Transitioning from mesophilic to thermophilic nitrification: shaping a niche for archaeal ammonia oxidizers

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ABSTRACT

The development of thermophilic nitrogen removal processes is a necessity for cost-effective biological treatment of warm nitrogenous wastewaters. Only one thermophilic nitrifying bioreactor was described so far, enriched for more than 300 days from compost samples and achieving 200 mg N L^{-1} d^{-1}. In practice, a more time-efficient development strategy based on mesophilic nitrifying sludge is preferred, since existing plants would be upgraded. This paper evaluates the adaptive capacities of mesophilic nitrifying sludge for several temperature transition strategies. Nitrification rates up to 150 mg N g^{-1} volatile suspended solids d^{-1} were achieved at a temperature up to 49°C by imposing a slow linear temperature increase to floccular sludge. This successful transition was related with a shift in ammonium oxidizing archaea dominating ammonia oxidizing bacteria. Furthermore, ex-situ temperature sensitivity measurements could be used to monitor and steer the process. Overall, this study reveals promising strategies to develop thermophilic nitrogen removal processes.

KEYWORDS: thermophilic, mesophilic, nitrification, ammonia oxidizing archaea.

INTRODUCTION

To sustain food production for the increasing global population and living standard, extensive production of inorganic nitrogen fertilizers is paramount (Erisman et al., 2008). This, however, resulted in the accumulation of reactive nitrogen species in many natural ecosystems, causing worldwide environmental problems (Galloway et al., 2014). Ammonia nitrogen is such a reactive species and is a major wastewater component, causing eutrophication and fish mortality when left untreated and discarded as such in water bodies (Camargo and Alonso, 2006). To alleviate the problems caused by ammonia, it must be removed from wastewater prior to discharge.

Nitrification is a key process in nitrogen removal and transforms ammonia to the less toxic nitrate. 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. Subsequently, nitrite-oxidizing bacteria (NOB) oxidize NO_2^- to NO_3^-, referred to as nitratation. To fully remove nitrogen from wastewater, nitrification is conventionally followed by denitrification to reduce NO_3^- to N_2 gas. Cost-beneficial short-cut nitrogen removal processes such as partial nitritation/anammox (PN/A), however, are gaining importance over the last years (Lackner et al., 2014, Vlaeminck et al., 2012).

Although nitrification is a well-studied process, used all over the world for the treatment of ammonia-containing wastewater, applications above 40°C represent a significant challenge. Thermophilic nitrogen converting organisms have been detected, thriving in natural environments with temperatures beyond 40°C such as hot springs (de la Torre et al., 2008). These organisms are proof that thermophilic nitrification is possible, revealing opportunities for thermophilic biotechnology for nitrogen removal. Experiences with carbon treatment 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). The development of thermophilic nitrogen removal could enable the treatment of wastewater in several niches such as (i) specific industries delivering warm wastewater linked to the production process or pre-treatment of the wastewater , (ii) sites where waste heat can be used to warm the wastewater, and (iii) exotic, low-income countries, where the climatic conditions give rise to several types of hot wastewater.

Two approaches can be considered to develop thermophilic nitrification: complementing a thermophilic microbial enrichment strategy with the implementation of the obtained communities in a thermophilic reactor and applying a temperature increase to a mesophilic process. 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, the enrichment of autotrophic thermophilic nitrifiers from compost samples and the subsequent successful application of these enrichments in a thermophilic nitrifying bioreactor at 50°C has recently been proven (Courtens et al., 2016a). Although proven, the low growth rate and/or low relative abundance of these nitrifiers in environmental samples may result in long enrichment processes. From a practical point of view, in which existing treatment facilities would be upgraded, the second strategy may prove more feasible where mesophilic nitrifying communities are adapted to elevated temperatures. Applying a stepwise temperature increase to a moving bed biofilm reactor (MBBR) from 30 to 40°C (10°C d^{-1}) was successful to maintain complete nitrification (Shore et al., 2012). A stepwise increase of 15°C d^{-1} from 30 to 45°C, however, proved unsuccessful resulting in a loss of nitrifying activity. Imposing smaller temperature differences can abate this issue and achieve higher nitrification temperatures. Imposing a temperature slope of 2.5°C d-1 from 40°C onward resulted in slightly higher temperatures (42.5°C) (Courtens et al., 2014). Moreover, salt amendment was brought forth as a tool for a more efficient temperature transition for nitrification.

Actual termophilic nitrification (>45°C), however, could not be reached using temperature slope >2.5°C d^{-1}, although short-term nitrification activity measured in batch-tests showed potential up to 50°C (Lopez-Vazquez et al., 2014).

Since previous research showed potential for thermophilic nitrification through adaptation of mesophilic sludge, a recent study explored the adaptive capacities of mesophilic nitrifying sludge to more gradual temperature increase patterns. In a first reactor experiment, a non-oscillating linear temperature increase (0.25 °C d^{-1}) was compared with an oscillating increase (amplitude 2°C) with the same final slope. In a second experiment, a linear temperature increase with a lower slope (0.08°C d^{-1}) was investigated and floccular growth (SBR) was compared with a biofilm based growth (MBBR). Finally, the nitrifying community was closely monitored by ex-situ batch activity tests and molecular analysis during the linear temperature increase to elucidate the adaptation process or shifts in the microbial community and selection towards thermophilic species. More details can be found in the paper, published in the ISME Journal (Courtens et al., 2016b).

MATERIAL AND METHODS

In a 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 a 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, filled with polyvinyl alcohol (PVA)-gel beads (Kuraray Japan) at a volumetric filling ratio of 15%. All reactors (2L) were inoculated with the same commercial nitrifying inoculum (Avecom NV, Ghent, Belgium) and fed with a synthetic medium 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 elements solution dissolved in tap water. The pH was controlled between 6.5 and 7.5 by dosage of 0.1 M NaOH/HCl.

During the second experiment, the abundance of selected key groups of nitrifying microorganisms was assessed along the temperature increase by means of qPCR. The overall community structure was analyzed using paired-end high-throughput sequencing (MiSeq Illumina platform). Sludge production was evaluated through the observed sludge yield, calculated using cumulative terms.

Temperature sensitivity measurements were performed at several reactor temperatures, in which the specific ammonia and nitrite oxidizing activities were measured at the respective reactor temperature ±2°C. For the SBR_3 sludge, 96-well plates with a working volume of 250 µL were used, while the MBBR carriers were transferred to 24-well plates with a working volume of 1.5-2.5 mL. Plates were incubated at the specific temperature, in which oxygen was provided through intensive shaking at 600 rpm. The buffer solution (pH 7) contained final concentrations of 2 g P L^{-1} (KH_2PO_4/K_2HPO_4), 1 g $NaHCO_3$ L^{-1} and 60 mg N L^{-1} ($(NH_4)_2SO_4$ or $NaNO_2$).

More details on the methodology can be found in the corresponding publication (Courtens et al., 2016b).

RESULTS AND DISCUSSION

Overall performance

The main results of the reactor performance during the different experiments, are summarized in Table 1. Overall, within the range of the tested parameters/combinations in this study, the highest temperature (49°C) with moreover the highest volumetric (800 mg N L^{-1} d^{-1}) and specific (170 mg N g^{-1} VSS d^{-1}) nitrification rates were achieved by implementing a slow, non-oscillating linear temperature increase (0.08°C d^{-1}) (SBR_3).

In parallel, a clear shift in optimum temperature was observed with the ex-situ activity measurements. These high-throughput activity tests could also predict the loss of ammonium and nitrite oxidation during the temperature transition, rendering it feasible to adjust the temperature slope when necessary and steer the temperature increase strategy.

Temperature increase pattern

Pre-exposure to a certain stress can result in an increased resilience to a secondary exposure (Li et al., 2014, Philippot et al., 2008). In this case, temperature is the stress factor and oscillations could induce the production of heat-shock proteins (HSP), which might protect the biomass during a subsequent temperature elevation and increase the adaptive capacity. This study, however, showed that the tested oscillating temperature pattern did not improve the adaptive capabilities of mesophilic nitrifying sludge towards higher temperatures. The tested amplitude of 2°C was possibly too high to observe beneficial effects. A linear temperature slope proved more successful, although it is evident that a low slope has to be applied, since 0.25°C d^{-1} resulted in a loss of activity at 45°C. Nevertheless, a linear increase was clearly more successful than a stepwise temperature pattern, reaching nitrification no higher than 40 and 42.5°C (Courtens et al., 2014, Shore et al., 2012).

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 (Courtens et al., 2016b). 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.

Reactor (type)	Experiment 1		Experiment 2	
	SBR_1	SBR_2	SBR	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	
Flow rate (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[a]				
Volumetric (mg N L^{-1} d^{-1})	26 ± 5	90 ± 3	794 ± 57	309 ± 30
Specific (mg N g^{-1} VSS d^{-1}	72[b]	139 ± 18	151 ± 7	67[b]

[a] In all cases, nitrite accumulation was negligible and nitrate formation >90% of ammonium removal.

[b] Only one biomass measurement available for the specific period.

Sludge growth mode

Thermophilic organisms exhibit a slow growth, by which biomass retention is essential. A biofilm reactor might favour the retention and the proliferation of thermophilic nitrifiers. Additionally, biofilms show an increased resistance to many types of environmental trials (Gilbert et al., 2002). The better resistance of biofilms towards antibiotics, for example, was mainly due to the restricted diffusion (Mah and O'Toole, 2001). Other factors, such as slow growth rate, high culture density and heterogeneity, were shown to influence the general stress response in biofilms as well (Mah and O'Toole, 2001, Ryall et al., 2012). Results obtained in this study, however, were not in line with the hypothesis, since nitrifying activity was lost around 46°C in the MBBR opposed to 49°C in the SBR_3.

Importance of functional community

The successful transition of SBR_3 seems to be related to a shift in abundance of ammonia oxidizing bacteria (AOB) to archaea (AOA), which was not completely achieved in the MBBR (Figure 1B). A shift towards AOA dominance is in accordance with literature, where most thermophilic ammonia oxidizing organisms described are archaeal (de la Torre et al., 2008, Hatzenpichler et al., 2008, Lebedeva et al., 2013). No shift in nitrite oxidizing bacteria was observed, *Nitrospira* was dominant in both reactors throughout the experiment.
Several hypotheses are brought forth that could explain the delayed AOA selection in the MBBR such as a stronger selection for fast growing AOB during stabilization, a lower AOA abundance after stabilization and differences in levels and dynamics of dissolved oxygen and NH_4^+ concentrations eliciting differential stimulation of AOA vs. AOB. Although the increasing trend in AOA did not fully came through in the MBBR, it does imply that the essential shift could occur with an even lower slope of temperature increase.

This dominance shift was further accompanied by an increase in observed biomass yield. 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.

The presence of AOA is, thus, of great importance to obtain thermophilic nitrification. The fact that AOA appear to be distributed in wastewater treatment facilities all over the world, in equal or higher abundance than AOB, displays the prospects for this transition strategy (Limpiyakorn et al., 2013).

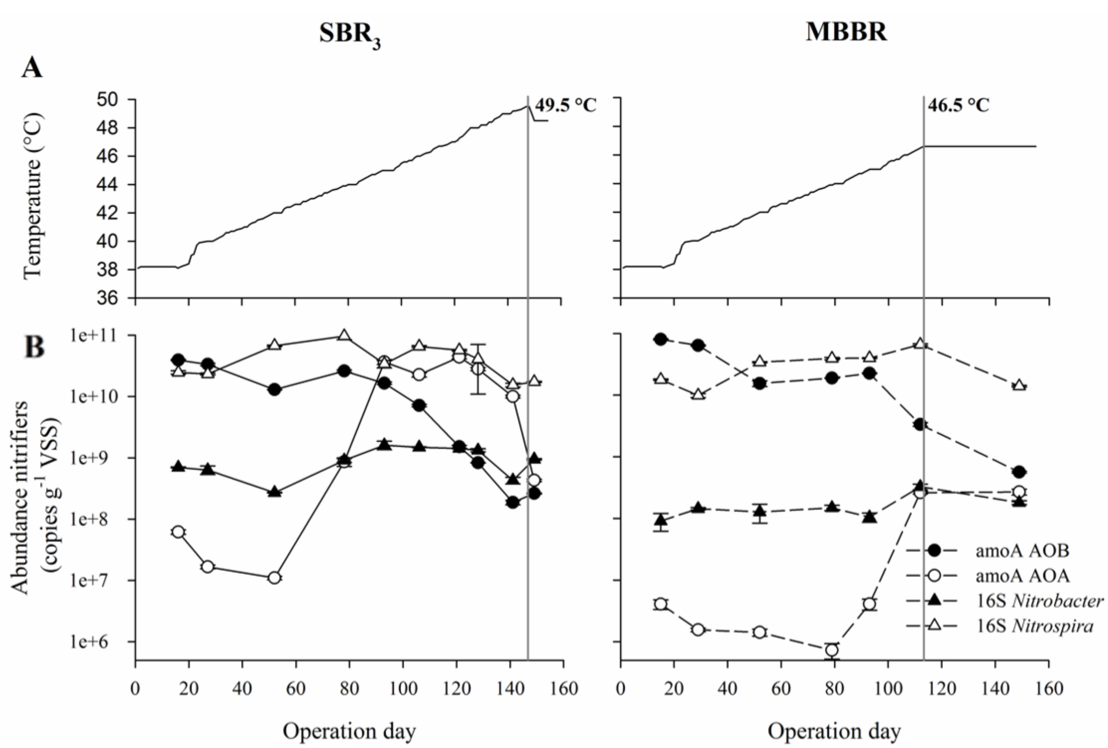

Figure 1. Operation and performance characteristics of SBR_3 (left) and MBBR (right). **(A)** Temperature increase patterns. **(B)** Abundance of nitrifiers as determined by qPCR.

CONCLUSION

Overall, this study achieved nitrification at 49°C within 150 days by gradual adaptation (0.08°C d^{-1}) of mesophilic sludge, related to a dominance shift from AOB to AOA. Furthermore it is shown that ex-situ temperature sensitivity screening can be used to monitor and steer the transition process. Upgrading existing mesophilic nitrifying wastewater plants to thermophilic systems can, thus, be feasible.

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