the FBBR. A first economic estimation revealed that thermophilic PN/A could be cost competitive to mesophilic PN/A. This novel process may present opportunities for the treatment of several wastewater types, for example reject water from thermophilic anaerobic digestion of sewage sludge.

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- A slow temperature increase on a mesophilic inoculum enabled thermophilic anammox
- A potentially novel species was highly abundant in the adapted community
- Stoichiometry and rescaled kinetics are low compared to mesophilic anammox cultures
- An attached and a suspended growth reactor were feasible
- Thermophilic partial nitritation/anammox may become economically cost-effective

Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: