

# The rise of thermophilic biotechnology for nitrogen removal

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## ABSTRACT

The development of thermophilic nitrogen removal processes is a necessity for cost-effective biological treatment of warm nitrogenous wastewaters. Furthermore, other thermophilic processes are known to be more stable, to achieve higher specific rates (smaller bioreactors), to produce less biological sludge and to accomplish better hygienization. These advantages render it possible to treat colder wastewater on sites with excess heat as well. Just until recently, there were no thermophilic nitrogen converting bioreactors described, the main thermophilic nitrogen removal mechanisms were assumed to be ammonia stripping and nitrogen assimilation into biomass. Two approaches were considered for the development of this new thermophilic process: (i) complementing a thermophilic microbial enrichment strategy with an implementation of the obtained communities in a thermophilic reactor ('synthetic combination strategy'), and (ii) applying a temperature increase to a mesophilic process ('temperature evolution strategy'). This paper provides an overview of the recent milestones reached in this development process.

**KEYWORDS:** thermophilic, mesophilic, nitrification, denitrification, wastewater treatment

## INTRODUCTION

To sustain food production for the increasing global population and living standard, extensive production of inorganic nitrogen fertilizers is critical (Erisman et al., 2008). However, the human disruption of the nitrogen cycle has by far exceeded the safety boundaries of our planet, rendering it impossible for Earth to cope with the massive N flows (Steffen et al., 2015). This resulted in the accumulation of reactive nitrogen species in many natural ecosystems, causing global environmental problems (Galloway et al., 2014). Ammonia nitrogen is one of the reactive nitrogen 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 ( $\text{NH}_4^+$ ) to nitrite ( $\text{NO}_2^-$ ), also known as nitritation. Subsequently, nitrite-oxidizing bacteria (NOB) oxidize  $\text{NO}_2^-$  to  $\text{NO}_3^-$ , referred to as nitrataion.

To fully remove nitrogen from wastewater, nitrification is conventionally followed by denitrification to reduce  $\text{NO}_3^-$  to  $\text{N}_2$  gas. Cost-beneficial short-cut nitrogen removal processes such as partial nitrification/anammox (PN/A), however, are gaining importance over the last years (Lackner et al., 2014, Vlaeminck et al., 2012).

Although complete nitrogen removal is a well-studied process, used all over the world for the treatment of ammonia-containing wastewater, applications above  $40^\circ\text{C}$  represent a significant challenge. The ways in which nitrogen is removed thermophilically, were assumed to be ammonia volatilization ( $65\pm 14\%$ ) and nitrogen assimilation into biomass ( $14\pm 4\%$ ) (Abeynayaka and Visvanathan, 2011a, b, Kurian et al., 2005, Yi et al., 2003). As ammonia stripping shifts the problem to the gas phase and the C/N ratio in high-strength nitrogenous wastewaters is too low to obtain sufficient nitrogen removal based on assimilation, an opportunity is revealed for thermophilic biotechnology for nitrogen removal.

Thermophilic nitrogen converting organisms have been found in extreme environments all over the planet and unravel a treasure for thermophilic nitrogen removing biotechnology. Denitrifiers have been isolated from soil (*Bacillus thermodenitrificans*), mud (*Geobacillus* sp.) and from manure amended soil (*Anoxybacillus pushchinensis*) (Mishima et al., 2009, Mora et al., 1998, Yamamoto et al., 2006). Besides the reductive process, representatives of the oxidative pathway of nitrification have been found as well. Both AOA (*Candidatus Nitrosocaldus yellowstonii*, *Candidatus Nitrososphaera gargensis*) and NOB (e.g. *Nitrospira calida*) have been isolated from warm environments (de la Torre et al., 2008, Hatzenpichler et al., 2008, Lebedeva et al., 2011). Up till now, these representatives of the thermophilic nitrogen cycle have been studied separately in batch cultures. Few actions have been taken to attempt a bioreactor, combining the thermophilic processes for biotechnological nitrogen removal in wastewater. Only one study accomplished a thermophilic denitrifying reactor for wastewater treatment, inoculated with thermal mud from a hot spring (Laurino and Siñeriz, 1991). Attempts to achieve thermophilic nitrification, using a mesophilic inoculum, never reached temperatures above  $42^\circ\text{C}$  (Shore et al., 2012). 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, thus, enable cost-effective 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.

This review provides an overview of the recent milestones achieved in the creation of thermophilic biotechnology for nitrogen removal. Two approaches were considered: (i) complementing a thermophilic microbial enrichment strategy with an implementation of the obtained communities in a thermophilic reactor ('synthetic combination strategy'), and (ii) applying a temperature increase to a mesophilic process ('temperature evolution strategy').

## **SYNTHETIC COMBINATION STRATEGY RESULTS IN THE FIRST EVER THERMOPHILIC NITRIFYING BIOREACTOR**

As stated earlier, thermophilic nitrogen converting organisms are ubiquitous. Representatives of both steps in the nitrification process, AOA and NOB, have been isolated and characterized. However, investigating the biotechnological potential of thermophilic nitrification requires the coupling of thermophilic ammonia and nitrite oxidizing organisms in a bioreactor. Only a few bioreactor studies focused on the long-term establishment of coupled thermophilic nitrification, but used mesophilic biomass and never reached temperatures higher than 42°C (Shore et al., 2012). Furthermore, the reported thermophilic nitrogen-converting organisms so far exhibit low substrate and product inhibition levels, rendering them rather unsuitable for the application in robust biotechnology (Hatzenpichler et al., 2008, Lebedeva et al., 2011). The newly characterized *Nitrolancea hollandica*, belonging to the phylum *Chloroflexi*, is an exception showing higher substrate inhibition levels (Sorokin et al., 2014).

A recent study, however, took a closer look to the coupling of thermophilic nitrifying organisms in biotechnology (Courstens et al., 2016a). It described the enrichment of autotrophic thermophilic nitrifiers from compost samples and the successful operation of a thermophilic nitrifying bioreactor with high biotechnological potential. Compost is known to contain archaeal amoA genes and even *Candidatus Nitrososphaera gargensis*-like sequences, a thermophilic AOA (Maeda et al., 2011, Oishi et al., 2012, Yamamoto et al., 2011, Zeng et al., 2011). However, no autotrophic thermophilic nitrifiers were enriched from compost yet, only one heterotrophic AOB related to *Bacillus halodurans* has been isolated from animal waste composting (Shimaya and Hashimoto, 2011).

### **Batch enrichments lead to a high-rate nitrifying bioreactor**

Different aerobic compost facilities were sampled during the thermophilic stage (50-70°C) (Courstens et al., 2016a). These samples served as inoculum for batch enrichment incubations at 50°C. The compost enrichments showing both  $\text{NH}_4^+$  and  $\text{NO}_2^-$  oxidation were transferred to a bioreactor (2L), operated in a sequencing batch feeding/withdrawal mode and fed with synthetic medium consisting of both ammonium and nitrite. The batch enrichment lasted about 1 year, after which the bioreactor was inoculated. In the first two months, volumetric nitrification rates were low ( $4.7 \pm 2.6 \text{ mg N L}^{-1} \text{ d}^{-1}$ ). Afterwards, an exponential increase led to high, stable nitrification rates after more than 1 year of reactor operation ( $>200 \text{ mg N L}^{-1} \text{ d}^{-1}$ ). A nitrogen balance confirmed that nitrification was the main process causing ammonia oxidation, since nearly all the removed  $\text{NH}_4^+\text{-N}$  was recovered as  $\text{NO}_3^-\text{-N}$  ( $93 \pm 4\%$ ). The nitrifying biomass was physiologically characterized and showed high specific oxidation rates of ammonia ( $198 \pm 10 \text{ mg N g}^{-1} \text{ VSS d}^{-1}$ ) and nitrite ( $894 \pm 81 \text{ mg N g}^{-1} \text{ VSS d}^{-1}$ ), both in the same order of magnitude as related nitrifiers (Kim et al., 2012, Nowka et al., 2015).

### **Selective FA/FNA inhibition opens up opportunities for short-cut nitrogen removal**

In addition, inhibitory levels of free ammonia (FA) and free nitrous acid (FNA) were evaluated (Courtens et al., 2016a). For the development of biotechnological applications and process control strategies, the differentiation was made between the inhibitory effect of  $\text{NH}_4^+$ /FA and  $\text{NO}_2^-$ /FNA. Since different operating conditions can lead to different FA/FNA concentrations at equal levels of  $\text{NH}_4^+$ / $\text{NO}_2^-$ , it is vital to make this distinction. Both AOA and NOB were sensitive to FA but insensitive to  $\text{NH}_4^+$ , resulting in half-maximal inhibitory concentrations ( $\text{IC}_{50}$ ) for FA of 7.5 and 5.0 mg  $\text{NH}_3\text{-N L}^{-1}$  respectively. Maximum inhibition of the *Candidatus Nitrososphaera gargensis*-like AOA in this bioreactor did not exceed  $63 \pm 5\%$  from 8.5 mg  $\text{NH}_3\text{-N L}^{-1}$  onward. At neutral pH and temperature of  $50^\circ\text{C}$ , this corresponds with a concentration of 300 mg  $\text{NH}_4^+\text{-N L}^{-1}$ , which is 7 times higher than the inhibitory  $\text{NH}_4^+$  concentration reported earlier for *Candidatus Nitrososphaera gargensis* (Hatzenpichler et al., 2008). An explanation for this high FA tolerance might be the fact that the initial samples originate from a nutrient-rich compost, in contrast with the oligotrophic conditions in the extreme environments. Indeed, AOA detected in cattle manure compost, showed a higher tolerance toward higher  $\text{NH}_4^+$  concentrations as well (Oishi et al., 2012). Regarding  $\text{NO}_2^-$ /FNA inhibition, AOA were clearly inhibited by  $\text{NO}_2^-$  but not by FNA, since equal levels of  $\text{NO}_2^-$  with different levels of FNA yielded the same inhibition response with increasing  $\text{NO}_2^-$  concentrations. However, sensitivity was very low, given the  $\text{IC}_{50}$  of 2117 mg  $\text{NO}_2^-\text{-N L}^{-1}$ . On the contrary, NOB were extremely sensitive to FNA and not to  $\text{NO}_2^-$  ( $\text{IC}_{50}$  of 0.001 mg  $\text{HNO}_2\text{-N L}^{-1}$ ). The higher sensitivity of NOB towards FA, and especially towards FNA, opens up opportunities for cost-efficient short-cut nitrogen removal processes such as nitrification/denitrification and partial nitrification/anammox.

### **Phylogeny, morphology and carbon incorporation prove the importance of autotrophic nitrifiers**

The thermophilic nitrifying community in the bioreactor was determined using 16S rRNA Illumina sequencing, and consisted of an AOA and NOB closely related to *Candidatus Nitrososphaera gargensis* and *Nitrospira calida* respectively (Courtens et al., 2016a), both originally isolated from the Garga hot spring (Russia) (Hatzenpichler et al., 2008, Lebedeva et al., 2011). No AOB could be found, suggesting a dominance of AOA over AOB at thermophilic temperatures. The presence of both species was confirmed using transmission electron microscopy, revealing cells with identical morphologies to *Nitrospira* spp. and *Candidatus Nitrososphaera gargensis*. Furthermore, autotrophic growth was demonstrated by  $^{13}\text{C}$ -labeled bicarbonate incorporation into crenarchaeol and 11-methyl C16:0, characteristic membrane lipids of the observed AOA and NOB respectively (Lipski et al., 2001, Pitcher et al., 2010). The  $^{13}\text{C}$  incorporation suggests that *Candidatus Nitrososphaera gargensis* and *Nitrospira calida* both played an important role in the biomass community for nitrification. However, an abundant cell type, embedded in a dense biofilms structure, could not be identified. Moreover, the abundance of the AOA over time was very heterogeneous. This might suggest that an uncharacterized ammonia-oxidizing organism was present as well, as was recently described in reactors with low dissolved oxygen concentrations (Fitzgerald et al., 2015).

Overall, the successfulness of the synthetic combination strategy for the development of thermophilic nitrification is demonstrated. Although not very time-efficient, it reports the first ever thermophilic coupled nitrifying bioreactor with high biotechnological potential. The biomass in the reactor, however, particularly grew on the reactor walls. It has been stated that thermophilic aerobic wastewater treatment suffers from poor settling behavior (Suvilampi and Rintala, 2003). The slow growth of thermophilic nitrifiers, initially low biomass concentration and the sequencing batch reactor (SBR) configuration might have hindered sufficient floccular biomass retention, causing a selective pressure for the organisms to grow in a biofilm on the reactor walls to prevent washout. To enable a more practical reactor for further research, the reactor was transformed to a continuous MBR configuration by inserting a submerged hollow fiber membrane unit.

## **SUCCESSFUL TEMPERATURE EVOLUTION STRATEGY FROM MESOPHILIC TO THERMOPHILIC NITRIFICATION**

From a practical point of view, in which existing plants would be upgraded, the synthetic combination strategy is rather time-consuming. A more time-efficient development would be preferred, in which existing mesophilic nitrifying communities are adapted to elevated temperatures. The investigation of the thermotolerance of mesophilic nitriiers and the quest for a successful transition strategy have been picked up by several researchers. Stepwise elevation of nitrification temperatures was the first attempted strategy. Short-term activity measurements showed nitrifying potential up to 50°C, displaying promising opportunities for the stepwise strategy (Lopez-Vazquez et al., 2014). Retaining nitrification activity on a long-term, however, proved to be more difficult. Imposing a stepwise temperature increase to a moving bed biofilm reactor (MBBR) from 30 to 40°C (10°C d<sup>-1</sup>) was successful to maintain complete nitrification (Shore et al., 2012). An increase of 15°C d<sup>-1</sup> from 30 to 45°C, however, resulted in a loss of nitrifying activity. Slightly higher temperatures (42.5°C) were reached by implementing smaller temperature differences (2.5°C) from 40°C on (Courstens et al., 2014a). Although these studies never achieved 'real' thermophilic (>45°C) nitrification, they reveal the importance of the magnitude of temperature increase.

Taking this into account, the most recent study explored the adaptive capacities of mesophilic nitrifying sludge to more gradual temperature increase patterns (Table 1) (Courstens et al., 2016b). In a first reactor experiment, a non-oscillating linear temperature increase (0.25 °C d<sup>-1</sup>) 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<sup>-1</sup>) 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.

**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 ( $\pm 3$  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 <sub>1</sub>	SBR <sub>2</sub>	SBR	MBBR
Linear temperature increase	Oscillating	Steady	Steady	
Linear slope ( $^{\circ}\text{C d}^{-1}$ )	0.25		<40 $^{\circ}\text{C}$ : 0.16	
			>40 $^{\circ}\text{C}$ : 0.08	
Oscillating amplitude ( $^{\circ}\text{C}$ )	2	n.a.	n.a.	
Oscillating frequency ( $\text{d}^{-1}$ )	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)	$2.1 \pm 0.2$		$2.1 \pm 0.3$	
HRT (d)	$1.0 \pm 0.2$		$1.0 \pm 0.2$	
Highest temperature ( $^{\circ}\text{C}$ )	42	42	49	45.5
Ammonium conversion rates <sup>a</sup>				
Volumetric ( $\text{mg N L}^{-1} \text{d}^{-1}$ )	$26 \pm 5$	$90 \pm 3$	$794 \pm 57$	$309 \pm 30$
Specific ( $\text{mg N g}^{-1} \text{VSS d}^{-1}$ )	$72^{\text{b}}$	$139 \pm 18$	$151 \pm 7$	$67^{\text{b}}$

<sup>a</sup> In all cases, nitrite accumulation was negligible and nitrate formation >90% of ammonium removal.

<sup>b</sup> Only one biomass measurement available for the specific period.

### **A low linear temperature increase as the best transition strategy for thermophilic nitrification**

In the first experiment, an oscillating pattern was chosen, since pre-exposure to a certain stress can sometimes increase the resilience towards that stress, as shown for copper stress in denitrifiers (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. However, it was shown that the adaptive capabilities were not improved using a oscillating temperature increase, since the oscillating reactor was more negatively affected by the temperature increase, resulting in a faster loss of activity. Up to 40 $^{\circ}\text{C}$ , no changes in ammonium removal rates were observed in both reactors. When further increasing the temperature, however, nitrifying activity dropped severely and more pronounced in the oscillating reactor. Both reactors eventually lost all activity at 45 $^{\circ}\text{C}$ , suggesting that the imposed slope of 0.25 $^{\circ}\text{C d}^{-1}$  was still too high.

Nitrifiers are known to withstand relatively high seasonal temperature changes in wastewater treatment plants in moderate climates (e.g. 10-30°C at DC Water, Washington, USA). In order to reach temperatures higher than 38-42°C, however, this study shows that a low temperature slope is necessary. Nevertheless, a linear increase was clearly more successful than a stepwise temperature pattern, reaching nitrification no higher than 40 and 42.5°C (Courstens et al., 2014a, Shore et al., 2012). At lower treatment temperatures (10-20°C), this finding was also reported, since a sudden temperature decrease had a more pronounced negative effect on nitrification than a gradual temperature decrease (Hwang and Oleszkiewicz, 2007).

Since a linear temperature increase proved to be more promising than an oscillating pattern and since a slope of 0.25°C d<sup>-1</sup> was too high, the second reactor experiment imposed a lower linear temperature slope in a comparison between a SBR and a MBBR. Thermophilic organisms exhibit a slow growth, by which biomass retention is essential. A biofilm reactor might favor 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). For this, the MBBR was hypothesized to better cope with a temperature transition by favoring the adaptive capacities of nitrifiers towards higher temperatures. The results obtained, however, were not in line with this hypothesis, since nitrifying activity was lost around 46°C in the MBBR opposed to 49°C in the SBR (Courstens et al., 2016b).

### **AOA as a key enabler of thermophilic nitrification**

Analyzing the abundance of the main groups of nitrifiers along the temperature increase by means of qPCR, revealed the importance of the functional community for a successful transition to thermophilic temperatures. Both reactors were inoculated with the same inoculum, containing a relatively well-balanced amount of AOB versus AOA (2.1x10<sup>9</sup> versus 3.5x10<sup>8</sup> *amoA* gene copies g<sup>-1</sup> VSS) and nitrite oxidizers *Nitrospira* spp. versus *Nitrobacter* spp. (6.7x10<sup>9</sup> versus 4.8x10<sup>10</sup> 16S gene copies g<sup>-1</sup> VSS). AOB abundance remained stable up to 45°C at a level of about 10<sup>10</sup> *amoA* gene copies g<sup>-1</sup> VSS, and then gradually decreased in both reactors. AOA abundance, however, evolved differently in both reactors. The MBBR sludge contained significantly less AOA after a stabilization period (before starting the temperature increase) compared with the SBR sludge. Moreover, the SBR sludge showed a steep increase in AOA abundance at about 44°C, rising with about 3 orders of magnitude from 1.0x10<sup>7</sup> to 2.9x10<sup>10</sup> copies g<sup>-1</sup> VSS. In contrast, the MBBR sludge only showed an increase by two orders of magnitude to 8x10<sup>8</sup> copies g<sup>-1</sup> VSS and this shift occurred a little later than in the SBR at 46°C. Conclusively, a clear dominance shift from AOB to AOA was demonstrated from 45°C on in the SBR, while this shift did not fully occur in the MBBR. A shift towards AOA dominance is in accordance with other reports, where most thermophilic ammonia oxidizing organisms described are archaeal (de la Torre et al., 2008, Hatzenpichler et al., 2008, Lebedeva et al., 2013).

Moreover, the first ever thermophilic nitrifying bioreactor, obtained via the synthetic combination strategy, didn't even contain AOB and, thus, also clearly shows the AOA dominance at higher temperatures (Courstens et al., 2016a). Although the increasing trend in AOA did not fully come through in the MBBR, it does imply that the essential shift could occur with an even lower slope of temperature increase. Potentially a stronger selection for fast growing micro-organisms happened in comparison with the SBR, giving more advantage to 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  $\text{NH}_4^+$  concentrations, as key substrates for ammonia oxidation and potentially niche differentiation. Both substrates fluctuated more frequently in the SBR than in the MBBR. Along with lower concentrations of  $\text{NH}_4^+$  in the SBR, these conditions might have favored AOA stimulation over AOB. 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).

### **Ex-situ high-throughput activity measurements as an effective tool for steering the temperature increase pattern**

In parallel with the reactor operation during the second experiment, ex-situ activity measurements were performed in 96-well plates to monitor the progress of the optimal temperature for both ammonia and nitrite oxidation. These small, fast, high-throughput activity tests, based on simple spectrophotometrical measurements, clearly could predict the loss of ammonium and nitrite oxidation in both reactors. Furthermore, an obvious shift in optimum temperature was observed throughout the temperature increase. When the optimum temperature of the community does not evolve with the imposed temperature increase, predicted using these measurements, the strategy can be steered and the slope lowered to allow an effective transition towards thermophilic nitrification.

### **Temperature transition strategy successfully reaches 50°C**

Although nitrifying temperatures did not exceed 49°C using the most successful temperature increase strategy, it was not the temperature increase itself that caused the activity to crash, a technical failure of the pH controller caused severe acidification (pH 3-4) and subsequent cease in activity. After a recovery period of about 50 days, AOA activity recovered up to 300 mg N L<sup>-1</sup> d<sup>-1</sup>, whereas NOB activity remained absent. The recovery phase was accompanied with a washout of biomass and, possibly, a selective pressure for the micro-organisms to grow in a biofilm, since the only remaining biomass grew in a biofilm on the bioreactor walls. To allow better biomass retention, the bioreactor was transformed to a continuous MBR configuration by inserting a submerged hollow fiber membrane unit, as was done for the thermophilic nitrifying bioreactor obtained via the synthetic combination strategy. After transformation to a MBR, NOB activity popped up again and a complete nitrifying reactor was recovered. After stabilization, the temperature increase was continued at the same gradual linear slope of 0.08°C d<sup>-1</sup>, finally reaching the initial aim of 50°C.



Overall, an efficient temperature transition strategy for thermophilic nitrification is available, bringing forth perspectives for upgrading current mesophilic systems. However prosperous the studies concerning thermophilic nitrification are, significant challenges still persist before the actual accomplishment of complete thermophilic nitrogen removal. One of the major tests yet to be investigated, is the effect of carbon on the autotrophic/heterotrophic competition. The presence of COD incurs the manifestation of faster growing heterotrophs which will potentially out-compete the autotrophs, resulting in a loss of nitrification and, thus, nitrogen removal. A second trial to overcome is the further coupling of nitrification to a reductive pathway such as denitrification or anammox. The maximum temperature at which anammox growth was observed is 43°C (Strous et al., 1999) and the enrichment of thermophilic anammox species has not yet succeeded (Itoh et al., 2013). However, the presence of anammox has been reported in several high temperature environments such as deep-sea hydrothermal vents (Byrne et al., 2009), hot springs (Jaeschke et al., 2009) and high temperature petroleum reservoirs (Li et al., 2010). Since this review shows how fast the detection of thermophilic nitrifiers can evolve in actual thermophilic bioreactors with high potential, it is not unthinkable that the same can hold for thermophilic anammox. Since denitrification is a more established and deeper studied process than anammox, this might be a more promising technology to couple with thermophilic nitrification.

## **RAPID DEVELOPMENT OF THERMOPHILIC DENITRIFICATION**

In order to completely remove nitrogen from warm wastewaters, thermophilic nitrification must be coupled to a reductive process. A possible partner in crime is denitrification, the reduction of nitrate to nitrogen gas. Thermophilic denitrifying organisms are widespread and have been isolated from soil (*Bacillus thermodenitrificans* sp.), mud (*Geobacillus* sp.) and manure amended soil (*Anoxybacillus pushchinensis*) (Mishima et al., 2009, Mora et al., 1998, Yamamoto et al., 2006). Besides these spore forming bacteria, archaea and non-spore forming bacteria such as *Thermus thermophilus* are known thermophilic denitrifiers as well (Bricio et al., 2011, Cabello et al., 2004). Two studies have already described a thermophilic denitrifying bioreactor for wastewater treatment. One of these applies more to the synthetic combination strategy and reports an upflow sludge blanket (USB) reactor at 55°C, inoculated with thermal mud originating from a hot spring. Maximum specific nitrogen removal rates were rather low (51 mg N g<sup>-1</sup> VS d<sup>-1</sup>) and a nitrate removal efficiency of 78.4% was obtained. A second, more recent study belonging to the temperature transition strategy, investigated whether a mesophilic denitrifying inoculum can be used for the start-up of a thermophilic SBR by applying an instant temperature increase step to 55°C (Courstens et al., 2014b).

### **Efficient start-up at thermophilic temperatures**

Two SBR reactors were inoculated with mesophilic denitrifying sludge (26°C). One reactor was set at a mesophilic temperature of 34°C, while the second was elevated instantly to 55°C to allow extensive comparison between mesophilic and thermophilic denitrification. Several functional aspects such as maximal specific nitrate removal rate, sludge production, sludge settleability and nitrous oxide production were evaluated.

Next to that, the phylogenetic diversity of the microbial community was compared for different substrate complexities, ranging from synthetic to real wastewater. An efficient start-up of both reactors, leading to high maximum specific activities within one week implies that mesophilic denitrifying sludge can adapt instantly to thermophilic temperatures. However, the maximum specific activity in the thermophilic reactor was only half of that in the mesophilic reactor ( $436 \pm 22$  vs.  $922 \pm 21$  mg N g<sup>-1</sup> VSS d<sup>-1</sup>). Nevertheless, the observed activity at high temperatures was about a factor 8.5 higher than obtained using the synthetic combination strategy (Laurino and Siñeriz, 1991). This difference might be attributed to the reactor configuration used in both studies, which results in different sludge aggregation states (floccular vs granular) and sludge densities ( $1.2 \pm 0.5$  g VSS L<sup>-1</sup> vs.  $25.8$  g VS L<sup>-1</sup>). Moreover, thermophilic denitrification rates were far higher than earlier mentioned thermophilic nitrification rates (Courtens et al., 2016a, Courtens et al., 2016b), rendering the lower denitrifying activity at thermophilic temperatures less important since it is not the rate-limiting step of thermophilic nitrogen removal.

### **Thermophilic denitrification is more robust towards carbon source and requires less carbon**

Both reactors suffered from an abrupt change in carbon source from synthetic acetate to more complex molasses. However, the thermophilic reactor showed a 26% higher resilience towards the shock, resulting in a higher nitrite build-up. Not only was the thermophilic reactor more robust for a change in carbon source, it also required a lower amount of COD than the mesophilic counterpart ( $2.8 \pm 0.3$  vs.  $3.6 \pm 0.3$  g COD<sub>removed</sub>/NO<sub>3</sub><sup>-</sup><sub>removed</sub>). A higher degree of endogenous respiration at thermophilic conditions can explain this feature, since it leads to lower apparent biomass yields and thereby lower consumed COD/N ratios (Peng et al., 2007). Additionally, the lower need for carbon can also be attributed to the availability of internal COD, which is higher at elevated temperatures due to higher cell decay and lysis. Besides acetate and molasses as carbon source, thermophilic denitrification of industrial wastewater, using the by-product from sugar refinery (molasses) as carbon source, was demonstrated as well.

### **Improved sludge settling at elevated temperatures**

Generally, studies report that sludge settling deteriorates when temperatures are increased in aerobic wastewater treatment (Suvilampi and Rintala, 2003). Anoxic thermophilic denitrification, however, seems to show an improved settling with a 3.6 times lower sludge volume index than mesophilic denitrifying sludge when treating real wastewater (Courtens et al., 2014b). Settling might have been related to the content of inorganics in the thermophilic sludge (54%) compared to the mesophilic sludge (13%), yielding higher specific densities. Several studies already reported an inverse correlation between both VSS/TSS ratio and density and VSS/TSS ratio and settling velocity (Schuler and Jang, 2007, Vlyssides et al., 2008). Furthermore, mesophilic sludge particle size was considerably higher than the thermophilic particle size, reaching a volume mean diameter of 381 μm compared to 87 μm, when treating real wastewater. Equal trends have been observed when comparing mesophilic with thermophilic aerobic wastewater treatment, reporting 150-500 μm flocs and 50-150 μm flocs respectively (Suvilampi et al., 2005).

Not only temperature affected the particle size, a higher complexity of the influent matrix clearly increased the particle size of mesophilic sludge, whereas only a small increase was seen for the thermophilic denitrifying sludge (Courstens et al., 2014b). The difference in particle size treating different carbon sources, might be explained by the effect of the carbon source on the production of extracellular polymeric substances (EPS). As for example glucose induces more EPS production than acetate (Li and Yang, 2007), the carbon source affects the amount of EPS. EPS is known to determine floc structure and formation, so more or less EPS due to the type of carbon source, can have an effect on sludge settleability. The difference between mesophilic and thermophilic sludge particle size, might be attributed to the fact that bioflocculation is determined by properties of specific EPS functional groups rather than the quantity of bound EPS (Liao et al., 2011). All things taken into account, the improved settling at thermophilic temperatures of denitrifying sludge can lead to the implementation of shorter settling times in a SBR configuration, or smaller settlers in continuous systems, leading to considerable savings in operational and capital expenditure. Even more savings can be accomplished since thermophilic denitrification produces up to 45% less sludge (Courstens et al., 2014b), substantially lowering sludge disposal cost, one of the major costs of a wastewater treatment facility.

Overall, thermophilic denitrification is more easily realized than thermophilic nitrification, leading to a very efficient start-up, higher specific rates than thermophilic nitrification and significant economic prospects concerning settling behavior and sludge production.

## **CONCLUSION**

This review elucidates the incredible potential of thermophilic nitrogen removal, displaying several key milestones recently achieved that pave the road to actual application in practice. A cost calculation based on three industrial case studies, revealed that total costs savings of 19 to 28% could potentially be achieved (CAPEX + OPEX) when upgrading mesophilic to thermophilic nitrogen removal and that the major share of cost savings is related to the reduced sludge production. However, several challenges still need answering along the way to practical implementation. Critical is the effect of the presence of carbon during thermophilic nitrification and the resulted competition between nitrifiers and aerobic heterotrophs. Besides coupling aerobic carbon removal and nitrification, the subsequent coupling with reductive denitrification and possibly even anammox must be completed. Only then can the phenomenon of thermophilic biotechnology for carbon and nitrogen removal compete with its mesophilic counterpart.

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## REFERENCES

- Abeynayaka, A. and Visvanathan, C. (2011a) Mesophilic and thermophilic aerobic batch biodegradation, utilization of carbon and nitrogen sources in high-strength wastewater. *Bioresource Technology* 102(3), 2358-2366.
- Abeynayaka, A. and Visvanathan, C. (2011b) Performance comparison of mesophilic and thermophilic aerobic sidestream membrane bioreactors treating high strength wastewater. *Bioresource Technology* 102(9), 5345-5352.
- Bricio, C., Alvarez, L., Gomez, M.J. and Berenguer, J. (2011) Partial and complete denitrification in *Thermus thermophilus*: lessons from genome drafts. *Biochemical Society Transactions* 39, 249-253.
- Byrne, N., Strous, M., Crepeau, V., Kartal, B., Birrien, J.L., Schmid, M., Lesongeur, F., Schouten, S., Jaeschke, A., Jetten, M., Prieur, D. and Godfroy, A. (2009) Presence and activity of anaerobic ammonium-oxidizing bacteria at deep-sea hydrothermal vents. *ISME Journal* 3(1), 117-123.
- Cabello, P., Roldan, M.D. and Moreno-Vivian, C. (2004) Nitrate reduction and the nitrogen cycle in archaea. *Microbiology-Sgm* 150, 3527-3546.
- Camargo, J.A. and Alonso, A. (2006) Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment. *Environment International* 32(6), 831-849.
- Courtens, E.N.P., Boon, N., De Schryver, P. and Vlaeminck, S.E. (2014a) Increased salinity improves the thermotolerance of mesophilic nitrification. *Applied Microbiology and Biotechnology* 98(10), 4691-4699.
- Courtens, E.N.P., Spieck, E., Vilchez-Vargas, R., Bode, S., Boeckx, P., Schouten, S., Jauregui, R., Pieper, D.H., Vlaeminck, S.E. and Boon, N. (2016a) A robust nitrifying community in a bioreactor at 50°C opens up the path for thermophilic nitrogen removal. *ISME J.*
- Courtens, E.N.P., Vandekerckhove, T., Prat, D., Vilchez-Vargas, R., Vital, M., Pieper, D.H., Meerbergen, K., Lievens, B., Boon, N. and Vlaeminck, S.E. (2016b) Empowering a mesophilic inoculum for thermophilic nitrification: Growth mode and temperature pattern as critical proliferation factors for archaeal ammonia oxidizers. *Water Research* 92, 94-103.
- Courtens, E.N.P., Vlaeminck, S.E., Vilchez-Vargas, R., Verliefde, A., Jauregui, R., Pieper, D.H. and Boon, N. (2014b) Trade-off between mesophilic and thermophilic denitrification: Rates vs. sludge production, settleability and stability. *Water Research* 63, 234-244.

de la Torre, J.R., Walker, C.B., Ingalls, A.E., Konneke, M. and Stahl, D.A. (2008) Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. *Environmental Microbiology* 10(3), 810-818.

Erisman, J.W., Sutton, M.A., Galloway, J., Klimont, Z. and Winiwarter, W. (2008) How a century of ammonia synthesis changed the world. *Nature Geoscience* 1(10), 636-639.

Fitzgerald, C.M., Comejo, P., Oshlag, J.Z. and Noguera, D.R. (2015) Ammonia-oxidizing microbial communities in reactors with efficient nitrification at low-dissolved oxygen. *Water Research* 70, 38-51.

Galloway, J.N., Winiwarter, W., Leip, A., Leach, A.M., Bleeker, A. and Erisman, J.W. (2014) Nitrogen footprints: past, present and future. *Environmental Research Letters* 9(11).

Gilbert, P., Maira-Litran, T., McBain, A.J., Rickard, A.H. and Whyte, F.W. (2002) *Advances in Microbial Physiology*, pp. 203-256, Academic Press.

Hatzenpichler, R., Lebedeva, E.V., Spieck, E., Stoecker, K., Richter, A., Daims, H. and Wagner, M. (2008) A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. *Proceedings of the National Academy of Sciences of the United States of America* 105(6), 2134-2139.

Hwang, J.H. and Oleszkiewicz, J.A. (2007) Effect of Cold-Temperature Shock on Nitrification. *Water Environment Research* 79(9), 964-968.

Itoh, Y., Sakagami, K., Uchino, Y., Boonmak, C., Oriyama, T., Tojo, F., Matsumoto, M. and Morikawa, M. (2013) Isolation and Characterization of a Thermotolerant Ammonia-Oxidizing Bacterium *Nitrosomonas* sp JPCCT2 from a Thermal Power Station. *Microbes and Environments* 28(4), 432-435.

Jaeschke, A., den Camp, H.J.M.O., Harhangi, H., Klimiuk, A., Hopmans, E.C., Jetten, M.S.M., Schouten, S. and Damste, J.S.S. (2009) 16S rRNA gene and lipid biomarker evidence for anaerobic ammonium-oxidizing bacteria (anammox) in California and Nevada hot springs. *Fems Microbiology Ecology* 67(3), 343-350.

Kim, J.G., Jung, M.Y., Park, S.J., Rijpstra, W.I.C., Damste, J.S.S., Madsen, E.L., Min, D., Kim, J.S., Kim, G.J. and Rhee, S.K. (2012) Cultivation of a highly enriched ammonia-oxidizing archaeon of thaumarchaeotal group I.1b from an agricultural soil. *Environmental Microbiology* 14(6), 1528-1543.

Kurian, R., Acharya, C., Nakhla, G. and Bassi, A. (2005) Conventional and thermophilic aerobic treatability of high strength oily pet food wastewater using membrane-coupled bioreactors. *Water Research* 39(18), 4299-4308.

- Lackner, S., Gilbert, E.M., Vlaeminck, S.E., Joss, A., Horn, H. and van Loosdrecht, M.C.M. (2014) Full-scale partial nitrification/anammox experiences - An application survey. *Water Research* 55, 292-303.
- Lapara, T.M. and Alleman, J.E. (1999) Thermophilic aerobic biological wastewater treatment. *Water Research* 33(4), 895-908.
- Laurino, C.N. and Siñeriz, F. (1991) Denitrification by thermophilic soil bacteria with ethanol as substrate in a USB reactor. *Biotechnology Letters* 13(4), 299-304.
- Lebedeva, E.V., Hatzenpichler, R., Pelletier, E., Schuster, N., Hauzmayer, S., Bulaev, A., Grigor'eva, N.V., Galushko, A., Schmid, M., Palatinszky, M., Le Paslier, D., Daims, H. and Wagner, M. (2013) Enrichment and Genome Sequence of the Group I. 1a Ammonia-Oxidizing Archaeon "Ca. Nitrosotenuis uzonensis" Representing a Clade Globally Distributed in Thermal Habitats. *Plos One* 8(11).
- Lebedeva, E.V., Off, S., Zumbargel, S., Kruse, M., Shagzhina, A., Lucker, S., Maixner, F., Lipski, A., Daims, H. and Spieck, E. (2011) Isolation and characterization of a moderately thermophilic nitrite-oxidizing bacterium from a geothermal spring. *Fems Microbiology Ecology* 75(2), 195-204.
- Li, H., Chen, S., Mu, B.Z. and Gu, J.D. (2010) Molecular Detection of Anaerobic Ammonium-Oxidizing (Anammox) Bacteria in High-Temperature Petroleum Reservoirs. *Microbial Ecology* 60(4), 771-783.
- Li, J., Zheng, Y.M., Liu, Y.R., Ma, Y.B., Hu, H.W. and He, J.Z. (2014) Initial Copper Stress Strengthens the Resistance of Soil Microorganisms to a Subsequent Copper Stress. *Microbial Ecology* 67(4), 931-941.
- Li, X.Y. and Yang, S.F. (2007) Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge. *Water Research* 41(5), 1022-1030.
- Liao, B.Q., Lin, H.J., Langevin, S.P., Gao, W.J. and Leppard, G.G. (2011) Effects of temperature and dissolved oxygen on sludge properties and their role in bioflocculation and settling. *Water Research* 45(2), 509-520.
- Limpiyakorn, T., Furhacker, M., Haberl, R., Chodanon, T., Srithep, P. and Sonthiphand, P. (2013) amoA-encoding archaea in wastewater treatment plants: a review. *Applied Microbiology and Biotechnology* 97(4), 1425-1439.

Lipski, A., Spieck, E., Makolla, A. and Altendorf, K. (2001) Fatty acid profiles of nitrite-oxidizing bacteria reflect their phylogenetic heterogeneity. *Systematic and Applied Microbiology* 24(3), 377-384.

Lopez-Vazquez, C.M., Kubare, M., Saroj, D.P., Chikamba, C., Schwarz, J., Daims, H. and Brdjanovic, D. (2014) Thermophilic biological nitrogen removal in industrial wastewater treatment. *Applied Microbiology and Biotechnology* 98(2), 945-956.

Maeda, K., Hanajima, D., Toyoda, S., Yoshida, N., Morioka, R. and Osada, T. (2011) Microbiology of nitrogen cycle in animal manure compost. *Microbial Biotechnology* 4(6), 700-709.

Mah, T.F.C. and O'Toole, G.A. (2001) Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology* 9(1), 34-39.

Mishima, M., Iwata, K., Nara, K., Matsui, T., Shigeno, T. and Omori, T. (2009) Cultivation characteristics of denitrification by thermophilic *Geobacillus* sp strain TDN01. *Journal of General and Applied Microbiology* 55(2), 81-86.

Mora, D., Fortina, M.G., Nicastro, G., Parini, C. and Manachini, P.L. (1998) Genotypic characterization of thermophilic bacilli: a study on new soil isolates and several reference strains. *Research in Microbiology* 149(10), 711-722.

Nowka, B., Daims, H. and Spieck, E. (2015) Comparison of Oxidation Kinetics of Nitrite-Oxidizing Bacteria: Nitrite Availability as a Key Factor in Niche Differentiation. *Applied and Environmental Microbiology* 81(2), 745-753.

Oishi, R., Tada, C., Asano, R., Yamamoto, N., Suyama, Y. and Nakai, Y. (2012) Growth of Ammonia-Oxidizing Archaea and Bacteria in Cattle Manure Compost under Various Temperatures and Ammonia Concentrations. *Microbial Ecology* 63(4), 787-793.

Peng, Y.Z., Ma, Y. and Wang, S.Y. (2007) Denitrification potential enhancement by addition of external carbon sources in a pre-denitrification process. *Journal of Environmental Sciences* 19(3), 284-289.

Philippot, L., Cregut, M., Cheneby, D., Bressan, M., Dequiet, S., Martin-Laurent, F., Ranjard, L. and Lemanceau, P. (2008) Effect of primary mild stresses on resilience and resistance of the nitrate reducer community to a subsequent severe stress. *Fems Microbiology Letters* 285(1), 51-57.

Pitcher, A., Rychlik, N., Hopmans, E.C., Spieck, E., Rijpstra, W.I.C., Ossebaar, J., Schouten, S., Wagner, M. and Damste, J.S.S. (2010) Crenarchaeol dominates the membrane lipids of

Candidatus Nitrososphaera gargensis, a thermophilic Group I. 1b Archaeon. *Isme Journal* 4(4), 542-552.

Ryall, B., Eydallin, G. and Ferenci, T. (2012) Culture History and Population Heterogeneity as Determinants of Bacterial Adaptation: the Adaptomics of a Single Environmental Transition. *Microbiology and Molecular Biology Reviews* 76(3), 597-+.

Schuler, A.J. and Jang, H. (2007) Density effects on activated sludge zone settling velocities. *Water Research* 41(8), 1814-1822.

Shimaya, C. and Hashimoto, T. (2011) Isolation and characterization of novel thermophilic nitrifying *Bacillus* sp. from compost. *Soil Science and Plant Nutrition* 57(1), 150-156.

Shore, J.L., M'Coy, W.S., Gunsch, C.K. and Deshusses, M.A. (2012) Application of a moving bed biofilm reactor for tertiary ammonia treatment in high temperature industrial wastewater. *Bioresource Technology* 112, 51-60.

Sorokin, D.Y., Vejmelkova, D., Lucker, S., Streshinskaya, G.M., Rijpstra, W.I.C., Damste, J.S.S., Kleebezem, R., van Loosdrecht, M., Muyzer, G. and Daims, H. (2014) *Nitrolancea hollandica* gen. nov., sp nov., a chemolithoautotrophic nitrite-oxidizing bacterium isolated from a bioreactor belonging to the phylum Chloroflexi. *International Journal of Systematic and Evolutionary Microbiology* 64, 1859-1865.

Steffen, W., Richardson, K., Rockstrom, J., Cornell, S.E., Fetzer, I., Bennett, E.M., Biggs, R., Carpenter, S.R., de Vries, W., de Wit, C.A., Folke, C., Gerten, D., Heinke, J., Mace, G.M., Persson, L.M., Ramanathan, V., Reyers, B. and Sorlin, S. (2015) Planetary boundaries: Guiding human development on a changing planet. *Science* 347(6223).

Strous, M., Kuenen, J.G. and Jetten, M.S.M. (1999) Key physiology of anaerobic ammonium oxidation. *Applied and Environmental Microbiology* 65(7), 3248-3250.

Suvilampi, J., Lehtomaki, A. and Rintala, J. (2005) Comparative study of laboratory-scale thermophilic and mesophilic activated sludge processes. *Water Research* 39(5), 741-750.

Suvilampi, J. and Rintala, J. (2003) Thermophilic aerobic wastewater treatment, process performance, biomass characteristics, and effluent quality. *Reviews in Environmental Science and Biotechnology* 2(1), 35-51.

Vlaeminck, S.E., De Clippeleir, H. and Verstraete, W. (2012) Microbial resource management of one-stage partial nitritation/anammox. *Microbial Biotechnology* 5(3), 433-448.

Vlyssides, A.G., Barampouti, E.M.P. and Mai, S.T. (2008) Simple estimation of granule size distribution and sludge bed porosity in a UASB reactor. *Global Nest Journal* 10(1), 73-79.



Yamamoto, M., Ishii, A., Nogi, Y., Inoue, A. and Ito, M. (2006) Isolation and characterization of novel denitrifying alkalithermophiles, AT-1 and AT-2. *Extremophiles* 10(5), 421-426.

Yamamoto, N., Asano, R., Yoshii, H., Otawa, K. and Nakai, Y. (2011) Archaeal community dynamics and detection of ammonia-oxidizing archaea during composting of cattle manure using culture-independent DNA analysis. *Applied Microbiology and Biotechnology* 90(4), 1501-1510.

Yi, Y.S., Kim, S., An, S., Choi, S.I., Choi, E. and Yun, Z. (2003) Gas analysis reveals novel aerobic deammonification in thermophilic aerobic digestion. *Water Science and Technology* 47(10), 131-138.

Zeng, G.M., Zhang, J.C., Chen, Y.N., Yu, Z., Yu, M., Li, H., Liu, Z.F., Chen, M., Lu, L.H. and Hu, C.X. (2011) Relative contributions of archaea and bacteria to microbial ammonia oxidation differ under different conditions during agricultural waste composting. *Bioresource Technology* 102(19), 9026-9032.