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Exploring the boundaries of biological nitrogen removal: Development strategies for thermophilic nitrification and denitrification

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

Titel van het doctoraat in het Nederlands: Verkenning van de grenzen van biologische stikstofverwijdering: Ontwikkelingsstrategieën voor thermofiele nitrificatie en denitrificatie.

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Notation Index

AD	Anaerobic digestion
AOA	Ammonium oxidizing archaea
(Aer)AOB	(Aerobic) ammonium oxidizing bacteria
AnAOB	Anoxic ammonium oxidizing bacteria
AMO	Ammonia monooxygenase
ATAD	Autothermal thermophilic aerobic digestion
BNF	Biological nitrogen fixation
CAPEX	Capital expenditures
C-BNF	Cultivation induced biological nitrogen fixation
BNR	Biological nutrient removal
CAS	Conventional activated sludge
DGGE	Denaturing gradient gel electrophoresis
DNRA	Dissimilatory nitrate reduction to ammonium
DO	Dissolved oxygen
EPS	Extracellular polymeric substances
GDGT	Glycerol dibiphytanyl glycerol tetraether lipid
HAO	Hydroxylamine oxidoreductase
FA	Free ammonia
FAME	Fatty acid methyl esters
FISH	Fluorescent *in-situ* hybridization
FNA	Free nitrous acid
HRT	Hydraulic retention time
HSP	Heat-shock protein
MBBR	Moving bed biofilm reactor

N	Nitrogen
N_r	Reactive nitrogen
N/DN	Nitrification/Denitrification
Nit/DeNit	Nitritation/Denitritation
NOB	Nitrite oxidizing bacteria
NXR	Nitrite oxidoreductase
OPEX	Operational expenditures
OTU	Operational taxonomic unit
PN/A	Partial nitritation/anammox
PVA	Polyvinyl alcohol
qPCR	Quantitative polymerase chain reaction
SBR	Sequencing batch reactor
SHARON	Single reactor system for High Ammonia Removal Over Nitrite
SRT	Sludge retention time
SVI	Sludge volume index
TEM	Transmission electron microscopy
TSS	Total suspended solids
USB	Upflow sludge blanket
VSS	Volatile suspended solids
WW	Wastewater
Y	Sludge yield

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CHAPTER 1:

INTRODUCTION

1. Nitrogen

Nitrogen (N) is a crucial building block of life. It is ubiquitous in all living organisms as an essential element in amino acids, proteins and nucleic acids and a prerequisite for photosynthesis as a fundamental atom in chlorophyll. Although 78% (v/v) of the Earth's atmosphere consists out of nitrogen gas (N_2), these strongly bound nitrogen atoms are unavailable to most organisms.

Two **natural processes** can provide the energy to convert N_2 into the biologically available 'reactive' forms of nitrogen (N_r), i.e. ammonia (NH_3), nitrite/nitrate (NO_2^-/NO_3^-) and nitrogen oxides (NO_X). **Lightning** (2%) and **biological nitrogen fixation** (BNF) (29/69% terrestrial/marine) result in in a natural creation rate of 203 Tg N $year^{-1}$ (Fowler et al., 2013; Vitousek et al., 2013). Only a number of specialized free-living or symbiotic prokaryotes, the so-called diazotrophs, possess the key enzyme nitrogenase reducing N_2 into ammonium (NH_4^+) (Stacey et al., 1992). For millennia, the formation of N_r by these natural processes was balanced by deep sedimentation and the conversion of N_r back to N_2 by several processes (e.g. denitrification), resulting in limited accumulation of N_r in environmental reservoirs (Galloway et al., 2014).

A growing human population in need of increased amounts of N_r for food and energy production however significantly altered this balanced N-cycle. The first main **anthropogenic** contribution to the increase in N_r was **food production**, starting at a relatively small scale in 1850 by **cultivation-induced BNF** (C-BNF) (15 Tg N $year^{-1}$, Figure 1.1). New N_r was generated during the cultivation of legumes (e.g. peas, beans), self- fertilizing the soil through symbiosis with diazotrophs, and during the cultivation of rice by cyanobacteria growing in anaerobic environments (Galloway et al., 2004). The food demand rapidly surpassed the traditional N_r sources such as biomass and manure, creating a need for synthetic fertilizers. The development of the **Haber-Bosch process** resulted in a doubling of the production of anthropogenic N_r per capita in less than 25 years (30 kg N yr^{-1}, Figure 1.1). This powerful process catalytically combines H_2 and N_2 to NH_3 under high pressure (15-25 MPa) and high temperature (300-350°C) (Chagas, 2007). Beside fertilizer production, Haber-Bosch nitrogen has been increasingly used as a cooling agent and in industrial products such as fibers, plastics

and paints (Gu et al., 2013). The increased demand for energy to run the Industrial Revolution moreover resulted in an increased **fossil fuel combustion**, emitting nitrogen as NO_x to the atmosphere as a waste product. Overall, in 2010, human N_r creation (220 Tg N $year^{-1}$) was more than three-fold greater than natural terrestrial N_r creation (Fowler et al., 2013; Vitousek et al., 2013). The Haber-Bosch process was responsible for more than half (120 Tg N yr^{-1}) of the human N_r creation, while C-BNF, fossil fuel combustion and industrial N_r contributed with 40, 40 and 25 Tg N yr^{-1}, respectively (Galloway et al., 2014). The enormous anthropogenic input of nitrogen imbalanced the natural nitrogen cycle, leading to accumulation of N_r in many natural ecosystems causing a worldwide environmental problem. Just as biodiversity loss and the biogeochemical P-flow from fertilizers to erodible soils, the anthropogenic distortion of the nitrogen cycle has by far exceeded the safety bounderies of our planet (Figure 1.2) (Rockstrom et al., 2009; Steffen et al., 2015).

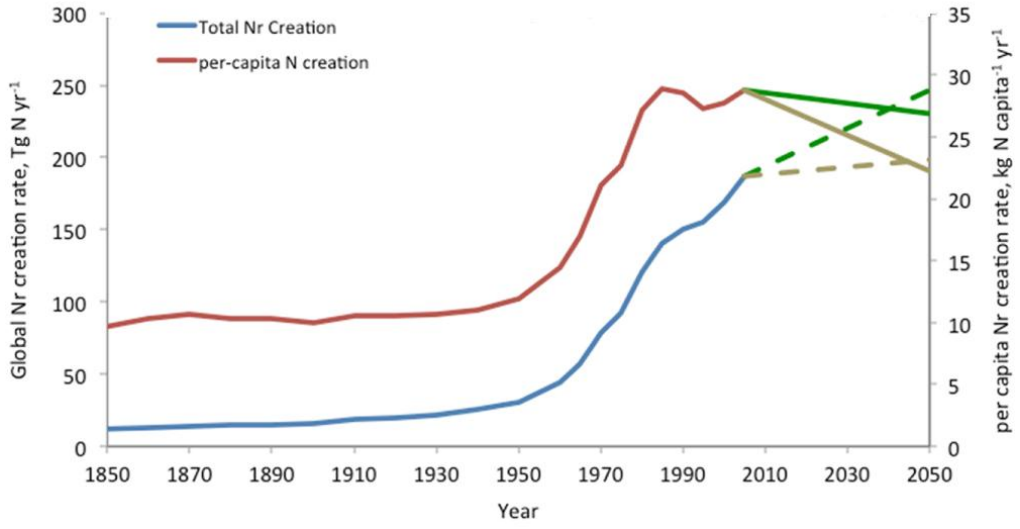

Figure 1.1 Temporal trends in global anthropogenic N_r creation on a total (left y-axis) and per-capita (right y-axis) basis. Adapted from Galloway et al. (2014). The green and brown lines represent future scenarios (2050) consisting of a baseline (without specific N_r mitigation) and a low estimate, respectively.

Reactive nitrogen is a main wastewater component in our global society, representing about 20 Tg N yr^{-1}, of which more than 99% is not treated and thus released as such in the environment (Galloway et al., 2008). Nitrogen in wastewater is commonly present as ammonium, in equilibrium with its unionized form free ammonia, which is toxic to aquatic macro-organisms at concentrations as low as 0.25 mg NH_3 L^{-1} (Randall and Tsui, 2002).

Increased nitrogen levels lead to algal blooms (eutrophication), causing oxygen depletion, a loss of biodiversity and even extensive fish mortality in case of toxic blooms (Camargo and Alonso, 2006). Microbial nitrification is furthermore enhanced by increased ammonium availability, resulting in additional oxygen depletion, subsequent fish mortality, the formation of nitrite and nitrate and a pH decrease. These toxic compounds can contaminate groundwater and drinking water, hereby playing a significant role in the development of health issues such as methemoglobinemia ('blue-baby syndrome'), certain types of cancer and other chronic health issues (Ward et al., 2005). A significant decrease of the nitrogen wastewater flux to the environment is thus crucial to prevent problems relevant to global ecology and society.

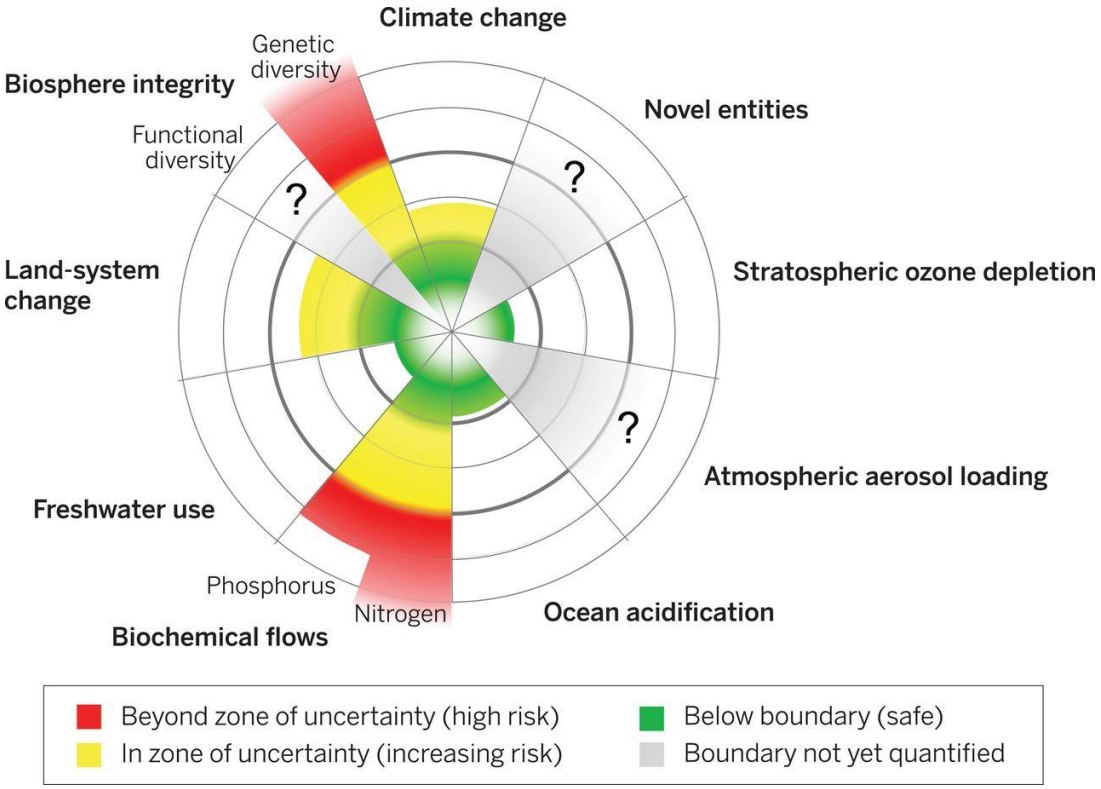

Figure 1.2 The current status of the control variables for seven of the nine planetary boundaries, where the inner green shading represents the proposed safe operating space. Anthropogenic perturbation levels of four of the earth system processes/features (climate change, biosphere integrity, biogeochemical flows, and land-system change) exceed the proposed planetary boundary (Steffen et al., 2015).

The nitrogen wastewater flux to the environment can be decreased via biological nitrogen removal (Section 2) or nitrogen recovery, e.g. air stripping, steam stripping, struvite precipitation, reverse osmosis and (bio)electrolysis. Both economical and sustainability

aspects determine the choice between nitrogen removal or recovery. From an energy point of view, nitrogen recovery could be more sustainable if the energy input is lower than the sum of nitrogen removal (0.9-2.3 kWh kg^{-1} N) and subsequent re-fixation through the Haber-Bosch process (9.6-12.4 kWh kg^{-1} N) (Mulder, 2003). However, as the fertilizer prices are still relatively low compared with the energy and chemical cost associated with nitrogen recovery technologies, biological nitrogen removal is the most cost-efficient option for wastewaters containing up to 5 g N L^{-1} according to Mulder et al. (2003). In some cases with current available technology, nitrogen recovery through ammonia stripping can even be cost competitive from 2 g N L^{-1} on (Menkveld and Broeders, 2015). New approaches such as urine separation resulting in very concentrated streams, however, could render recovery more efficient (Maurer et al., 2003).

2. Biological nitrogen removal (BNR)

2.1. Key functional processes

In accordance with the natural microbial nitrogen cycle (Figure 1.3), in biological nitrogen removal (BNR), four important functional processes are linked to four groups of bacteria enabling the transformation of N_r to N_2. The oxidation of ammonia (NH_3) to nitrite (NO_2^-), i.e. nitritation (Section 2.1.1) and subsequently nitrate (NO_3^-), i.e. nitratation (Section 2.1.2), is commonly known as nitrification. Further reduction of NO_2^-/NO_3^- to N_2 is carried out through denitrification (Section 2.1.3) or anaerobic ammonium oxidation or anammox (Section 2.1.4).

Nitrate can furthermore also be reduced by the dissimilatory nitrate reduction to ammonium or DNRA process. DNRA has been found to be the main nitrate reduction pathway in anaerobic digesters and in other methanogenic environments (Percheron et al., 1999), especially when high COD/NO_3 ratios (> 58) occur (Akunna et al., 1992). No literature is available on DNRA in conventional nitrification/denitrification systems, however, as usually lower COD/NO_3 ratios are present, DNRA is considered negligible in these systems and not discussed further.

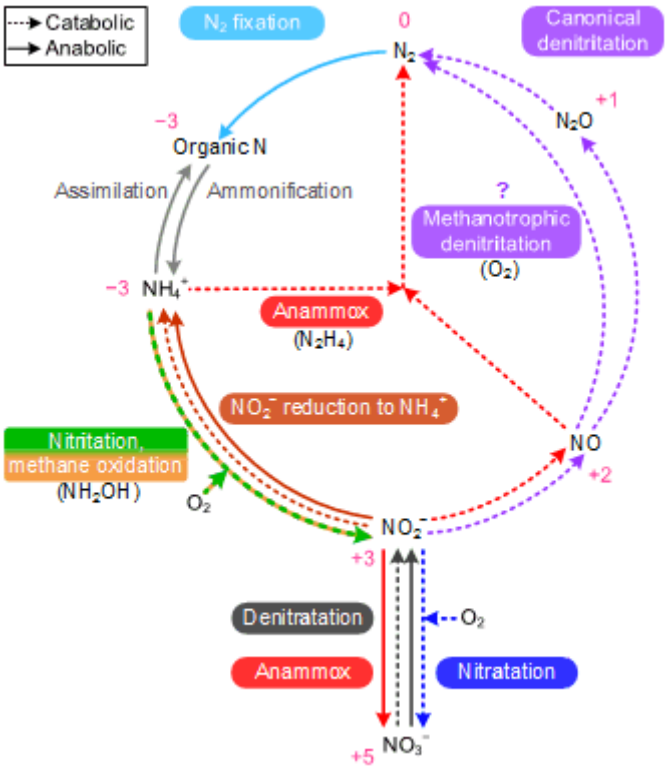

Figure 1.3 The microbial nitrogen cycle with pathways related to catabolism (dashed arrows) and anabolism (full arrows). Oxidation states of the nitrogen compounds are indicated in pink, and intermediates are shown between brackets. Adapted from Vlaeminck et al. (2011).

2.1.1. Nitritation

Aerobic microbial oxidation of ammonia to nitrite is the first step of nitrification and for decades, the **ammonium oxidizing bacteria (AOB)** were seen as the only protagonists. AOB catalyze this catabolic reaction ($\Delta G^{\circ\prime}$= -271 kJ mol^{-1}) in two sequential steps (Sayavedra-Soto and Arp, 2011). Ammonia is first oxidized to hydroxylamine (NH_2OH) with the membrane bound enzyme ammonia monooxygenase (AMO). The periplasmatic hydroxylamine oxidoreductase (HAO) then oxidizes hydroxylamine to nitrite, hereby providing the two reducing equivalents for the first step. The two other produced electrons are used for respiratory purposes, i.e. reducing oxygen (O_2) by a terminal oxidase, thereby generation a proton (H^+) motive force.

$$NH_3 + O_2 + 2\,H^+ + 2\,e^- \xrightarrow{AMO} NH_2OH + H_2O$$
$$NH_2OH + H_2O \xrightarrow{HAO} NO_2^- + 5\,H^+ + 4\,e^-$$
$$0.5\,O_2 + 2\,H^+ + 2\,e^- \xrightarrow{\text{terminal oxidase}} H_2O$$

$$NH_3 + 1.5\,O_2 \longrightarrow NO_2^- + H^+ + H_2O \qquad (\Delta G^{\circ\prime} = -271\ kJ\ mol^{-1})$$

The fundamental axiom that AOB, belonging to the β-and γ-Proteobacteria, were the only protagonists for nitritation was however challenged by the discovery that mesophilic archaea have ammonia oxidizing potential. The isolation of the marine aerobic **ammonium oxidizing archaeon (AOA)** *Nitrosopumulus maritimus* (Könneke et al., 2005), which fall within a novel archaeal phylum now known as *Thaumarchaeota*, broadened the phylogenetic capability of aerobic ammonium oxidation and led to a fundamental shift in our view of the nitrogen cycle. In particular because archaeal AMO-like gene sequences were found in almost every environment on Earth, including oceans, estuaries, soils, and animal gut and they outnumber their bacterial counterparts in many habitats, even in some wastewater treatment plants (Schleper, 2010; Limpiyakorn et al., 2013). Although ammonia limitation, low dissolved oxygen concentrations, pH and mixotrophy have been suggested as factors providing niche specialisation and differentiation between AOA and AOB (Hatzenpichler, 2012; Stahl and de la Torre, 2012), current data from genomes, cultures, field studies, and microcosms suggest that no single factor discriminates between AOA and AOB (Prosser and Nicol, 2012).

While it is generally accepted that ammonia (NH_3) is the actual substrate of AOB, the term ammonium (NH_4^+) is commonly used instead to refer to AOB activity, as is done throughout this thesis. However, it is unclear whether archaeal AMO catalyzes the same reaction as its bacterial counterpart. Although characterized AOA produce hydroxylamine as an intermediate in the ammonia oxidation pathway (Vajrala et al., 2013), no HAO homologue, cytochrome *c*, or enzymes for the detoxification of NH_2OH have been found in any AOA genome (Hatzenpichler, 2012). Even though the archaeal pathway is considered unresolved at this time, recent studies showing nitric oxide (NO) production during ammonia oxidation, suggest that NO may be an intermediate or function as a redox shuttle delivering electrons to the AMO (Stahl and de la Torre, 2012; Martens-Habbena et al., 2015).

2.1.2. Nitratation

The second step of nitrification is the oxidation of nitrite to nitrate ($\Delta G°' = -54$ kJ mol^{-1}), catalysed by a nitrite oxidoreductase (NXR) delivering two electrons which are transferred to oxygen with a terminal oxidase (Starkenburg et al., 2011):

$$NO_2^- + H_2O \xrightarrow{NXR} NO_3^- + 2\,H^+ + 2\,e^-$$

$$0.5\,O_2 + 2\,H^+ + 2\,e^- \xrightarrow{\text{terminal oxidase}} H_2O$$

$$NO_2^- + 0.5\,O_2 \longrightarrow NO_3^- \qquad (\Delta G°' = -54 \text{ kJ mol}^{-1})$$

The majority of the known nitrite oxidizing bacteria (NOB) belong either to the Proteobacteria (*Nitrobacter* (α-), *Nitrococcus* (γ-), *Nitrospina* (δ-) and *Nitrotoga* (β-)) or to the genus *Nitrospira* of the phylum Nitrospirae (Koops and Pommerening-Roser, 2001; Alawi et al., 2007). However, recently, a nitrite oxidizing bacterium belonging to the phylum Chloroflexi, *Nitrolancetus hollandicus*, was isolated from a nitrifying reactor (Sorokin et al., 2012). *Nitrospira* related NOB appear to be mostly prevailing in sewage treatment plants (Daims et al., 2006). So far, no nitrite oxidizing archaea (NOA) have been discovered yet.

2.1.3. Denitrification

The denitrification process, comprising the stepwise reduction of nitrate/nitrite to dinitrogen gas, can be performed by autotrophic or heterotrophic denitrifiers (Matějů et al., 1992). Denitrification by chemolithoautotrophic bacteria using e.g. hydrogen or sulfur as an electron donor is successfully used in the treatment of drinking water. In wastewater treatment, heterotrophic denitrifying bacteria are mainly used since adequate amounts of carbon are usually present. Most denitrifying species are facultative aerobes able to use oxygen in an aerobic metabolism, and, in the absence of oxygen, to reduce nitrate in an anoxic metabolism. Therefore, the same biomass can be used in a combined aerobic/anoxic process for carbon and nitrate removal. Heterotrophic denitrifiers reduce nitrate/nitrite according to the following biochemical pathway (Zumft, 1997):

$$NO_3^- \xrightarrow{NAR} NO_2^- \xrightarrow{NIR} NO \xrightarrow{NOR} N_2O \xrightarrow{NOS} N_2$$

The enzymes involved in each of these reduction steps are nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS) with a Gibbs free energy of -161, -76, -306 and -340 kJ mol^{-1}. Heterotrophic denitrifiers include some archaea and many bacteria spread over the Actinobacteria, the Bacteroidetes, the Firmicutes and four of the five subclasses of the Proteobacteria (Zumft, 1997).

2.1.4. Anammox

Ammonium and nitrite can anaerobically be converted to dinitrogen gas by the anammox process according to the following catabolic reactions ($\Delta G^{\circ\prime}$= -358 kJ mol^{-1}) (Strous et al., 1998; Strous et al., 2006):

$$NO_2^- + e^- + 2\,H^+ \xrightarrow{NIR} NO + H_2O$$
$$NH_4^+ + NO + 3\,e^- + 2\,H^+ \xrightarrow{HH} N_2H_4 + H_2O$$
$$N_2H_4 \xrightarrow{HAO} N_2 + 4\,H^+ + 4\,e^-$$
$$\overline{\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad}$$
$$NO_2^- + NH_4^+ \longrightarrow N_2 + H_2O$$

Nitrite is first reduced to nitric oxide by a nitrite reductase (NIR), which is subsequently combined with ammonium to hydrazine (N_2H_4) by a hydrazine hydrolase (HH). Finally, a hydroxylamine oxidoreductase (HAO) like enzyme oxidizes hydrazine to dinitrogen gas. Nitrate is produced during anabolism (Table 1.1), since nitrite oxidation is thought to generate reducing equivalents for carbon dioxide fixation (van de Graaf et al., 1996).

Anaerobic ammonium oxidizing bacteria (AnAOB) are exclusively monophyletic members of the phylum Planctomycetes (Strous et al., 1999).

Table 1.1 Overall stoichiometry of the four key functional processes (nitritation, nitratation, anammox and denitrification) as well as the combinations conventionally applied in biological nitrogen removal (N/DN, Nit/DeNit and PN/A) (Vlaeminck, 2009).

Process	Number	Sub reaction	Stoichiometry
Nitritation	1	Substrates	$NH_4^+ + 1.382\ O_2 + 0.091\ HCO_3^-$
		Products	$0.982\ NO_2^- + 1.891\ H^+ + 0.091\ CH_{1.4}O_{0.5}N_{0.2} + 1.36\ H_2O$
Nitratation	2	Substrates	$NO_2^- + 0.488\ O_2 + 0.003\ NH_4^+ + 0.013\ HCO_3^-$
		Products	$NO_3^- + 0.013\ CH_{1.4}O_{0.5}N_{0.2} + 0.008\ H_2O$
Anammox	3	Substrates	$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+$
		Products	$1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O$
Denitrification	4	Substrates	$NO_3^- + 1.080\ CH_3OH$
		Products	$0.476\ N_2 + OH^- + 0.760\ CO_2 + 0.325\ CH_{1.4}O_{0.5}N_{0.2} + 1.440\ H_2O$
Denitritation	5	Substrates	$NO_2^- + 0.53\ CH_3OH$
		Products	$0.48\ N_2 + OH^- + 0.33\ CO_2 + 0.20\ CH_{1.4}O_{0.5}N_{0.2} + 0.56\ H_2O$
Nitrification/Denitrification (N/DN)	1+2+4	Substrates	$NH_4^+ + 1.856\ O_2 + 1.058\ CH_3OH$
		Products	$0.457\ N_2 + 1.010\ H^+ + 0.641\ CO_2 + 0.421\ CH_{1.4}O_{0.5}N_{0.2} + 2.349\ H_2O$
Nitritation/Denitritation (Nit/DeNit)	1+5	Substrates	$NH_4^+ + 1.382\ O_2 + 0.52\ CH_3OH$
		Products	$0.47\ N_2 + 0.998\ H^+ + 0.235\ CO_2 + 0.287\ CH_{1.4}O_{0.5}N_{0.2} + 2.349\ H_2O$
Partial nitritation/Anammox (PN/A)	1+3	Substrates	$NH_4^+ + 0.792\ O_2 + 0.080\ HCO_3^-$
		Products	$0.435\ N_2 + 1.029\ H^+ + 0.111\ NO_3^- + 0.052\ CH_{1.4}O_{0.5}N_{0.2} + 0.028\ CH_2O_{0.5}N_{0.15} + 1.460\ H_2O$

2.2. Different process configurations

2.2.1. Conventional nitrogen removal

Conventional BNR is based on the combination of autotrophic nitrification and heterotrophic denitrification (N/DN). The overall stoichiometry of the separate functional microbial processes, as separately discussed in sections 2.1.1-2.1.3, and the commonly applied combinations in BNR are presented in Table 1.1. Typical mesophilic specific nitrification and denitrification rates in conventional activated sludge systems are in the order of magnitude of 0.025 and 0.25 g N g^{-1} VSS d^{-1}, respectively (Henze et al., 2008). Dedicated nitrification/denitrification reactors on concentrated nitrogen streams can however reach considerable higher rates of e.g. 230 and 1000 mg N g^{-1} VSS d^{-1} for nitrification and denitrification, respectively (Fux et al., 2003). As nitrification and denitrification are performed under different conditions and by different microorganisms, the processes have to be separated in time or space to function effectively. An overview of the different established N/DN process configurations, operated as one- or two stage systems are presented in Table 1.2. and described below (Henze et al., 2008; Zhu et al., 2008).

Table 1.2 Overview of the different conventional nitrification/denitrification (N/DN) process configurations (Henze et al., 2008; Zhu et al., 2008).

Process	N/DN separation	Stages
Post-denitrification	Space	2
Pre-denitrification = modified Ludzack-Ettinger (MLE) system	Space	2
Bio-denitro	Space	2
Bardenpho system	Space	4
Step feeding	Space	>2
Simultaneous N/DN	Space/Time	1
Sequential batch reactor	Time	1

If separated in space, the position of the anoxic zone in the biological reactor significantly affects the denitrifying performance. In the simplest method, the **post-denitrification system**, the first reactor is aerobic and the second anoxic. Complete nitrification as well as oxidation

of biodegradable organics occur in the first reactor, while denitrification occurs in the second. However, often an additional external carbon source is needed to achieve complete denitrification. Utilization of the influent organics as carbon source for denitrification can be achieved with a **pre-denitrification system**, including recycling of the nitrate rich effluent of the aerobic nitrification reactor to the anoxic reactor. In order to overcome the deficiency of incomplete nitrate removal in the standard 2 stage pre-denitrification system, the **4-stage Bardenpho system** was proposed, including a secondary anoxic reactor and a reaeration reactor. The need of large reactor volumes can however be avoided by alternative processes such as the **Bio-denitro process**, where in general two tanks working in an alternating mode of operation, or a **step feeding process**, where wastewater is introduced to several points along the aeration basin.

In a one stage system, nitrification and denitrification can be both separated in space or in time. In **simultaneous N/DN** systems (e.g. an oxidation ditch), a natural separation of nitrifiers and denitrifiers occurs as a result of the slow oxygen diffusion in sludge flocs. Nitrifiers located on the outside of the floc scavenge the oxygen and nitrate formed diffuses together with soluble organics inside the floc, supporting denitrification in the anoxic core. As aeration in those systems is usually controlled by nitrate sensors, also a separation in time is provided. Similarly, in a **sequential batch reactor** (SBR) both processes are separated in time by applying different aeration and non-aeration phases in the same reactor. It had to be noted however that in practice the separation of aerobic/anoxic processes is not straight forward as there are space limitations in mixing. This can result in locally different conditions as anoxic zones in aerobic phases and vice versa.

2.2.2. Shortcut nitrogen removal

Shortcut biological nitrogen removal refers to two processes that convert ammonia to nitrogen gas via nitrite: nitritation/denitritation (Nit/DeNit) and partial nitritation/anammox. Nit/DeNit halts the oxidation step at nitrite and denitrifies nitrite directly to nitrogen gas. Partial nitritation/anammox is the process where half of the ammonia is oxidized to nitrite combined with oxidation of the remaining ammonia using nitrite as electron acceptor via the

anammox reaction. These processes not only reduce the aeration demand, but also the carbon requirement and the sludge production.

Preventing the oxidation of nitrite to nitrate, thus the suppression of NOB, is the critical factor for successful short-cut nitrogen removal and can be achieved by several control strategies. The higher affinity of AOB for oxygen compared with NOB leads to out-competition of NOB and thus nitrite accumulation at **low dissolved oxygen (DO) concentrations** at side stream conditions (1-1.5 mg L^{-1}) (Blackburne et al., 2008). In other cases, however, the effect of DO on the AOB/NOB balance may be different as recently shown for mainstream conditions (Regmi et al., 2014). Maximum growth rates of AOB and NOB species vary with temperature. Elevated **temperatures** (> 20°C) not only promotes the growth of certain AOB species but can also expand the growth rate differences between certain AOB and NOB species (Balmelle et al., 1992) and so, eventually, enable NOB washout by imposing a **low sludge retention time** (SRT) (Hellinga et al., 1998). Furthermore **intermittent aeration** patterns are known to outcompete NOB due to the NOB lag phase after the anoxic period (Yoo et al., 1999; Kornaros et al., 2010; Gilbert et al., 2014b) and **elevated free ammonia (FA)/free nitrous acid (FNA)** concentrations (Anthonisen et al., 1976) were found to selectively inhibit NOB.

For a successful parital nitritation/anammox, it is also crucial to sustain the growth of the slow growing anammox bacteria in the system. As the doubling time of denitrifiers is about 100 times shorter than the doubling time of the anammox bacteria, they might be outcompeted if too much organic carbon is present (biodegradable COD (bCOD)/N > 3), hereby limiting the PN/A application area (Lackner et al., 2008). The advantages compared with N/DN systems (1) no requirement of external carbon source, (2) 60% lower aeration demand and (3) 75% lower sludge production already resulted in about 100 full scale installations until 2014 (Lackner et al., 2014).

3. Thermophilic biological nitrogen removal

As stated in textbooks, it is common knowledge that opposed to other biological processes in wastewater treatment, thermophilic nitrification above 40°C is not possible (Figure 1.4) (Henze et al., 1997). Investigation of nitrification and denitrification in activated sludge was thus mainly focused within the temperature range of 5-35°C (Dawson and Murphy, 1972; Painter and Loveless, 1983; Shammas, 1986; Antoniou et al., 1990). Even during the last two decades, only 1.8% of the publications concerning BNR appear to investigate nitrogen removal in the thermophilic range (Web of Science search based on the keywords 'biological nitrogen removal' and 'thermophilic', 2015). However, different microbes performing one step in the thermophilic nitrogen cycle (e.g. AOA and NOB) were previously separately isolated or enriched from oligotrophic samples such as hot springs and hydrothermal vents, representing a hidden treasure of natural resources (See section 3.3). Although thermophilic carbon treatment already pointed out several advantages (See section 3.2), the transposition capability into useful communities for biotechnological applications was however still unexplored and were investigated in this thesis.

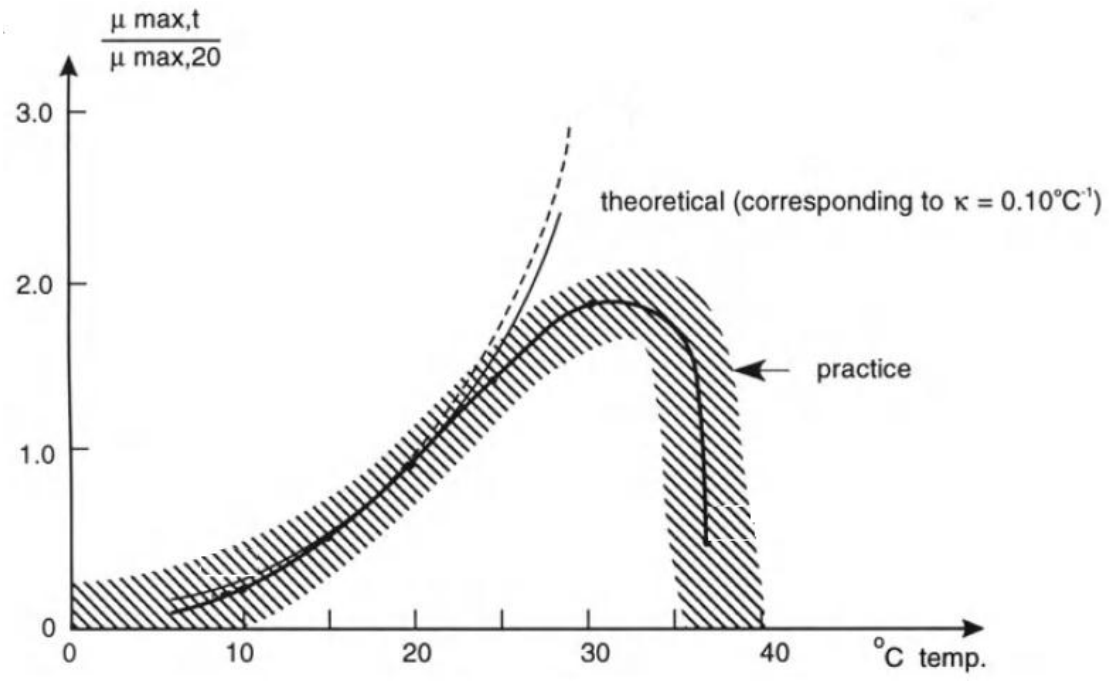

Figure 1.4 Nitrification as a function of temperature. Adapted from Henze et al. (1997)

3.1. Life at high temperature

Thermophiles are commonly defined as organisms living within a temperature range of 50-60°C (Brock, 1986). Limits are however not well defined and from a wastewater treatment perspective, temperatures above 45°C are widely accepted to determine thermophilic conditions (Lapara and Alleman, 1999). The upper temperature limit of microbes is mainly governed by molecular instability and hydrothermal decomposition of the standard high-molecular weight components of the cell: nucleic acids (section 3.1.1), proteins (section 3.2.1), lipids and other compounds used to build up membranes (section 3.1.3) (Jaenicke and Sterner, 2006).

3.1.1. Nucleic acids

Elevated temperatures induce either strand separation ('melting', T_m) of nucleic acids and chemical damage of the nucleotide constituents or, at the extreme, breakage of backbone phosphodiester bonds (Grogan, 1998; Daniel and Cowan, 2000). The guanine-cytosine (GC) pair in nucleic acids is bound by three hydrogen bonds while the adenine-thymine (AT) pairs are bound by two hydrogen bonds. Hence, T_m of DNA and RNA is known to increase with increased G+C content and could thus be a possible adaptation mechanism to thermophilic conditions. A strong positive correlation between the optimum growth temperatures of prokaryotes and the G+C content of tRNAs/rRNAs was observed (Galtier and Lobry, 1997). The genomic DNA is however not correlated with the growth temperature. In contrary, the G+C content of some of the most hyperthermophilic archaea revealed to be remarkably low (31 mol%) and an average of 45 mol% for all known (hyper)thermophilic bacteria and archaea was described (Stetter, 1996; Grogan, 1998). Additional extrinsic factors such small metabolites (polyamines), proteins or salts are thus necessary to stabilize the DNA double helix. High salt concentrations (e.g. Mg^{2+}- and Na^{+}-salts) namely mask the destabilizing charge repulsion between the two negatively charged phosphodiester backbones hereby increasing the base-interaction strength.

3.1.2. Proteins

The biochemical limit of viability of water-soluble proteins is around 130-140°C (Jaenicke and Sterner, 2006). Natural amino acids decompose hydrothermally and the solvent properties of water change with increasing temperature. As a consequence, the differences between polar and non-polar residues is masked, thus interfering with the 'hydrophobic collapse' as the initial step of protein folding. An increased **intrinsic stability** can be obtained by additional stabilizing **intra- and intermolecular interactions**. Ion pairs, hydrogen bonds, and disulphide bonds stabilize proteins, occurring from primary to quaternary structure of proteins. Generally, (hyper)thermophilic proteins are known to (1) show a decreased content of uncharged polar amino acids, (2) contain an increased content of charged amino acids, being involved in stabilizing ion pairs at the protein surface and (3) are on average significantly smaller than the mesophilic homologues (Jaenicke and Sterner, 2006).

Beside the increased intrinsic stability of proteins at higher temperature, **extrinsic stabilizing mechanisms** such as ligand binding and crowding, but more important, preferential solvation in the presence of high concentrations of compatible solutes and the actions of 'molecular chaperones' play a crucial role. **Compatible solutes** are small organic solutes that many organisms accumulate in response to different stress conditions, but do not compromise any cellular functions (e.g. amino acids, sugars, polyols, methylamines) (Santos and da Costa, 2002). Stabilizing compounds are preferentially excluded from the protein surface as the protein has a higher affinity for water than for those solutes. Consequently, the proteins are preferentially hydrated, favouring the native state and makes unfolding more difficult. Beside compatible solutes, **molecular chaperones** play a crucial role in stabilisation of thermophilic proteins, better known as heat-shock proteins (HSP). They are omnipresent in the cell and can be defined as 'any protein that transiently interacts with/stabilizes an unstable conformer of another protein, facilitating its folding, assembly and interaction with other cellular components, as well as its intracellular transport or proteolytic degradation' (Leroux and Hartl, 2000).

3.1.3. Lipids and membranes

The cell membrane is a barrier between the cytosol and environment consisting of lipid layers with embedded proteins that generate specific and vital solute concentration gradients over the membrane. Active transport mechanisms or passive diffusion helps the penetration of small solutes across the membrane. As passive diffusion is accelerated with increasing temperature, thermophilic membranes do not only have to be **thermostable**, but do require specific mechanisms to limit the permeability of ions, especially of protons, because of the essential role of proton gradients in ATP synthesis. This is also known as the '**homeoproton permeability adaptation**' (van de Vossenberg et al., 1995).

In contrast with nucleic acids and proteins, where the thermophilic cells consist of the same building block as mesophilic cells, a clear difference in chemical composition of lipids/membranes can be observed (Table 1.3, Figure 1.5). Generally, the cell membrane is composed of inner and outer hydrophilic surfaces (polar head groups) enclosing a hydrophobic interior (long hydrophobic chains). All bacterial lipids are composed of **esters** between glycerol and fatty acid chains, whereas archaeal lipids are composed of **ethers** between glycerol (or another alcohol) and branched C_{20}-hydrocarbon sidechains (Schouten et al., 2013).

Table 1.3. Overview of fundamental difference in the chemical composition of lipids and membranes of bacteria and (thermophilic) archaea.

	Lipid type	Glycerol	Side chain	membrane
Bacteria	glycerol fatty acyl di**esters**	1	Fatty acid	Bilayer
Archaea	diphytanylglycerol di**ether** = archaeol	1	C_{20}-hydrocarbon	Bilayer
Thermophilic archaea	dibiphytanylglycerol tetra**ether** = caldarchaeol	2	C_{20}-hydrocarbon*	Monolayer

* possibly modified with cyclopentane rings

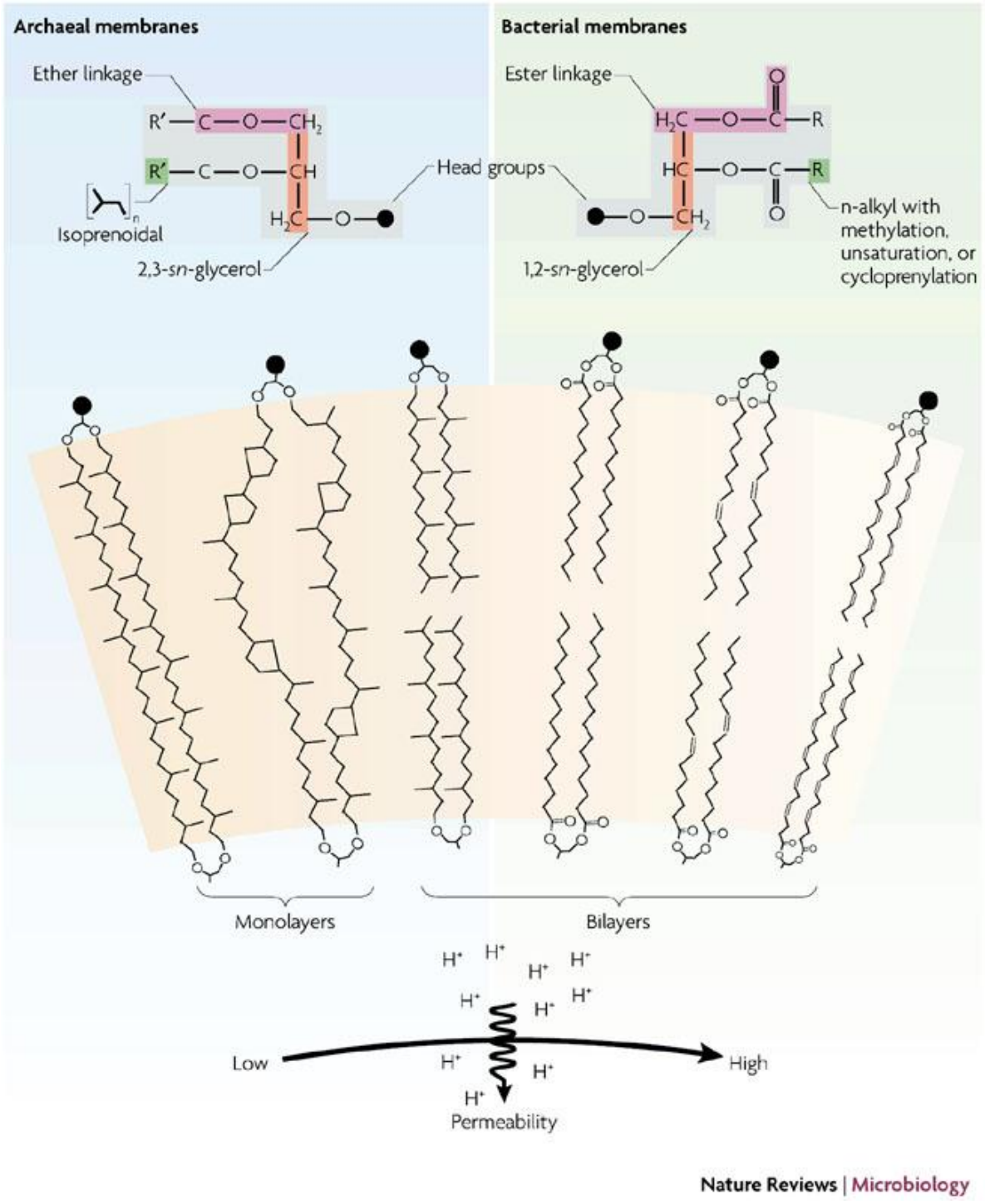

Figure 1.5. The basic chemical structures of archaeal (left) and bacterial (right) membrane lipids. Examples of intact membrane structures used by archaea (left) and bacteria (right) are shown below, including the monolayers that are produced by some archaea and the highly unsaturated membranes produced by some bacteria. The arrow (bottom) indicates a general trend of increasing permeability to ions such as protons and sodium. (Valentine, 2007).

The hydrocarbon chains within archaeal lipids consist of repeated isoprenoid units with a methyl group at every 4th carbon atom in the backbone, hereby restricting the mobility of the chains and so, stabilizing them and restricting ion permeability. The side chains can be either ether-linked with one glycerol unit (archaeol) as found in all archaea or with two glycerol units (caldarchaeol), only found in thermophilic archaea (Table 1.3). Caldarchaeols can furthermore be modified with cyclopentane rings in the side chains, together with the ether bonds, being crucial for ensuring the low ion permeability at high temperatures (Jaenicke and Sterner, 2006). Caldarchaeols moreover form monolayers that, in contrast with the typical bilayer structure of bacterial membranes, result in smaller diameters and are much more stable due to their covalent character. A relevant example of a thermophilic archaeal membrane compound is crenarchaeol, dominating the membrane lipids of the ammonium oxidizing archaea (AOA) *Candidatus Nitrososphaera gargensis* (Figure 1.6) (Pitcher et al., 2010).

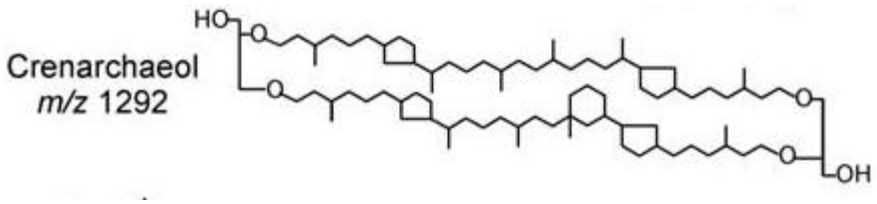

Figure 1.6. Molecular structure of Crenarchaeol, a dibiphytanylglycerol tetraether, the main membrane lipid component of the of the AOA *Candidatus Nitrososphaera gargensis* (Pitcher et al., 2010).

Beside the intrinsic differences, both bacteria and archaea can adjust the chemical composition of their membranes in function of temperature fluctuations, ensuring constant stability and permeability. Archaea respond to elevated temperatures by increasing the degree of saturation, cyclization of the side chains and by replacing diether to tetratether lipids. Bacteria act by increasing the average length of the lipid acyl side chains, the degree of saturation and the ratio of iso/anteiso branched fatty acids (Koga, 2012).

3.2. Thermophilic carbon treatment

3.2.1. Thermophilic aerobic treatment

The first aerobic thermophilic biological treatments, referred to as 'fluidized composters', emerged from the composting process, commonly used to treat moist organic solids (Rozich and Bordacs, 2002). In the **autothermal thermophilic aerobic digestion** (**ATAD**) process, the microbial mediated breakdown of organics results in a heat release of sufficient magnitude to maintain reactor temperatures of 45-65°C. The ATAD process initially evolved from a pasteurization process for sewage sludge and animal slurries as it produces a biologically stable product suitable for disposal in the environment (Layden et al., 2007). Full scale ATAD processes have been in operation since 1977, particularly in Canada and the United States (Layden et al., 2007). Beside the ATAD process for 'aerobic digestion' of sludge, **thermophilic aerobic wastewater treatment** for the treatment of industrial wastewaters (pulp and paper, brewery, slaughterhouse,…) has extensively been studied in laboratory and pilot-scale reactors and a few full scale plants (Suvilampi and Rintala, 2003).

As predicted by the van't Hoff-Arrhenius equation, thermophilic **biodegradation rates are higher** compared with mesophilic rates. Thermophilic aerobic processes are consequently known to operate under markedly high loading rates up to 25 kg COD m^{-3} d^{-1}, decreasing the required reactor volume and reducing the construction costs. Elevated biodegradation rates allow rapid recovery from reactor failing conditions hereby **improving process stability** (Lapara and Alleman, 1999). Furthermore, the **sludge yield** is generally accepted to be **lower** at thermophilic compared with mesophilic conditions (Suvilampi et al., 2006).

The settling of small particles (e.g. free bacteria) should theoretically be enhanced with increasing temperatures due to lower water viscosity (Schwarzenbach et al., 2005). However, the common opinion is that thermophilic aerobic processes suffer from **poor sludge settling properties** due to a poor floc formation at high temperatures (Suvilampi et al., 2006).

3.2.1. Thermophilic anaerobic digestion

Similar with the ATAD process, anaerobic digestion (AD) has historically mainly been associated with the treatment of animal manure and sewage sludge as a hygienization/stabilization practice prior to land application. However, increased environmental awareness together with the demand for renewable energy and new waste management strategies, have broadened the application domain of AD. In AD, organic material is converted to biogas (mainly methane and carbon dioxide), that can be converted to heat and electrical energy by a combined heat and power (CHP) unit. This resulted in a tripling of the gross green power production in Flanders (Belgium) from various biogas technologies over the 5 last years (De Geest et al., 2012).

Although most of the installed anaerobic digesters until now are mesophilic, thermophilic (55°C) AD is gaining more attention. Thermophilic AD shows a **higher reduction of pathogens** and can achieve **higher loading** rates compared with mesophilic AD (De la Rubia et al., 2013). **Higher biogas production** rates and **higher volatile solids reduction** can be achieved. However also higher toxic, free ammonia concentrations and accumulation of volatile fatty acids were observed and if a lot of energy is needed for heating, thermophilic AD might be energetically less favourable.

3.3. Thermophilic nitrogen converting microorganisms

Thermophilic microorganisms, including nitrogen-converting species, are considered the pioneers of life on our planet (Nisbet and Sleep, 2001). Nitrogenase, which after Rubisco is the next most important enzyme, consists of an iron protein and a molybdenum–iron protein including sulfur. The presence of molybdenum, iron and sulfur suggest a hydrothermal heritage (Nisbet and Sleep, 2001). The recent discovery of thermophilic AOA and NOB (sections 3.3.1 and 3.3.2), and the detection of anammox sequences in thermophilic environments (section 3.3.4), show that even nowadays, thermophilic nitrogen cycling might be crucial for hydrothermal and geothermal life.

3.3.1. Nitritation

An overview of the different ammonium oxidizing strains isolated from thermophilic environments is presented in Table 1.4. Beside a morphological observation of *Nitrosomonas* at 50°C (Golovacheva, 1976) and the isolation of some thermophilic heterotrophic AOB (**Table 1.4**), thus far, only one thermophilic autotrophic AOB was isolated and characterized. It was isolated from activated sludge in a thermal power station, is related to *Nitrosomonas nitrosa*, and has a 'moderately' thermophilic optimum temperature (42°C) (Itoh et al., 2013). Autotrophic ammonium oxidation at higher temperatures seems to be dominated by AOA. Four thermophilic AOA isolations/enrichments are described so far (Table 1.4), of which *"Candidatus* Nitrosocaldus yellowstonii*"*, enriched from a Yellowstone hot spring, oxidizes ammonium at the highest temperature (74°C) (de la Torre et al., 2008). The difficulty of cultivating the slow growing AOA at elevated temperature under laboratory conditions resulted in low number of enrichments. However, many archaeal ammonia monooxygenase (*amoA*) genes have been detected in high-temperature habitats such as deep-sea hydrothermal vents (Wang et al., 2009b; Baker et al., 2012), subsurface thermal springs (Spear et al., 2007; Weidler et al., 2008) and terrestrial hot springs (Reigstad et al., 2008; Dodsworth et al., 2011). In addition to these oligotrophic ecosystems, the *amoA* gene was also measured in nutrient-rich high-temperature engineered environments such as petroleum reservoirs (Li et al., 2011) and composting facilities (Zeng et al., 2011).

Table 1.4 Overview of the thermophilic ammonium oxidizing strains isolated from thermophilic environments. Het: heterotrophic. n.d.: not determined.

Micro-organism	Strain	T_{opt} (°C)	T_{max} (°C)	Origin	Reference
AOB	*Nitrosomonas* sp. JPCCT2	42	48	activated sludge (thermal power station)	(Itoh et al., 2013)
Het AOB	*Bacillus* sp. strain T3	50	n.d.	compost (cattle manure)	(Shimaya and Hashimoto, 2011)
Het AOB	*Bacillus* sp. and *Thermus* sp.	65	75	hydrothermal vent (Mid-Atlantic Ridge)	(Mevel and Prieur, 1998)
AOA	*Nitrosospaera viennensis* strain EN76	42	47	garden soil (Vienna, Austria)	(Tourna et al., 2011)
AOA	*"Candidatus* Nitrososphaera gargensis*"*	46	50	hot spring sediment (Baikal rift zone, Russia)	(Hatzenpichler et al., 2008)
AOA	*"Candidatus* Nitrosotenuis uzonensis*"*	46	52	hot spring sediment (Kamchatka, Russia)	(Lebedeva et al., 2013)
AOA	*"Candidatus* Nitrosocaldus yellowstonii*"*	72	74	hot spring sediment (Yellowstone, USA)	(de la Torre et al., 2008)

3.3.2. Nitratation

Nitrospira spp. are the most dominant nitrite oxidizers up to 60°C. *Nitrospira calida* was isolated from a microbial mat of a terrestrial geothermal spring and maximally oxidizes nitrite at 46-52°C (Lebedeva et al., 2011). Thus far, other detected/enriched NOB from geothermal springs are all closely related with *Nitrospira calida* (Marks et al., 2012; Edwards et al., 2013). The recently discovered *Nitrolancetus hollandicus* belonging to the phylum Chloroflexi is considered a thermotolerant nitrite oxidizer with a maximum growth temperature of 46°C, and nitrite oxidizing activity up to 63°C (Sorokin et al., 2012).

3.3.3. Denitrification

In contrast with the relative recent discovery of thermophilic nitrifying micro-organisms, the first thermophilic denitrifying bacteria was isolated in 1913, originally named *Denitrobacterium thermophilum* (Ambroz, 1913), later renamed as Bacillus thermo-denitrificans and then reclassified as *Geobacillus thermodenitrificans* (Coorevits et al., 2012). The majority of the described thermophilic denitrifiers belong to the *Geobacillus* lineage, of which most of the known are presented in Table 1.5. Beside these Gram positive, spore forming *Geobacilli*, also archaea and non-spore forming bacteria such as *Thermus thermophiles* are known to denitrify under thermophilic conditions (Table 1.5) (Oshima and Imahori, 1974; Cabello et al., 2004).

3.3.4. Anammox

The maximum temperature at which anammox growth was observed is 43°C (Strous et al., 1999). Although anammox enrichments at thermophilic temperatures did not succeed so far (Itoh et al., 2013), the presence of anammox has been reported in different high temperature environments. Amplification of 16S rRNA gene sequences related to known anammox bacteria, ladderanes lipids analysis and isotope experiments showed the occurrence of anammox in deep-sea hydrothermal vents (Byrne et al., 2009), hot springs (Jaeschke et al., 2009) and high temperature petroleum reservoirs (Li et al., 2010).

Table 1.5 Examples of different thermophilic denitrifying bacteria and archaea isolated from thermophilic environments. red: reduction

	Strain	NO_3^- red	NO_2^- red	T_{opt} (°C)	T_{max} (°C)	Origin	Reference
Bacteria							
	Geobacillus thermodenitrificans	+	+	55	70	soil	(Manachini et al., 2000; Nazina et al., 2001)
	Geobacillus stearothermophilus	+	+	60	80	soil	(Ho et al., 1993; Nazina et al., 2001)
	Geobacillus thermoglucosidasius	+	-	62	69	soil	(Suzuki et al., 1983; Nazina et al., 2001)
	Geobacillus uzenensis.	+	-	55	65	oilfield	(Nazina et al., 2001)
	Geobacillus subterraneus	+	+	58	70	oilfield	(Nazina et al., 2001)
	Geobacillus toebii	+	+	60	70	hay compost	(Sung et al., 2002)
	Anoxybacillus pushchinensis	+	+	55	65	manure amended soil	(Yamamoto et al., 2006)
	Thermus thermophilus	+	-	65	85	thermal water	(Oshima and Imahori, 1974)
	Hydrogenobacter thermophiles	+	+	70	75	thermal water	(Suzuki et al., 2001)
	Petrobacter succinatimandens	+	-	50	60	oil well	(Salinas et al., 2004)
Archaea							
	Pyrobaculum aerophilum	+	+	100	102	boiling marine water hole	(Volkl et al., 1993)
	Haloarcula marismortui	+	+	45	50	Dead sea	(Oren et al., 1990)
	Haloferax denitrificans	+	+	50	55	salt lake	(Tomlinson et al., 1986)

3.4. Thermophilic nitrogen biotechnology

Different nitrogen containing warm wastewater streams could potentially be treated with thermophilic nitrogen removal: domestic wastewater in tropical regions with high seasonal temperatures, industrial wastewaters from e.g. steel industry and fertilizer industry and thermophilic digestates (e.g. sludge, biorefinery (Agler et al., 2008)). However, thermophilic nitrogen biotechnology is an unexplored territory. So far, four studies focused on the biological nitrogen removal performance at thermophilic temperatures, of which half of them only investigated the short-term effect of elevated temperatures on nitrification (N) or denitrification (DN). Both, nitrifying as well as denitrifying activity was measured up to 50°C in short-term (hours) batch activity tests in activated sludge from an oil refinery (Lopez-Vazquez et al., 2013) and manure treatment plant, respectively (Willers et al., 1998). These short-term tests only clarify the effect of temperature on the intrinsic N or DN rate, while long term exposure to thermophilic conditions can induce a loss of N/DN capacity due to biomass decay. However, the mesophilic population may adapt to high temperatures after long-term exposure.

Shore et al. (2012) investigated the temperature transition of two nitrifying moving bed biofilm reactors (MBBR) at 30°C to 40°C and 45°C, respectively. The temperature increase to 40°C initially inhibited nitrification in the MBBR, but recovered within four weeks reaching a maximum nitrification rate of 144 mg N L^{-1} d^{-1}. At 45°C, however, no nitrification could be achieved. In contrast with the relatively low maximum reactor temperature of 40°C for nitrification, a denitrifying upflow sludge blanket (USB) reactor was described at 55°C (Laurino and Sineriz, 1991). The USB reactor was inoculated with thermal mud originating from a hot spring and started-up in batch mode for 15 days. After switching to continuous mode, a maximum nitrogen removal rate of 1317 mg N L^{-1} d^{-1} with a nitrate removal efficiency of 78.4% was observed.

Beside these two research articles focusing on N/DN at elevated reactor temperatures, thermophilic nitrogen removal has mainly been investigated as a part of studies focusing on thermophilic activated sludge. As the main goal in those studies was the oxidation of organic material, the exact fate of nitrogen in these thermophilic systems remained unclear in most

cases. Limited data with regard to the nitrogen balance is available but the main nitrogen removal mechanisms are assumed to be ammonia stripping and nitrogen assimilation into biomass (Table 1.6). The detection of nitrous oxide (N_2O) in the gas phase of a lab-scale aerobic thermophilic bioreactor treating swine waste suggests that nitrogen was partially removed by biological nitrogen conversion. Nitrous oxide is a well-known intermediate of the (anoxic) denitrification process (see section 2.1.3), but can also be formed by autotrophic nitrifiers during nitrification or 'nitrifier denitrification' (Wrage et al., 2001). However, as neither nitrite nor nitrate were ever measured in thermophilic aerobic bioreactors, there is no evidence that nitrification took place in these thermophilic systems (Juteau, 2006).

Table 1.6 Reported nitrogen balances in different aerobic thermophilic reactor studies. n.d.: not determined. * not measured (estimated in the respective study through calculation of the nitrogen balance)

Influent			Nitrogen mass balance (% N)					Reference
Type	mg N L^{-1} (g COD/ g N)	T (°C)	Effluent	Bio-mass	NH_3	N_2O	N_2	
Industrial	1817 (9.2)	45	21	13	66*	n.d.	n.d.	(Kurian et al., 2005)
Pig waste	4680 (7.5)	50	40	4.8	35	20	n.d.	(Yi et al., 2003)
Pig waste	4718 (7.4)	60	34	n.d.	28	9	29	(Lee et al., 2004)
Synthetic	765 (14.6)	60	46	9	28*	n.d.	17*	(Abeynayaka and Visvanathan, 2011a)
Synthetic	320 (14.7)	60	64	5	24*	n.d.	7*	(Abeynayaka and Visvanathan, 2011b)

Although ammonia stripping is assumed to be the main nitrogen removal mechanism in current warm streams, according to Table 1.6, still 21-64% of the ammonium remains in the effluent and needs further treatment. Ammonia stripping efficiencies can be increased with caustic dosage with current dedicated technologies up to 85-90% (Menkveld and Broeders, 2015). Though, depending on the nitrogen discharge limits, a biological nitrogen removal is needed as it can achieve removal efficiencies > 95%. The cost effectiveness of nitrogen recovery through ammonia stripping furthermore mainly depends on the ammonium concentration in the influent (> 2-5 g N L^{-1}), scale of the installation, and the local value and market for ammonium sulphate. Although some streams are eventually enough concentrated (Table 1.6), it thus not always make sense to recover depending on the discharge limits and ammonium sulphate market and so, thermophilic nitrogen removal is needed.

4. Objectives and outline of this research

Thermophilic nitrogen cycling microorganisms were separately isolated, enriched or detected, representing a hidden treasure of natural resources. The transposition capability into useful, biotechnological communities for wastewater treatment is however still unexplored. Therefore, four research chapters were elaborated in this work to develop thermophilic nitrogen removal processes (Figure 1.7).

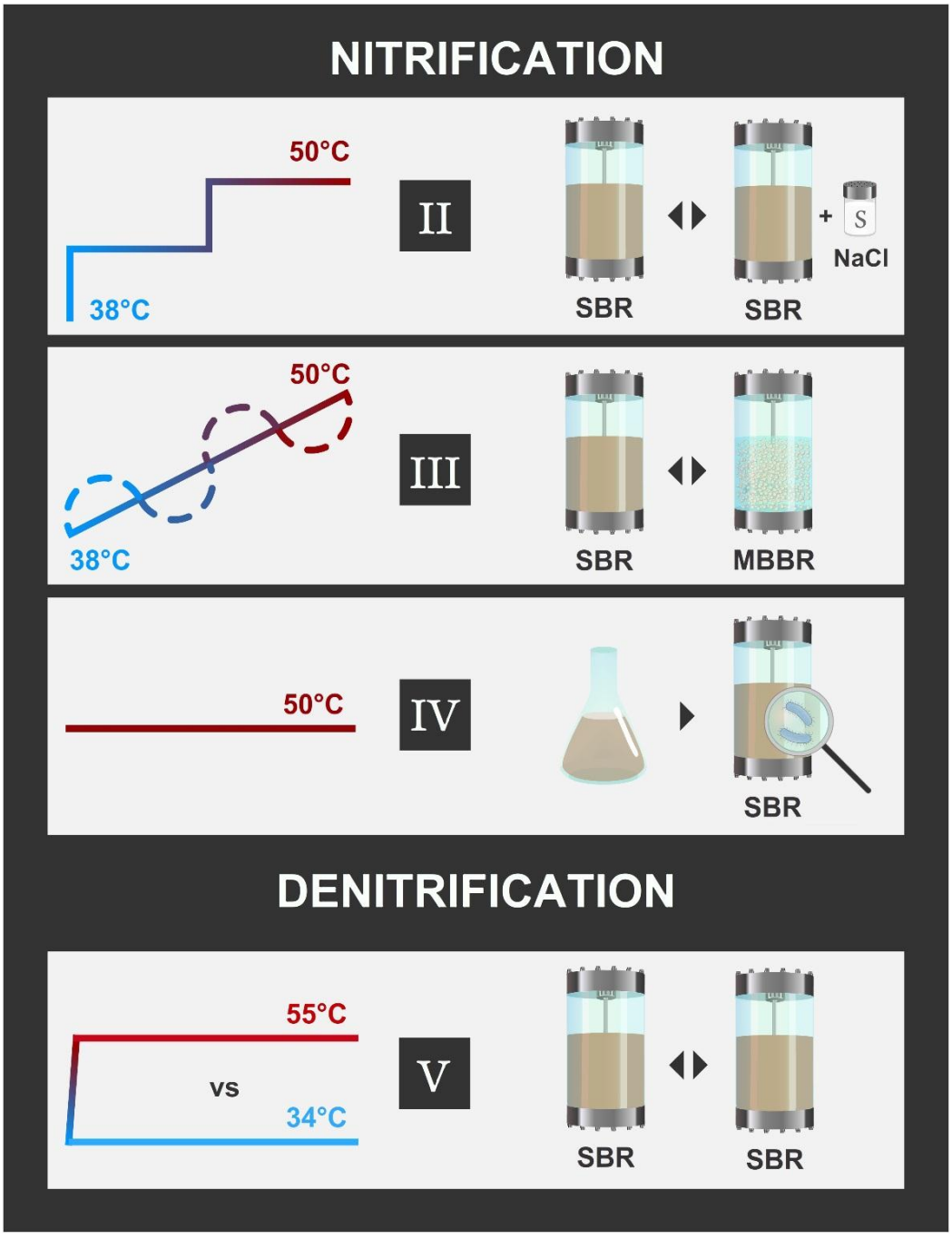

Figure 1.7. A graphical overview of the different research chapters

As nitrification is known as the most sensitive- and rate limiting step in conventional biological nitrogen removal, this research focused on development strategies for thermophilic nitrification. Three research papers were elaborated with two fundamentally different strategies to achieve thermophilic nitrification. The first strategy involved the transition of an established mesophilic nitrifying community to elevated temperatures (Chapter 2 and 3), whereas the second strategy involved the selective enrichment of thermophilic nitrifiers from natural thermophilic environments (Chapter 4).

Thermophiles have faster growth rates than mesophiles, however, due to even higher maintenance energy and decay rates, this results in lower net microbial growth (Lapara and Alleman, 1999). The low sludge growth suggest that long adaptation/acclimatization periods will be necessary to achieve thermophilic nitrogen removal. This PhD research therefore developed strategies to shorten down the adaptation period, mainly by the use of a pre-exposure to stress. Pre-exposure to a certain stress can in some cases namely result in an increased resistance towards this, or another, subsequent stress (Fletcher and Csonka, 1998; Philippot et al., 2008). With regard to an increasing temperature stress in this case, heat-shock proteins (HSP) are known to play a crucial role (Jaenicke and Sterner, 2006). Interestingly, beside a temperature shock, HSP production and subsequent heat-shock response can also be induced by osmotic stress (Feder and Hofmann, 1999). Therefore both salt stress (Chapter 2) and temperature stress (Chapter 3), in the form of a temperature oscillation, was evaluated to facilitate the transition process. The low sludge growth furthermore suggest the need of a good biomass retention and was investigated through the implementation of different sludge growth modes (Chapter 3).

- **Chapter 2**: Salt stress was anticipated to improve the thermo-elasticity of the mesophilic sludge. Short term batch tests evaluated different salt concentrations at different temperatures and for different sludge types. The use of salt amendment as a tool to achieve more efficient temperature transitions for mesophilic sludge was subsequently investigated in a long term reactor subjected to a **stepwise temperature increase.**

- **Chapter 3**: The thermo-elasticity of a suspended growth reactor system was evaluated for an **oscillating** and **gradual temperature increase**, in which it was compared with a biofilm based system. The adaptive capacities were closely monitored with parallel batch activity tests and the abundance of key nitrifiers was followed over time.

- **Chapter 4**: Samples from composting facilities were used as inocula for the batch-wise enrichment of thermophilic (50°C) nitrifying communities. The objective of this research was to assess the biotechnological potential of this nitrifying enrichment in a continuous bioreactor and to provide a phylogenetic, physiological and morphological characterization of the nitrifying community.

In the second part of this work, thermophilic denitrification was extensively compared with mesophilic denitrification (Chapter 5).

- **Chapter 5**: This chapter assessed the start-up of a thermophilic denitrifying reactor (55°C) in continuous mode with a mesophilic inoculum (26°C). Reactor performance, sludge characteristics and microbial community were compared with a mesophilic denitrifying reactor (34°C) for different feeding periods ranging from synthetic to real wastewater.

In a final general discussion the main results are discussed, after which future perspectives and research suggestions are proposed, followed by an overall conclusion (Chapter 6).

CHAPTER 2:

INCREASED SALINITY IMPROVES THE THERMOTOLERANCE OF MESOPHILIC NITRIFICATION

This chapter has been redrafted after:

Courtens, E.N.P., Boon, N., De Schryver, P. & Vlaeminck S.E. 2014. Increased salinity increases the thermotolerance of mesophilic nitrification. Applied Microbiology and Biotechnology, 98(10), 4691-4699.

1. Introduction

The adaptive capacities of microbial communities to changing temperatures have been described, for instance for activated sludge systems (Suvilampi and Rintala, 2003). Acclimatization periods are mostly needed, but some factors can improve resistance to higher temperatures. In food preservation for example, high osmolarity induced an increased thermotolerance in *Salmonella* (Fletcher and Csonka, 1998). As a response to osmotic stress, microorganisms accumulate osmoprotectants. The role of those compatible solutes can also protect the cell or cell components from other environmental constraints such as freezing, desiccation, oxygen radicals, and high temperature (Welsh, 2000). These findings suggest that, although a salt shock is known to inhibit both AerAOB and NOB (Moussa et al., 2006), the associated induction of compatible solutes may also help nitrifiers to maintain their activity at higher temperatures.

This study investigated whether increased salinity can enhance the thermotolerance of a mesophilic nitrifying community. Firstly, in batch activity tests, both the effect of different salt concentrations at 40°C as the effect of 5 g NaCl L^{-1} at different temperatures was explored, measuring not only immediate activity, but also activity after 48 h. Subsequently, a continuous experiment with fed-batch reactors was used to evaluate salt amendment (7.5 g NaCl L^{-1}) between 34 and 50°C. Besides physicochemical measurements, the nitrifying community was closely monitored by microbial as well as molecular analyses.

2. Materials and methods

2.1. Activity batch tests

Aerobic batch experiments were performed as described by Windey et al. (2005). Initially, a screening test at 34 and 40°C with a salinity shock of 5 g NaCl L^{-1} was performed on two different inocula containing AerAOB and NOB. Conventional activated sludge (CAS) and sludge from a biological nutrient removal (BNR) reactor were used. Beside the nitrifying AerAOB and NOB, the CAS sludge contained additional aerobic heterotrophs and the BNR sludge additional anammox bacteria (AnAOB). The CAS sludge was freshly collected from a sewage treatment

plant (Destelbergen, Belgium), operating at a yearly average temperature of 15°C, while the BNR sludge was harvested from a mature lab-scale rotating biological contactor operating at 34°C for ten years and fed with synthetic influent (1 g N L^{-1} $(NH_4)_2SO_4$, 7.5 g $NaHCO_3$ g^{-1} N and 308 mg KH_2PO_4 L^{-1} (Pynaert et al., 2003). As only the BNR sludge showed significant effects, further tests were performed with this biomass. Firstly, at 40°C, the effect of different salinity shocks (0, 2, 5 and 8 g NaCl L^{-1}) was tested. Secondly, a salinity shock of 5 g NaCl L^{-1} was tested at three different temperatures (31, 40 and 45°C) and both the direct activity as well as the activity after 48h was determined. The aerobic tests were all performed in open Erlenmeyer flasks with ammonium as substrate (NH_4Cl, 50 mg N L^{-1}) and a pH 7 buffering solution with final concentrations of 1 g $NaHCO_3$ L^{-1}, 4.2 g KH_2PO_4 L^{-1} and 5.8 g K_2HPO_4 L^{-1}. Ammonium, nitrite and nitrate concentrations were monitored for a period of 8 hours after substrate addition. The direct specific activity was calculated based on the partial nitritation/anammox stoichiometry (eq 1+3 in Table 1.1) (Vlaeminck et al., 2012) after the first substrate addition (substrate added after ±1 hour acclimatization of the sludge to the tested temperature), whereas the activity after 48h was calculated after a second substrate addition. In short, the nitrogen loss was assigned to AnAOB activity and AerAOB activity was calculated based on the ammonium removal taking into account the contribution of the AnAOB activity. Similarly, the NOB activity was calculated based on the nitrate production taking into account the nitrate production by the anammox (0.26 mol $NO_{3\ produced}^-$/mol $NH_{4\ removed}^+$).

$$r\_AerAOB\ (mg\ N\ L^{-1}\ d^{-1}) = P_{NO2} + NOB + 1.32/2.32 \times (R_N + (0.11/0.89 \times R_N))) \quad (1)$$

$$r\_NOB\ (mg\ N\ L^{-1}\ d^{-1}) = P_{NO3} - (0.11/0.89 \times R_N) \quad (2)$$

Herein, R_N is nitrogen removal rate by AnAOB (mg N L^{-1} d^{-1}), P_{NO2} the part of the AerAOB nitrite production rate that was not consumed by NOB or AnAOB (mg N L^{-1} d^{-1}) and P_{NO3} the nitrate production rate produced by NOB and AnAOB (mg N L^{-1} d^{-1}). The constants of 0.11/0.89 are derived from the nitrate production in PN/A stoichiometry (i.e. eq 3 in Table 1.1), whereas the constants 1.32/2.32 are derived from the anammox stoichiometry itself in which 1.32 mole nitrite is combined with one mole of ammonium (eq. 3 in Table 1.1). All tests were performed in triplicate on a shaker (100 rpm), including an abiotic reference for stripping and a reference without NaCl addition for each temperature.

2.2. Reactor set-up and operation

The two parallel fixed-bed (AnoxKaldnes K1 carriers, filling ratio 50%) reactors (salt and control) had an effective liquid volume of 2 L, an inner diameter of 12 cm (Figure 2.1). The reactors were inoculated with BNR biofilm sludge originating from a rotating biological contactor described by Pynaert et al. (2003) at an initial biomass concentration of 3.3 ± 0.1 g VSS L^{-1}. The reactors were operated in a sequential batch feeding/withdrawal mode to allow for both biofilm as suspended growth. The one hour cycle consisted of a 50 min aerobic reaction period including a 6 min feeding period at the beginning of the cycle, followed by a 5 min settling period and a 5 min decanting period. In order to estimate the amount of NH_3 stripping during operation, hydraulic tests (without biomass) were performed in the reactors before start-up at pH 8, 50°C and varying NH_4^+ concentrations. Reactors were fed with synthetic medium consisting of $(NH_4)_2SO_4$ (50 mg N L^{-1}), 7 g $NaHCO_3$ g N^{-1} (1.17 mol $NaHCO_3$ mol^{-1} N) and KH_2PO_4 (10 mg P L^{-1}) dissolved in tap water resulting in a salinity of 2 g L^{-1} NaCl equivalents. The influent of the salt reactor was supplemented with 7.5 g NaCl L^{-1}. As less bicarbonate was dosed in the reactor influent compared with the batch tests, a higher NaCl concentration was dosed here in order to reach the same final conductivity as in the batch tests.

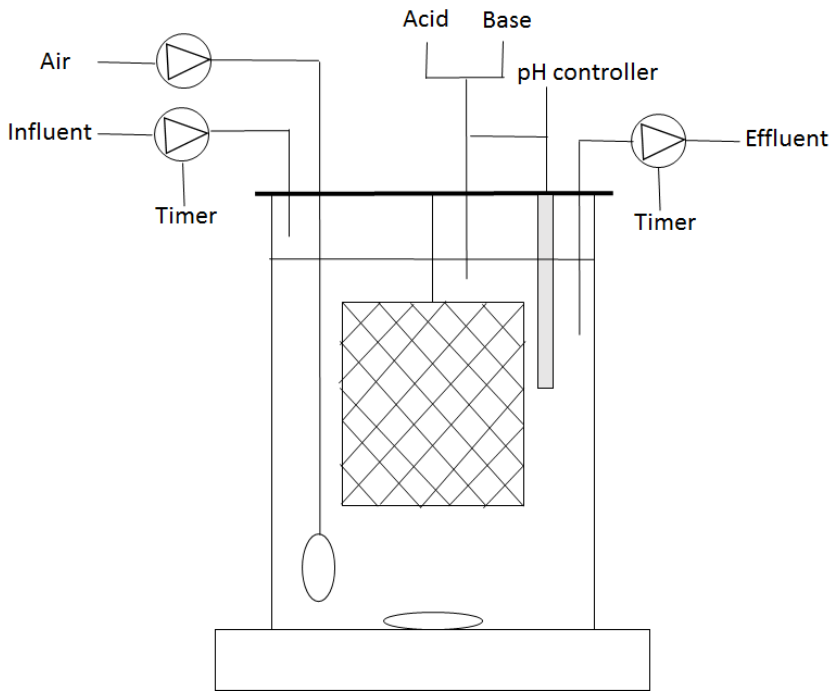

Figure 2.1 Reactor set-up of the fixed bed reactors (filling ± 50%).

The reactor vessels were jacketed, and the temperature was controlled with a circulating thermostatic water bath. After a start-up period of 14 days at 34°C, the temperature of both was gradually increased to 40, 42.5, 45, 47.5 and 50°C on days 14, 88, 130, 180 and 193, respectively. Temperature was increased after 2 weeks of stabilization, except for the 40°C period, where each reactor suffered from a pH control failure and a longer stabilization period was applied. At 40 and 42.5°C, a 2 week period where no NaCl was added to the feed of the salt reactor was also included.

In principle the influent nitrogen concentrations were kept stable. However, from the moment that AerAOB activity decreased, the influent NH_4^+ concentration was lowered from 50 to 20 mg N L^{-1} to avoid higher free ammonia (FA) concentrations. FA concentrations were calculated based on pH, T and ionic strength, as described in Bell et al. (2008) and reached 0.30 ± 0.23 and 0.19 ± 0.35 mg N L^{-1} in the control and salt reactor, respectively. From the moment AerAOB activity decreased, 50 mg N L^{-1} nitrite was additionally dosed in the influent as $NaNO_2$ to prevent NOB substrate limitation and thus underestimation of the NOB activity, as done from day 23 on in the control reactor and from day 58 on in the salt reactor (Figure 2.5 B).

The reactor pH was controlled between pH 6.8 and 7.0 by dosage of 0.1 M NaOH/HCl in order to minimize FA stripping and avoid high FA concentrations at higher temperatures. Averages pH values of 6.88 ± 0.18 and 6.91 ± 0.16 for the control and salt reactor were reached, respectively. High pressure air pumps provided aeration through a diffuser stone at an average superficial air flow rate of 1.33 m^3 m^{-2} h^{-1}, resulting in the dissolved oxygen (DO) concentrations of 3.94 ± 1.23 and 4.06 ± 1.14 mg O_2 L^{-1} for the control and salt reactor, respectively. Flow rates were not significantly different (7.5 ± 1.0 and 7.8 ± 1.2 L d^{-1}, for control and salt reactor respectively), resulting in similar hydraulic retention times (HRT) of 6.51 ± 0.80 and 6.12 ± 0.60 h, respectively. Consistent with the batch tests, the AerAOB and NOB volumetric activities were calculated based on the partial nitritation/anammox stoichiometry (eq 1,2) and average activities were calculated at stable periods of minimum 5 days.

2.3. Chemical analyses

Ammonium (Nessler method) and volatile suspended solids (VSS) were measured according to standard methods (Greenberg et al., 1992). Nitrite and nitrate were determined on a 761 Compact Ion Chromatograph (Metrohm, Switzerland) equipped with a conductivity detector. DO and pH were measured with, respectively, a HQ30d DO meter (Hach Lange, Germany) and an electrode installed on a R305 pH-controller (Consort, Belgium). Fatty acid methyl esters (FAME) from the biomass were prepared by transesterification for identification by a gas chromatograph (Coutteau and Sorgeloos, 1995).

2.4. Molecular analyses of the microbial communities

Fluorescent *in-situ* hybridization (FISH) was used to determine the NOB genus, i.e. distinguish between *Nitrobacter* and *Nitrospira*, as a supporting analysis for the NOB target choice in the following qPCR analysis. Inoculum and endpoint samples were examined by FISH as described by Vlaeminck et al. (2010). **Quantitative polymerase chain reaction** (qPCR) was used to quantify the abundance of AOA, AerAOB, NOB and AnAOB over time. Biomass samples were taken from the inoculum and before each change in temperature or salt concentration. DNA extraction and qPCR were performed according to De Clippeleir et al. (2012) targeting the functional *amoA* gene for AerAOB and AOA, and the 16S rRNA genes of the AnAOB *(Kuenenia* and *Brocadia)*, and *Nitrospira* sp. **Denaturing gradient gel electrophoresis** (DGGE) was used to evaluate the AerAOB community evolution. An inoculum sample was compared with samples of both reactors at the end of each temperature period. Nested PCR, and DGGE were performed based on the primers CTO189ABf, CTO189Cf, and CTO653r for β-proteobacterial AerAOB (Pynaert et al., 2003). The obtained DGGE patterns were subsequently processed with BioNumerics software (Applied Maths, Belgium).

3. Results

3.1. Batch activity tests

Increased salinity could possibly enhance the thermotolerance of mesophilic nitrifiers through the induction of compatible solutes that can help nitrifiers to maintain their activity at higher temperatures. The batch screening experiments with CAS sludge at 34 and 40°C, however, did not show any beneficial effect of salt addition (Figure 2.2). At 34°C, addition of 5 g NaCl L^{-1} namely inhibited AerAOB and NOB by 37% and 91% respectively. Moreover, at 40°C, nearly complete inhibition of NOB was observed. The CAS sludge was not used further.

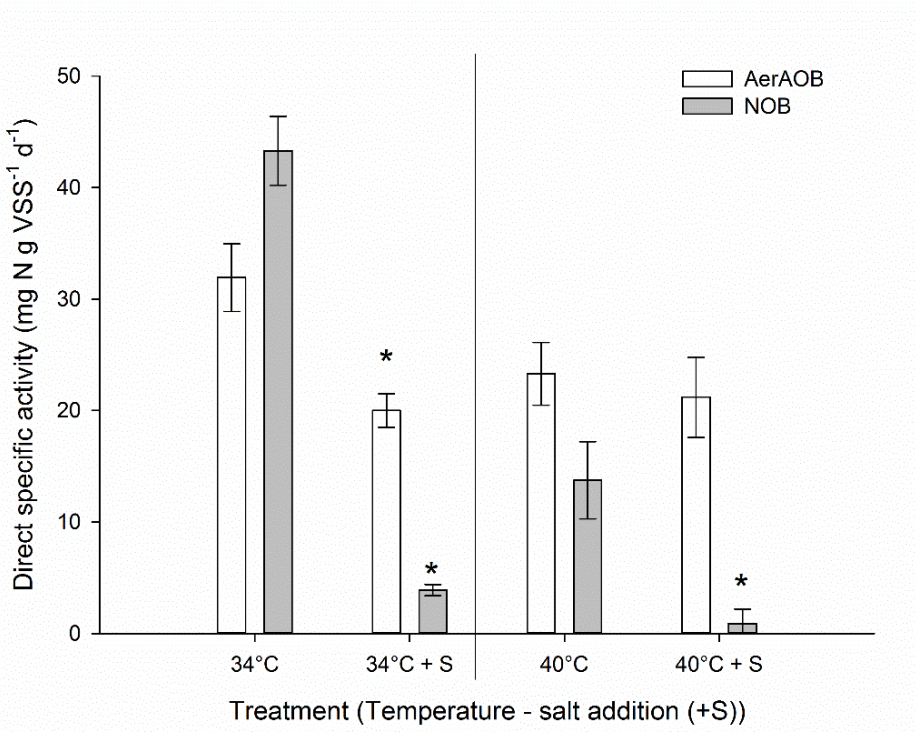

Figure 2.2 Effect of salt addition (5 g NaCl L^{-1}) on the direct specific AerAOB and NOB activities of the CAS. All experiments were performed in triplicate, and statistically significant differences ($p<0.05$) between the control and the salt addition are indicated with an asterisk.

The effect of different NaCl concentrations (0-2-5 and 8 g L^{-1}) were tested on the BNR sludge at 40°C (Figure 2.3). Although no effect could be observed on the NOB activity, the AerAOB activity increased with increasing NaCl concentration until a maximum at 5 g NaCl L^{-1}. At 8 g $NaCl^{-1}$, activity declined, showing no significant difference to the control treatment (Figure 2.3). Consequently, a concentration of 5 g NaCl L^{-1} was used to test its effect at three different temperatures (Figure 2.4).

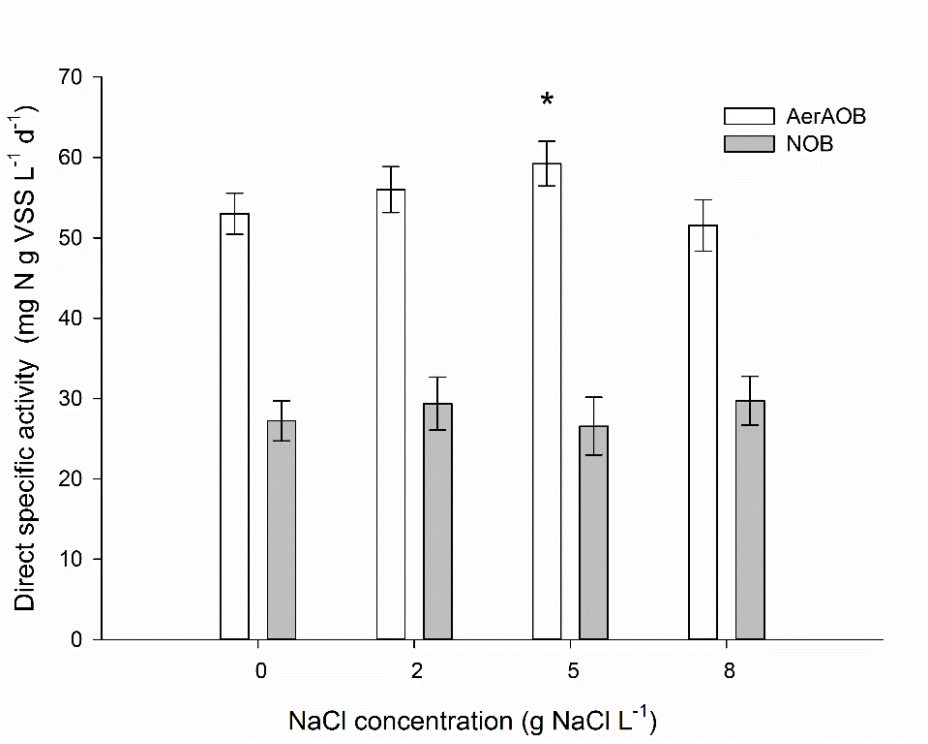

Figure 2.3 Effect of different salt concentrations on the direct specific AerAOB and NOB activities of the BNR sludge. All experiments were performed in triplicate, statistically significant differences ($p<0.05$) between the control and the salt addition are indicated with an asterisk.

In both the control and salt treatment of 5 g NaCl L^{-1}, specific activities decreased with increasing temperature. The lowest temperature tested was 34°C, the temperature corresponding to the temperature of the BNR reactor. At 34°C, salt addition induced no significant immediate changes for AerAOB and NOB activity. Also after 48 h, no salt effect could be detected. Whereas NOB activity was not affected by salt addition at 40°C, the immediate AerAOB activity increased by 21% compared to the control treatment at 40°C. However, after 48h, no significant activity effect of salt addition could be observed anymore. The same phenomenon was observed at 45°C, where the salt addition increased the immediate AerAOB activity by 20%. While for the AerAOB salt addition had a positive effect on the activity, for the NOB salt addition decreased the direct activity at 45°C by 83%. After 48h at 45°C, no AerAOB activity could be detected anymore. Consequently, as no nitrite was added in this test and not produced by the AerAOB, the NOB activity could not be determined. Beside AerAOB and NOB, the BNR sludge also contained AnAOB. The presence of anoxic zones resulted in a nitrogen loss of 37±11%. Both at 34 and 40°C, the addition of salt decreased the AnAOB activity. After 48h, AnAOB activity could only be detected at 34°C. To confirm the

observed salt-induced thermotolerance on AerAOB and exclude a possible starvation effect, a long term reactor experiment was conducted.

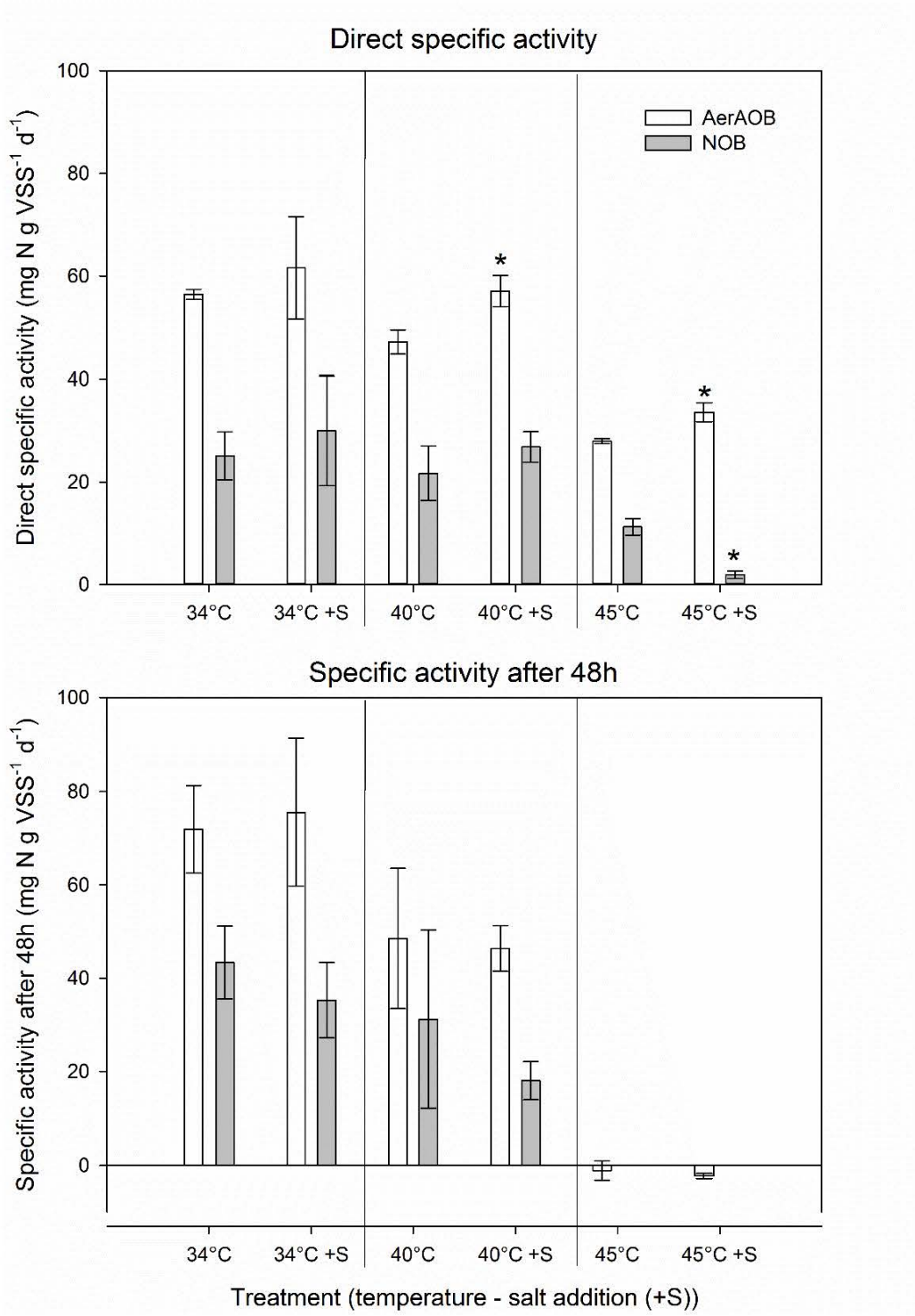

Figure 2.4 Effect of salt addition (5 g NaCl L^{-1}) on the specific AerAOB and NOB activities of the BNR sludge at different temperatures (34, 40 and 45 °C). Both the direct specific activity (top) and the specific activity after 48 h (bottom) were determined. All experiments were performed in triplicate, and statistically significant differences ($p<0.05$) between the control and the salt addition treatment are indicated with an asterisk.

3.2. Parallel reactor tests

A control reactor and a salt reactor were set up in parallel to investigate the longterm effect of salt addition on the thermotolerance of AerAOB and NOB. The imposed temperature profile, salt dosage and influent nitrogen concentrations are shown in Figure 2.5 **A** and **B**, respectively. Figure 2.5 **C** and **D** show the measured effluent nitrogen concentration and the resulting calculated AerAOB and NOB volumetric activities. During the elevated temperature periods (>40°C), 99 ± 3% (control reactor) and 91 ± 6% (salt reactor) of the removed ammonium was recovered as nitrite or nitrate, indicating that nitrification was the main process involved. Denitrification, especially by AnAOB, had thus a minor effect on ammonia conversion compared with aerobic ammonia oxidation. Moreover, hydraulic stripping tests in the reactors reveal that 6 mg N L^{-1} d^{-1} is stripped at pH 8, 55°C and 20 mg NH_4^+ L^{-1}. Since the reactors were controlled at pH 7 and the temperature was mostly much lower than 50°C, stripping accounted for << 4% of the nitrogen loading. Furthermore, assimilation of nitrogen into biomass is minimal for autotrophic nitrifiers (Barnes and Bliss, 1983), accounting for about 2.3% of the converted nitrogen.

After a start-up period of 14 days, stable nitrification rates of 229 ± 40 and 134 ± 10 mg N L^{-1} d^{-1} were reached for the control and the salt reactor, respectively (Figure 2.5 **D**). Increasing the temperature from 34 to 40°C (day 15, Figure 2.5 **A**) initially decreased the specific AerAOB and NOB activities in the control reactor by respectively 90 and 88% resulting in a build-up of ammonium up to 46 mg N L^{-1} (Figure 2.5 **C**). The salt reactor was more resistant to this temperature shock as the immediate decrease in activities for AerAOB and NOB were only 25 and 51%, respectively. Together these data refer to a 65% (= 90% - 25%) and 37% (= 88% - 51%) reduced activity loss for nitritation and nitratation, respectively. The nitrification in the salt reactor restored nearly completely after 20 days. However, in the control reactor, only the NOB could restore entirely, while the AerAOB only regained 54% of their activity present before the temperature increase. While at 34°C, no significant differences in FA could be observed between both reactors, at 40°C, the control reactor reached significantly higher FA values as a consequence of the strongly reduced ammonium oxidation activity. These elevated FA concentrations in the control reactor probably did not facilitate the recovery of the AerAOB.

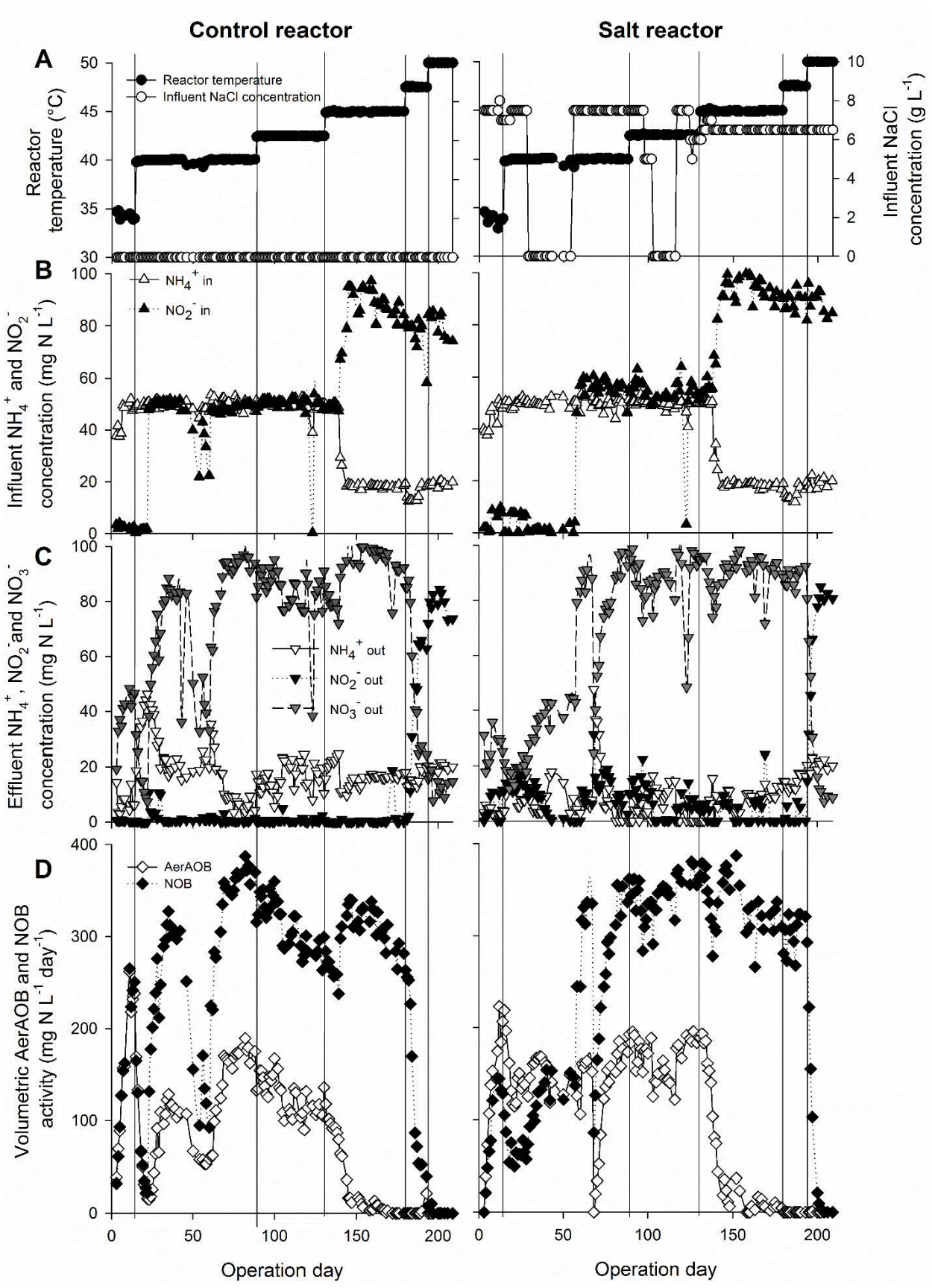

Figure 2.5 Operation and performance characteristics of control (left) and salt reactor (right). (**A**) Temperature and influent NaCl dosage (**B**) Influent nitrogen concentrations (**C**) Effluent nitrogen concentrations (**D**) Volumetric AerAOB and NOB activity.

During the 40°C period, both reactors suffered from a pH control failure resulting in a temporary drop in activity at day 43 and 68 for the control and salt reactor, respectively.

Raising the temperature from 40 to 42.5°C on day 88 (Figure 2.5 **A**) showed a similar salt protection trend. No significant changes in activity were observed in the salt reactor while in the control reactor, AerAOB and NOB activity decreased by respectively 36 and 21% (Figure 2.5 **D**).

After the further temperature increase to 45°C, the AerAOB activity disappeared completely in both reactors rendering 42.5°C the maximum cultivation temperature for AerAOB in this study with ammonium oxidation rates of 113 ± 13 and 184 ± 9 mg N L^{-1} d^{-1} in the control and salt reactor, respectively (Figure 2.5 **D**). For the NOB, transition from 42.5 to 45°C resulted in an activity drop. However, this was transient and entirely due to nitrite limitation deriving from the AerAOB's activity loss. Indeed, increased dosage of nitrite in the influent (Figure 2.5 **B**) restored the NOB activity at the level before temperature increase.

Raising the temperature from 45 to 47.5°C, led to a constant NOB activity for the salt reactor (302 ± 21 mg N L^{-1} d^{-1}), yet the control reactor practically lost all nitratation activity achieving a steady state of about 5 days at 49 ± 7 mg N L^{-1} d^{-1}. The salt reactor finally lost its nitratation activity after the final increase to 50°C. Overall, NOB activity could be maintained until 47.5°C with a six fold higher activity in the salt reactor than in the control reactor (Figure 2.5 **D**).

In order to investigate whether the salt was essential in the functioning of the salt reactor, a period without salt addition was included, both at 40 and 42.5°C (Figure 2.5 **A**). At 40°C, removing the salt had no effect on the AerAOB activity but did increase the NOB activity (Figure 2.5 **D**). At 42.5°C, removal of the salt did not affect the NOB, but affected the AerAOB activity with a decrease of 19% (Figure 2.5 **D**). Restarting salt addition, however, increased AerAOB activity to the original level. This decrease is however small compared with the AerAOB activity decrease of 90% after the first temperature transition, indicating that salt addition is more essential during the temperature transition than for a good performance after the temperature raise.

3.3. Fatty acid methyl ester (FAME) profiles

The FAME profiles allow studying biomass fatty acid composition, reflecting among others the membrane fluidity. No major differences could be observed in the FAME results between both reactors at day 148 (Table 2.1). Over time, some clear changes occurred in the reactors compared to the inoculum, indicating a clear temperature effect. The saturated fatty acid 16:0 almost doubled in abundance while some unsaturated fatty acids such as 14:1(n-5) and 16:1(n-7) decreased, suggesting hydrogenation of unsaturated fatty acids. This phenomenon is clearly illustrated in the evolution of the ratio saturated upon unsaturated fatty acids (sat/unsat) over time (Figure 2.6). The ratio evolved similarly in both reactors, starting at 1 and gradually increasing to 2, and thus doubling the level of saturation.

Table 2.1 Relative long-chain fatty acid composition of the inoculum biomass and biomass from control and salt reactor at day 148, as determined through FAME analysis. Compounds below 1% in all samples were omitted from the table.

	Inoculum	**Control reactor**	**Salt reactor**
14:0	5.8	1.8	2.0
14:1(n-5)	15.4	2.9	2.1
15:1(n-5)	0.1	4.5	2.4
16:0	33.1	56.6	57.8
16:1(n-7)	16.0	12.3	12.4
17:1(n-7)	2.2	1.5	3.3
18:0	4.8	3.4	3.5
18:1(n-9)	5.1	3.8	2.7
18:1(n-7)	2.9	0.9	1.5
19:1(n-9)	0.3	1.6	2.3
18:4(n-3)	0.5	3.2	2.7
20:0	1.3	0.6	0.8
21:0	1.1	1.9	1.5
20:3(n-3)	1.1	0.0	0.0
22:0	1.0	0.5	0.9
22:6(n-3)	1.2	0.2	0.4

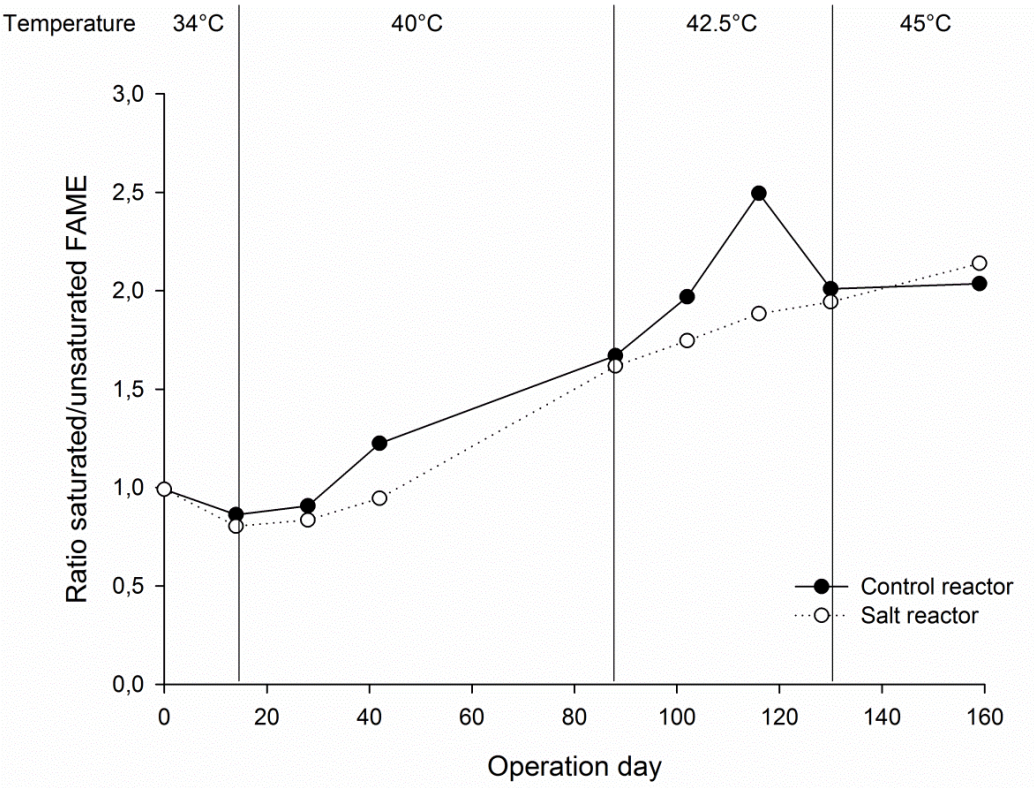

Figure 2.6 Evolution of the saturated/unsaturated fatty acid ratio of the microbial biomass in the control and the salt reactor, as determined through FAME analysis.

3.4. Molecular analyses of the microbial communities

FISH revealed that only *Nitrospira* NOB was present in the inoculum and both reactors at day 148, and that *Nitrobacter* could not be retrieved in any sample (data not shown).

For qPCR, AerAOB, AOA, AnAOB and the NOB *Nitrospira* were targeted (Figure 2.7). AOA were not detected in the inoculum, nor in one of the reactors. AerAOB, AnAOB and NOB evolved similarly in both reactors (Figure 2.7). AerAOB and *Nitrospira* copies ng^{-1} DNA increased with respectively one and two log units. The AnAOB copies ng^{-1} DNA however decreased with one log unit. When the last qPCR result (Figure 2.7, day 158) is compared with the corresponding specific activities (Figure 2.4 **D**), it has to be noted that although no AerAOB activity was detected for almost one week, still 3×10^5 and 1×10^6 AerAOB copies ng^{-1} DNA were measured.

The DGGE profiles for AerAOB confirmed that only some evolution had occurred at the species level, suggesting that salinity did not induce strong selection towards specialized bacteria

(Figure 2.8). The effect of temperature on the microbial community was thus similar for both reactors.

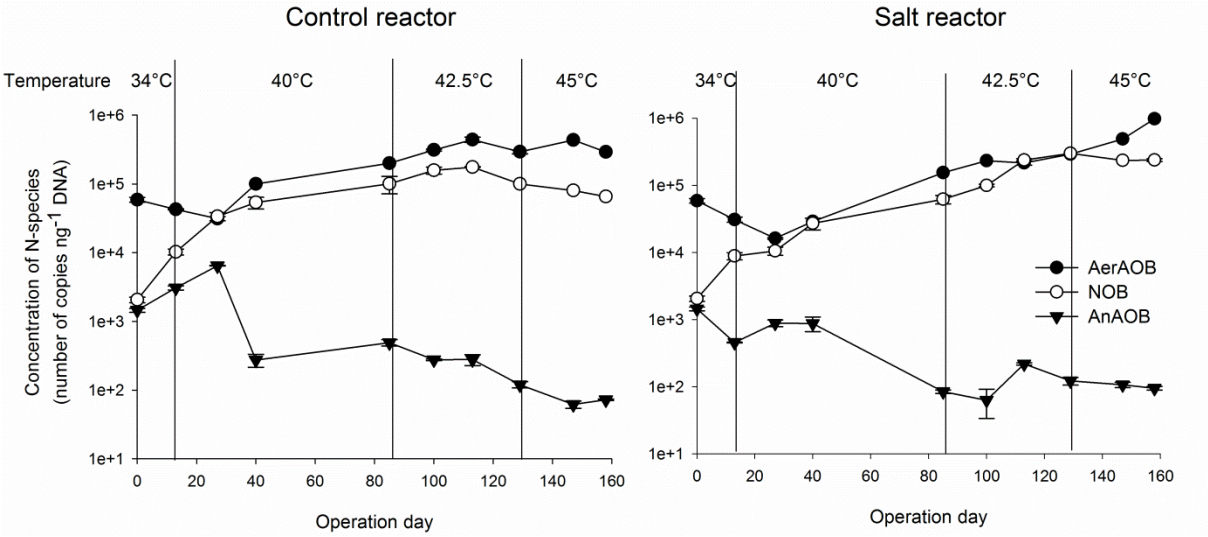

Figure 2.7 qPCR abundance of AerAOB, NOB-*Nitrospira*, AnAOB, expressed as copies ng^{-1} DNA, in the control and salt reactor. AOA were under the detection limit of 120 copies ng^{-1} DNA.

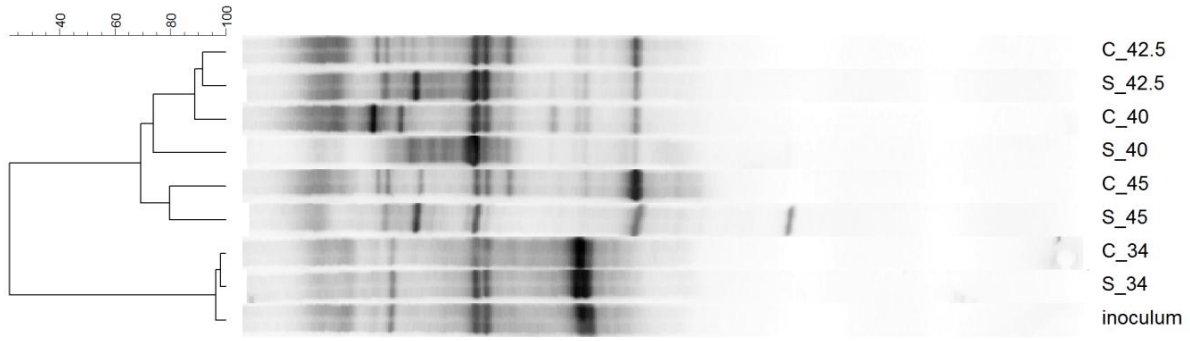

Figure 2.8 DGGE for β-proteobacterial AerAOB. Biomass samples were taken at the end of a temperature period (34, 40, 42.5 and 45°C). Similarities were calculated using the Pearson correlation coefficient. C: control reactor; S: salt reactor.

4. Discussion

This study showed that mesophilic nitrifying sludge can gradually adapt to 42.5°C and 47.5°C for nitritation and nitratation, respectively. It was shown that a more efficient temperature transition and eventually higher temperatures can be reached through addition of NaCl at 5 to 7.5 g L^{-1}.

4.1. Thermophilic nitrogen removal

Numerous thermophilic AOA have already been enriched (de la Torre et al., 2008; Hatzenpichler et al., 2008), which might derive from the pioneering role under the more extreme conditions of the early Earth (Vlaeminck et al., 2011). In this study however, no AOA could be detected in the inoculum and in both reactors after 148 days. AerAOB and NOB have also been detected and/or enriched in batch cultures from for example hot springs (Golovacheva, 1976; Lebedeva et al., 2005). The applications of those thermophilic nitrogen converting organisms for the treatment of nitrogenous wastewater in reactors have been scarce, however. The main nitrogen removal mechanisms in thermophilic activated sludge experiments have been ammonia stripping and nitrogen assimilation, accounting for 80 to 100% of the total nitrogen removal (Kurian et al., 2005; Abeynayaka and Visvanathan, 2011b). The amount of stripping in this study was minimal, since the nitrogen balance in the elevated temperature periods (>40°C) could be closed for >99% and >91% in the control and salt reactor, respectively. The main nitrogen loss (± 19%) occurred during start-up of the salt reactor at 34°C (Figure 2.5 **B** and **C**), while a negligible nitrogen loss was measured in the control reactor. This is the period where the AerAOB and NOB were inhibited by the salt addition, so that both ammonium and nitrite became available. Since the reactors were inoculated with AnAOB containing BNR biofilm, providing anoxic zones, and ammonia stripping and nitrogen assimilation were minimal, the nitrogen loss during the start-up of the salt reactor can thus at least be attributed to AnAOB activity.

Lopez-Vaquez et al. (2013) detected nitrifying activity in mesophilic biomass in short-term batch tests up to 50°C, yet the activity after a longer incubation period was not investigated. The results of the present study however showed a complete loss of AOB activity upon 48

hours incubation at 45°C, demonstrating the need for longer term reactor experiments to investigate thermophilic nitrification. Similarly, Sudarno et al. (2011) showed that mesophilic nitrifying biomass could not recover at 22.5°C after an incubation at 50°C. In the cited study, ammonium and nitrite oxidation rates in batch assays increased from 12.5°C to 40°C and were almost zero at 50°C. Until now, only Shore et al. (2012) focused on the temperature transition of a nitrifying moving bed biofilm reactor, only testing at 40 and 45°C. At 40°C, nitrification was still observed at maximum 144 mg N L^{-1} d^{-1}, which is comparable to our study. At 45°C, no nitrification could be achieved. Our study shows that nitrification in engineered systems is also possible above 40°C. Mesophilic nitrifying sludge could be adapted to 42.5°C with nitrification rates of 184 mg N L^{-1} d^{-1}. Moreover, by using salt addition, NOB could even be further adapted until 47.5°C with a nitrite oxidation rate of 302 mg N L^{-1} d^{-1}. Cooling of warm wastewaters for nitrification could thus partly be avoided using transient salt addition, hereby reducing both investment and operating costs.

4.2. Effect of salt on nitrification

In wastewater treatment, salt is considered as a common stress factor inhibiting the autotrophic nitrification (Moussa et al., 2006). Dincer and Kargi (1999) showed that both AerAOB and NOB activity linearly decrease with increasing salt concentrations, with NOB seeming to be more sensitive. Indeed, after the start-up period of the continuous reactors, both AerAOB and NOB activities were lower in the salt reactor compared with the control reactor (Figure 2.5 **D**). Moreover, the decrease in NOB activity in the salt reactor is more pronounced, reflecting the higher sensitivity of the NOB towards a salt shock. Nitrifiers can nevertheless adapt to salt concentrations up to 20 g NaCl L^{-1} (Bassin et al., 2012), explaining the absence of NOB inhibition after some weeks (Figure 2.5 **D**). In the batch activity tests with BNR sludge, NOB showed inhibition with addition of 5 g NaCl L^{-1} at 45°C, while AerAOB were stimulated at 40 and 45°C (Figure 2.4). Interestingly, the NOB inhibition was only observed at 45°C and not at 34 and 40°C suggesting that, next to the applied NaCl concentration, temperature has an important role in the salt shock response of NOB. Indeed, the CAS sludge, used at operating temperatures of about 15°C, already showed 91% NOB inhibition to a salt addition of 5 g NaCl L^{-1} at 34°C (Figure 2.2). The higher sensitivity of the CAS sludge towards

salt stress can potentially also be explained by the lower conductivity of the CAS influent compared with the BNR sludge treating high-strength nitrogenous wastewater.

4.3. Stress response versus adaptation and/or selection

The batch activity tests demonstrated the short-term salt and temperature stress responses. Addition of salt showed an increased direct AerAOB activity at elevated temperatures (Figure 2.4), suggesting an interconnection between osmotic and thermal stress response. Bacteria immediately react to stress conditions by expressing those genes whose products are required to deal with the damaging nature of the stress. Firstly, exposure to high osmolarity involves the accumulation of compatible solutes in order to counteract the osmotic stress (Welsh, 2000). The role of these small organic compounds is however not limited to osmoprotection and can protect temperature-sensitive enzymes from denaturation and stimulate their renaturation (Caldas et al., 1999). Secondly, osmotic stress can also induce the synthesis of heat-shock proteins (HSP) and hereby initiating the heat-shock response (Feder and Hofmann, 1999).

While AerAOB activity in the BNR sludge increased with salt addition at elevated temperatures, the activity of NOB in the same test decreased at 45°C (Figure 2.4). As NOB seem more sensitive to salt inhibition (Dincer and Kargi, 1999), it is likely that the inhibition effect predominated over the indirect protective effect through the production of HSP or compatible solutes. This was probably also the case for both the AerAOB and the NOB in the CAS sludge, where salt addition showed no positive effect. On the contrary, a positive NOB effect could be observed in the continuous experiment where NOB were adapted to the added salt concentration. The salt reactor reached NOB activity until 47.5°C while the control reactor lost all nitratation activity at that temperature.

In contrast to the direct stress response on the short term, the process of adaptation and/or selection is dominating on a longer term and is clearly reflected in the FAME analysis (Table 2.1 and Figure 2.6). Conventionally, FAME profiles are routinely used as a biomarker for identification or characterization of microbial communities (Cavigelli et al., 1995), as some FAME profiles can be linked to certain microbial groups, or genera. Recently however, FAME analyses have been used to evaluate the adaptation of certain microorganisms to a particular

environmental condition, such as acid environments (Quivey et al., 2000). Indeed, regulation of membrane fluidity through fatty acid alterations is a way for the bacterial membrane to restore the balance between bilayer and non-bilayer forming lipids when challenged with environmental disturbances such as temperature (Denich et al., 2003). In this study, higher temperatures resulted in a doubling of the ratio of saturated upon unsaturated fatty acids suggesting adaptation (Figure 2.6). However, since the DGGE profiles indicates some changes in the microbial community with increasing temperature (Figure 2.8), it is inconclusive whether adaptation and/or specialization towards specific species occurred and identification was thus not pursued.

Although a clear temperature effect was observed in the microbial community over time, there were no clear differences between the control and salt reactor, neither in DGGE, qPCR or FAME data. The communities in both reactors hence responded similarly to higher temperatures, despite the addition of salt. These observations consequently confirm that salt addition interacts mainly on a short term, in the direct stress response, and did not remarkably influence long term community adaptation/selection at higher temperatures. Further research should clarify this salt-stress response on the gene expression level and identify the development of possible specialized species.

5. Conclusions

Overall, this study demonstrates salt addition as a tool for a more efficient temperature transition for mesophilic sludge (34°C) and the achievement of higher nitrification temperatures as:

- Addition of 5 g NaCl L^{-1} increased the activity of AerAOB in batch activity tests at 40 and 45°C by 20-21%.
- In a long-term continuous reactor test, the AerAOB activity showed 65 and 37% higher immediate resistance in the salt reactor (7.5 g NaCl L^{-1}) for the temperature transitions from 34 to 40°C and 40 to 42.5°C.

- The control reactor lost NOB activity at 47.5°C, while the salt reactor only lost activity at 50°C.

6. Acknowledgments

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CHAPTER 3:

EMPOWERING A MESOPHILIC INOCULUM FOR THERMOPHILIC NITRIFICATION: GROWTH MODE AND TEMPERATURE PATTERN AS CRITICAL PROLIFERATION FACTORS FOR ARCHAEAL AMMONIUM OXIDIZERS

This chapter has been redrafted after:

Emilie N. P. Courtens, Tom Vandekerckhove, Delphine Prat, Ken Meerbergen, Bart Lievens, Nico Boon* and Siegfried E. Vlaeminck*. Empowering a mesophilic inoculum for thermophilic nitrification: growth mode and temperature pattern as critical proliferation factors for archaeal ammonium oxidizers. *equally contributed *Submitted*

1. Introduction

Thermophilic nitrification could be achieved by the adaptation of existing mesophilic nitrifying communities to elevated temperatures. Shore et al. (2012) achieved complete nitrification at 40°C applying a stepwise temperature step from 30 to 40°C (10°C d^{-1}) to a moving bed biofilm reactor (MBBR). In a parallel MBBR the temperature was increased from 30 to 45°C (15°C d^{-1}), however, losing all nitrifying activity. Slightly higher nitrification temperatures (42.5°C) were reached in Chapter 1, in which smaller temperature differences (2.5°C d^{-1}) were imposed from 40°C on. However, from those studies it is clear that no 'real' thermophilic (>45°C) nitrification can be achieved through a stepwise temperature increase pattern (> 2.5°C d^{-1})**,** although short-term activity measurements of mesophilic sludge (34°C) showed nitrifying potential up to 50°C (Lopez-Vazquez et al., 2014).

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

2. Materials and methods

2.1. Reactor set-up and operation

An overview of the two reactor experiments and associated reactor parameters is presented in Table 3.1. In the first experiment with two identical lab-scale sequential batch reactors (SBR), a linear temperature increase (0.25°C d^{-1}) with (SBR_1) and without (SBR_2) an oscillation

(amplitude 2°C, frequency 0.088 d^{-1}) were compared. In the second reactor experiment, a lower linear temperature increase (0.08°C d^{-1}) was investigated, in which a SBR (SBR_3) was compared with a MBBR. The majority of the process and feeding parameters were equal in all reactors to investigate the effect of temperature pattern and/or sludge flocculation state (flocs versus biofilm) (Table 3.1).

Table 3.1 Overview of reactor parameters and temperature increase patterns in the two different reactor experiments. n.a.: not applicable, SBR: sequencing batch reactor, MBBR: moving bed biofilm reactor, VER: volumetric exchange ratio, HRT: hydraulic retention time.

	Experiment 1		**Experiment 2**	
Reactor(type)	SBR_1	SBR_2	SBR_3	MBBR
Linear temperature increase	Oscillating	Steady	Steady	
Linear slope (°C d^{-1})	0.25		<40°C: 0.16 >40°C: 0.08	
Oscillating amplitude (°C)	2	n.a.	n.a.	
Oscillating frequency (d^{-1})	0.088	n.a.	n.a.	
Experimental periods				
Stabilization (d)	7		79	
Temperature increase (d)	50		150	
VER (%)	25		20	
Cycle duration (h)	6		4	
Flowrate (L)	2.1 ± 0.2		2.1 ± 0.3	
HRT (d)	1.0 ± 0.2		1.0 ± 0.2	

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

The carrier material of the MBBR consisted of polyvinyl alcohol (PVA)-gel beads (Kuraray, Japan) at a volumetric filling ratio of 15%. All reactors were inoculated with the same commercial nitrifying inoculum (Avecom NV) at an initial biomass concentration of 2.4 ± 0.1 g

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

2.2. Ex-situ nitrification activity tests

In parallel with the second reactor experiment, batch activity tests were performed with the SBR_3 sludge and MBBR carriers to monitor the evolution of the optimal temperature for both ammonium and nitrite oxidation. At the reactor temperature of 38°C, 40°C, 42°C, 44°C, 46°C and 48°C, the specific ammonium 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 in 24-well plates with a working volume of 1.5-2.5 mL. Plates were incubated in a MB100-4A Thermoshaker (Hangzhou Allsheng Instruments, China) 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_2HPO4), 1 g $NaHCO_3$ L^{-1}, 0.1 mL L^{-1} trace element solution (Kuai and Verstraete, 1998) and $(NH_4)_2SO_4$ or $NaNO_2$ (60 mg N L^{-1}). The sensitivity of ammonium and nitrite oxidation for free ammonia (FA) was also evaluated by determining the specific activity at different ammonium concentrations (25-200 mg N L^{-1}). All treatments were performed in sextuple, and liquid samples (2 µL) were taken over time for NH_4^+ and NO_2^- analysis. These high-throughput activity measurements were highly optimized for each sludge type prior to the actual tests. A validation experiment was performed in which the obtained rates were not significantly different with rates obtained in conventional 250 mL Erlenmeyer batch tests.

2.3. Sludge production and settleability

The sludge yield (Y) was calculated to evaluate the sludge production, taking into account the growth and death of the biomass. Calculations were performed using cumulative terms:

$$Y_{obs} = \frac{\sum VSS\ produced}{\sum COD\ removed} = \frac{\sum(F_w.VSS_w + F_{ef}.VSS_{ef} + \Delta VSS_{system})}{\sum(F_{in}.(COD_{in} - COD_{ef}))} = \frac{kg\ VSS}{kg\ COD}$$

where F_w, F_{in} and F_{ef} correspond to the waste, influent and effluent flows (L d^{-1}), respectively; COD_{in} and COD_{ef} correspond to soluble organic matter in the influent and effluent (g COD L^{-1}), respectively; VSS_W and VSS_{eff} correspond to the VSS in the waste and the effluent (g VSS L^{-1}), respectively; and ΔVSS_{system} corresponds to the biomass accumulation in the system (g VSS L^{-1}). Biomass settleability of the floccular sludge was measured through the determination of the sludge volume index (SVI*) with an in house, not standard, protocol. The sludge height variation was monitored for 5 min instead of 30 min in a 1 L Imhoff cone to prevent extensive cooling of the sludge.

2.4. Functional community analysis

Biomass samples of the inoculum and the reactors (SBR_3 and MBBR) were collected over time, and total DNA was extracted using the Fast-Prep24 instrument (MP-BIO, Germany) as described previously (Vilchez-Vargas et al., 2013). DNA quality and quantity were analysed electrophoretically on 1% (w/v) agarose gels and spectrophotometrically by determination of the absorbance at 260nm and the absorbance ratios at 260 nm and 280 nm, using NanoDrop ND-1000 (Thermo Scientific), respectively. The SYBR Green assay (Power SyBr Green, Applied Biosystems) was used to quantify the 16S rRNA of *Nitrospira* spp. and *Nitrobacter* spp. and the functional *amoA* gene for β-proteobacterial AOB and AOA (Table 3.2).

Table 3.2 Overview of the primers sets and conditions used for determination of the abundance of β-proteobacterial AOB, AOA, *Nitrospira* spp. and *Nitrobacter* spp. with qPCR.

Functional group	Target gene	Primers	Melting temp (°C)	Reference
AOB	*amoA* gene	amoA-1F	54.1	(Rotthauwe et al., 1997)
		amoA-2R	59.2	
AOA	*Crenarchaeal*	CrenamoA23f	51.2	(Tourna et al., 2008)
	amoA gene	CrenamoA616R	54.4	
***Nitrospira* spp.**	16S rRNA	NSR1113F	56.3	(Dionisi et al., 2002)
		NSR1264R	57.6	
***Nitrobacter* spp.**	16S rRNA	Nitro1198F	57.8	(Graham et al., 2007)
		Nitro1423R	60.4	

Nitrifiers were identified using pair-end high-throughput sequencing (MiSeq Illumina platform) of the regions V5-V6 of the 16S rRNA gene, using the primers 807F and 1050R previously described (Bohorquez et al., 2012). Amplification, sequencing and bioinformatic processing of sequences was done according to Camarinha-Silva et al. (2014) with some modifications. Raw sequences were assembled (Cole et al., 2014) and subsequently aligned using MOTHOR (gotoh algorithm with the SILVA reference database) prior to preclustering. Only phylotypes exhibiting a cumulative abundance of at least 0.1% (sum of percentage normalized data from all samples; "gut filter") and a sequence length >200bp were considered for follow-up analysis. Phylogenetic analyses were performed with MEGA5 (Tamura et al., 2011) using the neighbor-joining method with Jukes-Cantor correction and pairwise deletion of gaps/missing data. A total of 1000 bootstrap replications were performed to test for branch robustness.

2.5. Chemical analyses

Ammonium (Nessler method), total suspended solids (TSS) and volatile suspended solids (VSS) were measured according to standard methods (Greenberg et al., 1992). Nitrite, nitrate, DO and pH were measured as discussed in Chapter 2. The biomass concentration in the MBBR was determined through extraction of the biomass from the PVA carriers and subsequent protein measurement. The protein content was then translated to a VSS concentration using the average protein content of the MBBR sludge, 0.31 g protein g^{-1} $VSS_{MBBR\ sludge}$ as determined. The carriers were cut in fine pieces and incubated in 1 M NaOH for 30 min at 46°C with regular mixing for biomass extraction. To determine the protein concentration in the extract, the method developed by Lowry was used with bovine serum albumin (BSA) as the standard. In the batch activity tests, the liquid samples for ammonium and nitrite determination were always immediately analyzed spectrophotometrically with the Berthelot and Montgomery reaction, including a triplicate standard curve for each analysis run. Measurements were obtained using a Tecan infinite plate reader (Tecan, Switzerland), and biomass was quantified through protein concentrations.

3. Results

3.1. Oscillating versus non-oscillating linear temperature increase

The adaptive capacities of mesophilic nitrifying sludge were first evaluated for two different gradual temperature increase patterns. An oscillating temperature increase with an amplitude of 2°C and a frequency of 0.088 d^{-1} was compared with an non-oscillating increase with the same linear slope (0.25°C d^{-1}) as shown in Figure 3.1. Prior to any temperature increase, the reactors were started up identically at 37°C reaching ammonium removal rates of 180 ± 14 mg N L^{-1} d^{-1} or 136 ± 10 mg N g VSS^{-1} d^{-1} after one week of stabilization. Nitrite accumulation was negligible in both reactors and nitrate production accounted for 95% of the ammonium removal. Up to 40°C, no changes in volumetric rates were observed in both reactors. Further temperature increase above 40°C, however, negatively affected the nitrifying activity in both reactors, with a more pronounced effect in the oscillating reactor. At 42°C, only 15% of the initial volumetric nitrifying activity remained in the oscillating reactor (26 ± 5 mg N L^{-1} d^{-1}) while 50% remained in the non-oscillating one (90 ± 3 mg N L^{-1} d^{-1}) (Figure 3.1). Although the non-oscillating reactor seemed to better resist the temperature increase, the decreasing trend pursued in both reactors finally resulting in an entire loss of activity at 45°C in both reactors, suggesting that the imposed slope of 0.25°C d^{-1} was too high.

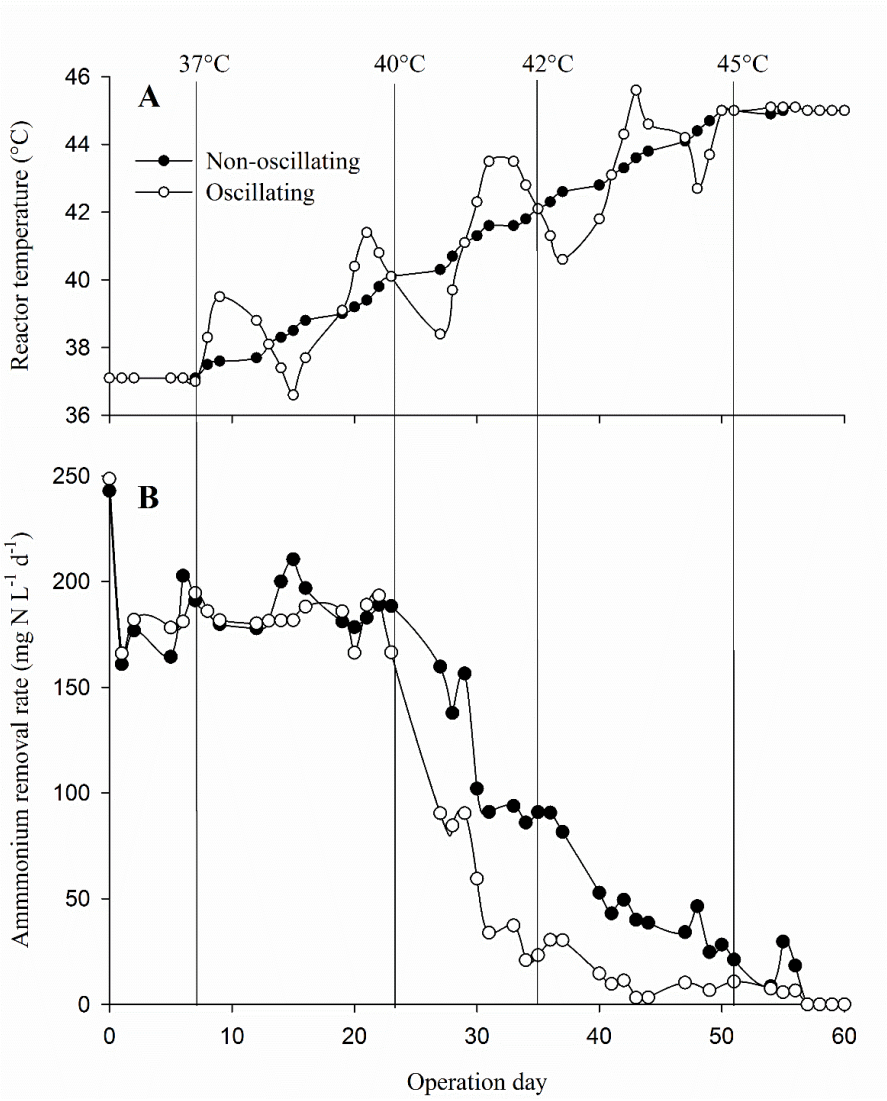

Figure 3.1 Temperature increase pattern **(A)** and ammonium removal rate **(B)** of two sequential batch reactors (SBR_1 and SBR_2) comparing an oscillating with a non-oscillating temperature increase (0.25°C d^{-1}).

3.2. Floccular versus biofilm based reactor system

3.2.1. Reactor performance

In the second reactor experiment, a linear temperature increase with a lower slope was investigated (0.08-0.16°C d^{-1}), in which a SBR (SBR_3) was compared with a MBBR (Table 3.1). A 79-day stabilization period at 38°C allowed sufficient acclimatization of the inoculum and, more specifically, biomass growth on the PVA gel carriers of the MBBR. The suspended biomass in the MBBR was gradually wasted during this period, while clear attached growth was observed (Figure 3.2).

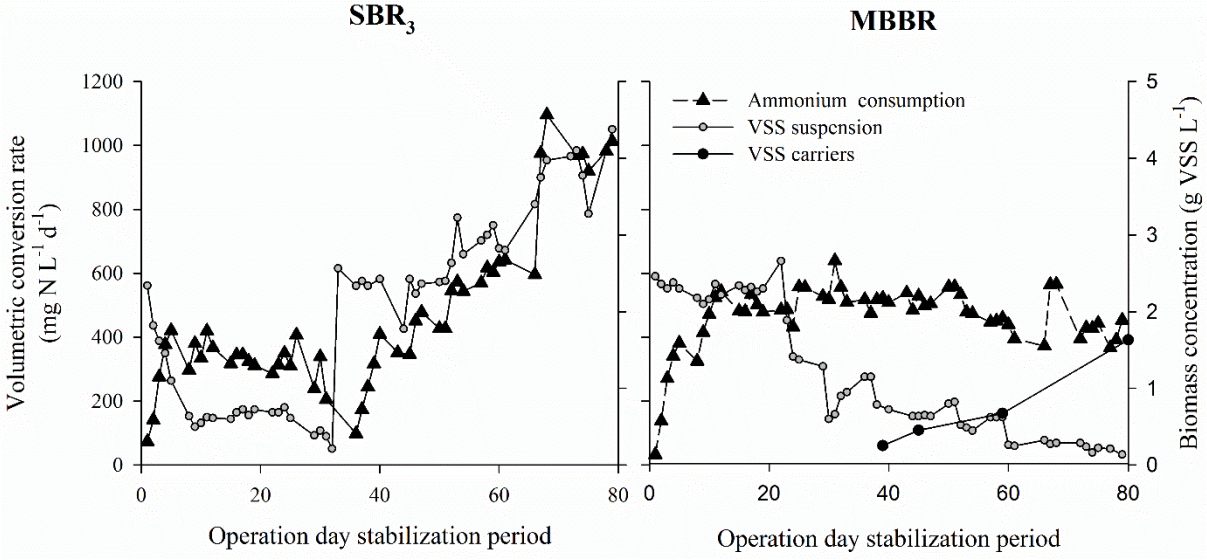

Figure 3.2 Ammonium consumption and biomass content (suspended and attached) of the sequential batch reactor (SBR_3) and the moving bed biofilm reactor (MBBR) during the start-up and stabilization period at 38°C.

At the start of the actual experiment the settling was excluded to waste all the suspended sludge. This resulted in about a doubling of the attached growth (Figure 3.3 **C**) and a further increase of the ammonium removal rate up to 580 ± 44 mg N L^{-1} d^{-1} (Figure 3.3 **B**). As the SBR_3 sludge content also sharply increased from about 3.3 g VSS L^{-1} (day 6) to 4.5 g VSS L^{-1} (day 16), eventually endangering settling behavior, about one third of the SBR_3 sludge was wasted before the start of the temperature increase. Concurrently, the loading was lowered by one third to prevent overloading, reaching comparable volumetric nitrification rates in both reactors (Figure 3.3 **B**). From day 20 on, temperature was gradually increased in both reactors at a slope of 0.16°C d^{-1} (Figure 3.3 **A**). In accordance with the first reactor experiment, from 38°C to 40°C, no negative effect on the nitrification performance was observed.

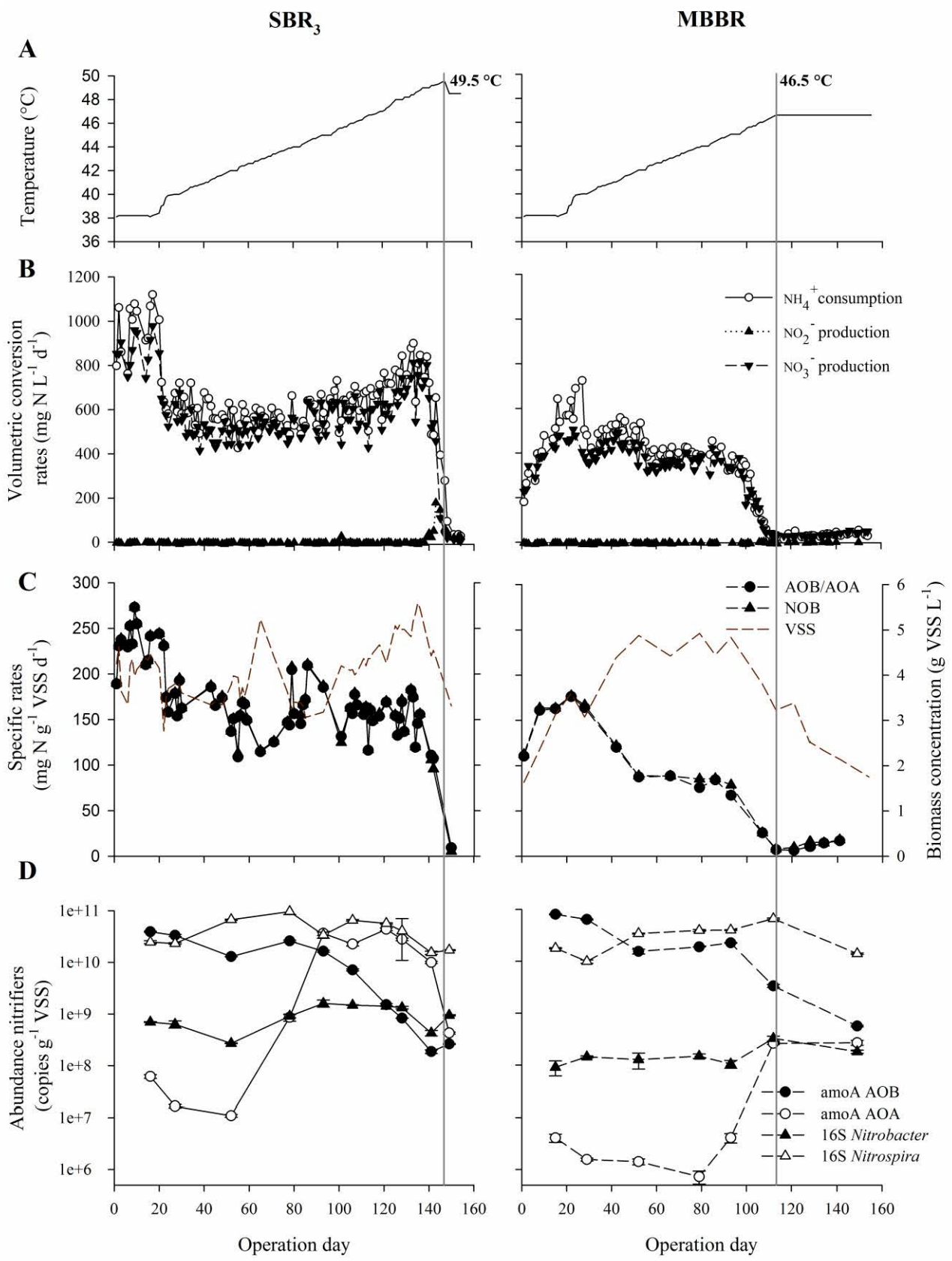

Figure 3.3 Operation and performance characteristics of SBR_3 (left) and MBBR (right). **(A)** Temperature increase patterns. **(B)** Volumetric ammonium removal and nitrite/nitrate production rates. **(C)** Specific rates and sludge content. **(D)** Abundance of nitrifiers as determined by qPCR.

On the contrary, volumetric rates slightly increased (Figure 3.3 **B**). As temperatures above 40°C initiated reactor failing in the first experiment (Figure 3.1), from 40°C on, the imposed slope was halved to 0.08°C d^{-1} (day 32). At that moment, a technical failure of the pH controller led to acidification (pH 5) in the MBBR resulting in a 18% decrease of nitrification performance. The MBBR recovered, even though temperature further increased. Stable ammonium removal rates of 563 ± 52 mg N L^{-1} d^{-1} (SBR_3) and 358 ± 40 mg N L^{-1} d^{-1} (MBBR) were observed in both reactors until 45°C. From 45.5°C on, however ammonium removal rates gradually decreased in the MBBR from 358 ± 40 to 23 ± 8 mg N L^{-1} d^{-1} at 46.5°C (Figure 3.3 **B**). As more than 90% of the activity was lost, temperature increase was ceased in the MBBR. In contrast, volumetric rates increased in the SBR_3 up to 776 ± 62 mg N L^{-1} d^{-1} at a temperature as high as 49°C, corresponding with a specific ammonium removal rate of 155 ± 24 mg N g VSS^{-1} d^{-1} (Figure 3.3 **C**). Nitrite accumulation was observed from temperatures higher than 49°C up to 200 mg N L^{-1}. As batch activity tests with SBR_3 sludge showed that nitrite concentrations up to 500 mg N L^{-1} did not have a significant effect on the ammonium oxidizing activity ($p<0.05$), the loading rate was not adjusted. At 49.5°C, a malfunctioning of the pH controller pump now also acidified the SBR_3 (pH 3-4), resulting in a decrease of ammonium removal activity to 30 mg N L^{-1} d^{-1}. The temperature in the SBR_3 was decreased to 48.5°C to allow for recovery of the SBR_3. Ammonium oxidation rates increased again reaching >300 mg N L^{-1} d^{-1} after 50 days, while nitrite oxidation could not be recovered. Overall, the highest temperature where complete and stable nitrification was observed was 45.5°C and 49°C in the MBBR and SBR_3, respectively.

3.2.2. Community adaptation

The adaptive capacity of the SBR_3 and MBBR sludge towards the imposed temperature increase was closely monitored with parallel batch activity tests. Every 2°C along the temperature increase, specific ammonium and nitrite oxidizing activities of both sludge types were measured at the respective reactor temperature and at plus and minus 2°C. The results of these batch activity tests are presented in Figure 3.4. Similar observations were made for both reactors up to 42°C. Although the differences were small, it appeared that between 38 and 42°C, the temperature with the highest ammonium oxidizing activity was 40°C. Although the ammonium oxidation optimum in the SBR_3 gradually shifted from 40°C towards 46-48°C

(Figure 3.4 **A**), the MBBR optimum did not get higher than 42°C (Figure 3.4 **B**). Moreover, at a reactor temperature of 44°C, no ammonium oxidation activity could be measured in the MBBR sludge at 46°C, clearly predicting the MBBR crash at 46°C (Figure 3.4 **B**). Despite the loss of ammonium oxidation at 46°C, the batch activity test indicate that the MBBR's nitrite oxidizers were still active up to 48°C (Figure 3.4 **D**). The nitrite oxidizers in the SBR_3 seemed to be adapted once the reactor reached 48°C, but a significant inhibition was observed at 50°C (Figure 3.4 **C**). Indeed, temperatures higher than 49°C led to nitrite accumulation in the SBR_3 (Figure 3.3 **B**).

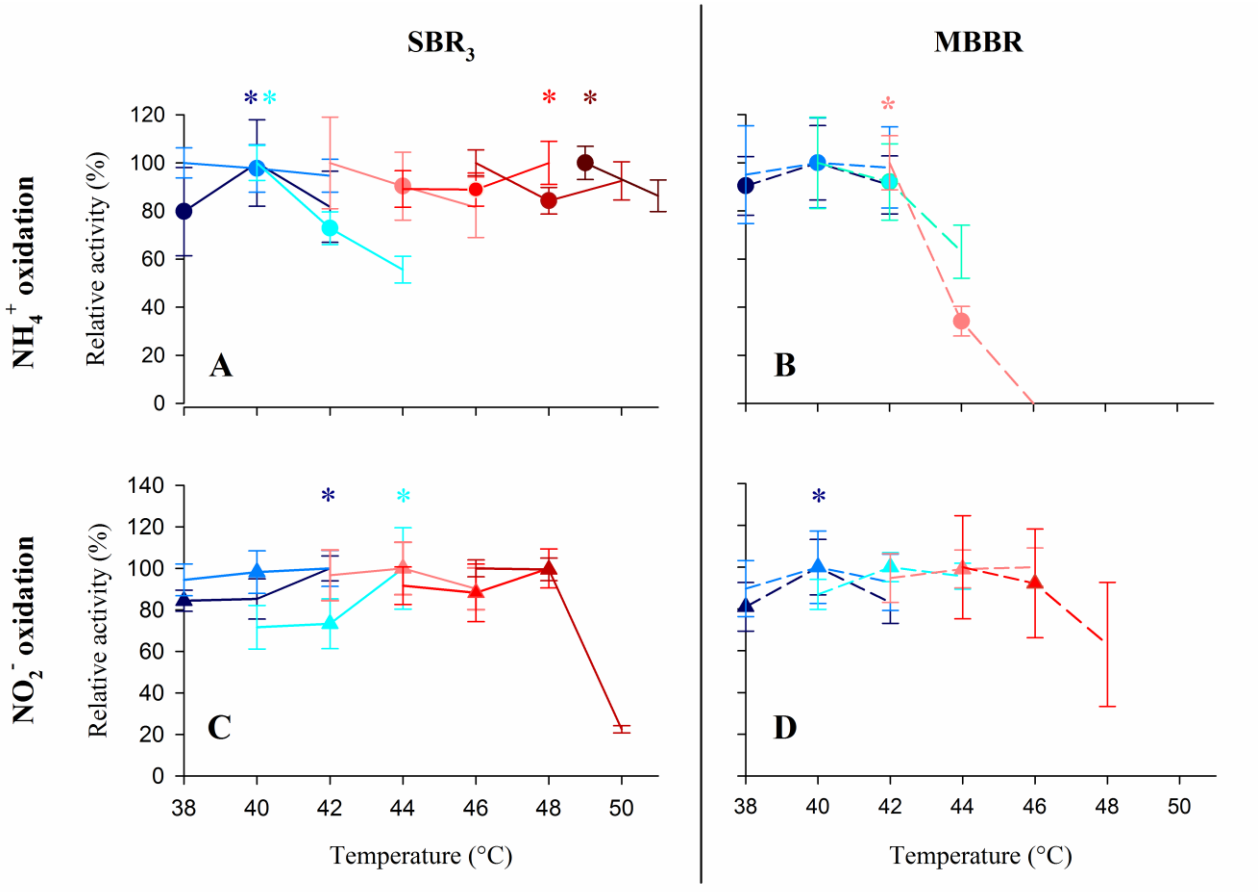

Figure 3.4 Relative temperature activity curves for ammonium **(A,B)** and nitrite **(C,D)** oxidation of the SBR_3 **(A,C)** and MBBR **(B,D)** sludge. Each curve represent a batch test performed at a certain reactor temperature, of which the temperature is indicated with a symbol. Per batch test, the temperature where the highest activity was measured was indicated as the 'optimum temperature' and assigned as 100%. All experiments were performed in sextuple, and statistically significant optima (student's t-test, $p<0.05$) are indicated with an asterisk.

3.2.3. Free ammonia sensitivity

Sensitivity of the nitrifying sludge for elevated free ammonia (FA) was evaluated along the temperature increase. No significant inhibition of ammonium oxidation could be measured in both reactors by FA up to 6 mg N L^{-1}, in contrast, ammonium oxidation was stimulated by elevated FA (Figure 3.5 **A** and **B**). The SBR_3's nitrite oxidizers were initially only slightly or not inhibited by FA up to 6 mg N L^{-1} at the lower operating temperatures (38-42°C), but were strongly inhibited at 46-48°C with a 50% (IC_{50}) and 100% (IC_{100}) inhibitory concentration of 0.67 ± 0.01 and 1.42 ± 0.08 mg NH_3-N L^{-1}, respectively (Figure 3.5 **C**). The opposite trend was observed in the MBBR. Nitrite oxidation was clearly inhibited at 38-40°C, with an IC_{50} of 0.48 ± 0.07 mg NH_3-N L^{-1}, while the inhibition by FA disappeared at elevated temperatures (44-46°C) (Figure 3.5 **D**), possibly due to an increased diffusion limitation as the biomass concentration in the MBBR and thus thickness of the biomass strongly increased over time/temperature.

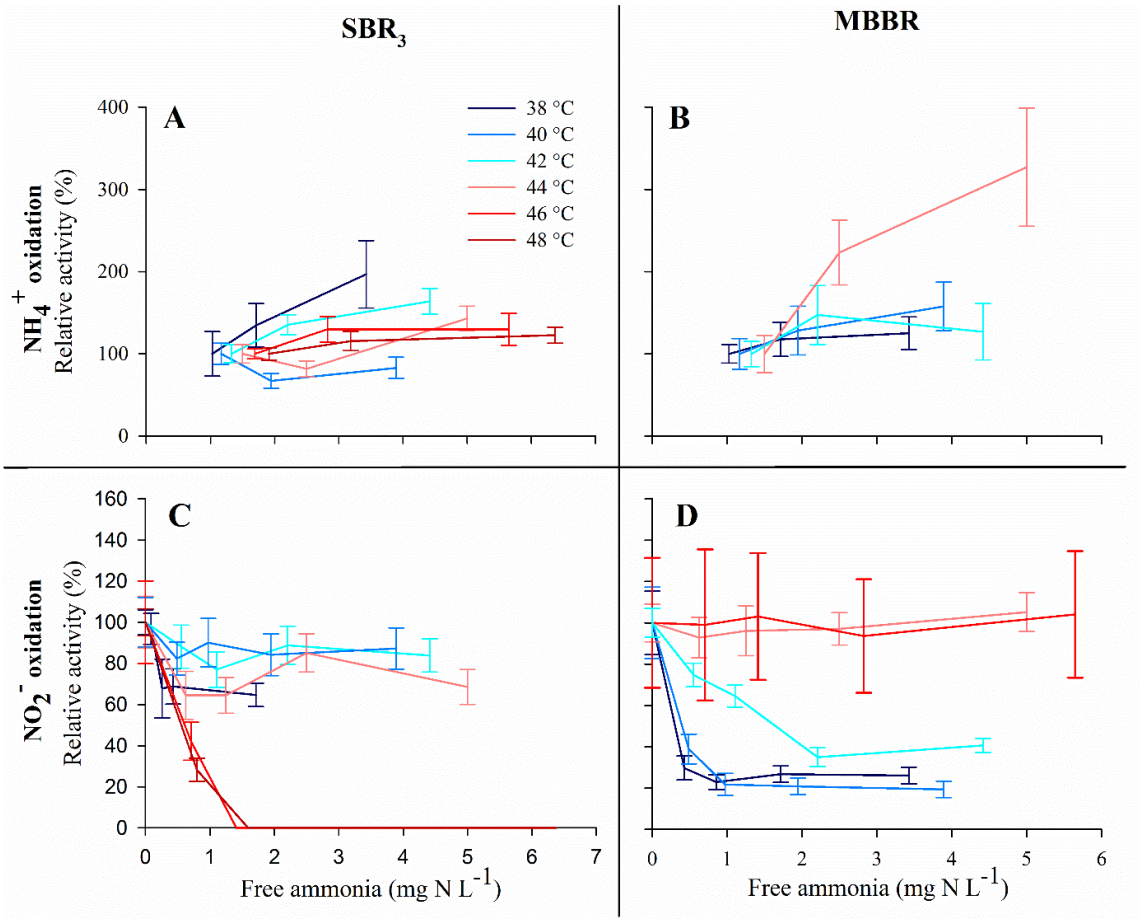

Figure 3.5 Effect of free ammonia (FA) on the ammonium **(A,B)** and nitrite **(C,D)** oxidizing activity of the SBR_3 **(A,C)** and MBBR **(B,D)** sludge, along the different reached reactor temperatures during the experiment. (n=6)

3.2.4. Sludge production and settleability

The increasing temperature initially induced a sharp decrease in sludge production in the SBR_3. The observed sludge yield halved from 0.074 to 0.035 g VSS g^{-1} N from 38°C to 42°C, whereas it increased again from 44°C to a yield of 0.067 ± 0.005 g VSS g^{-1} N up to 48°C (Figure 3.6). In contrast, sludge production in the MBBR amounted 0.11 g VSS g^{-1} N until 42°C, whereupon it decreased and finally became negative at 46°C as a result of biomass die off (Figure 2.3 **C**). Settling behavior of the SBR_3 sludge was stable up to 44°C, with a SVI_5* of 241 ± 38 mL g^{-1}, and improved at 46-48°C with a SVI_5* of 154 ± 2 mL g^{-1} (Figure 3.6). The sludge residence time (SRT) in the SBR_3 was 92 ± 7 days, while the SRT of the MBBR was considered infinite as nearly no suspended sludge could be measured in the effluent.

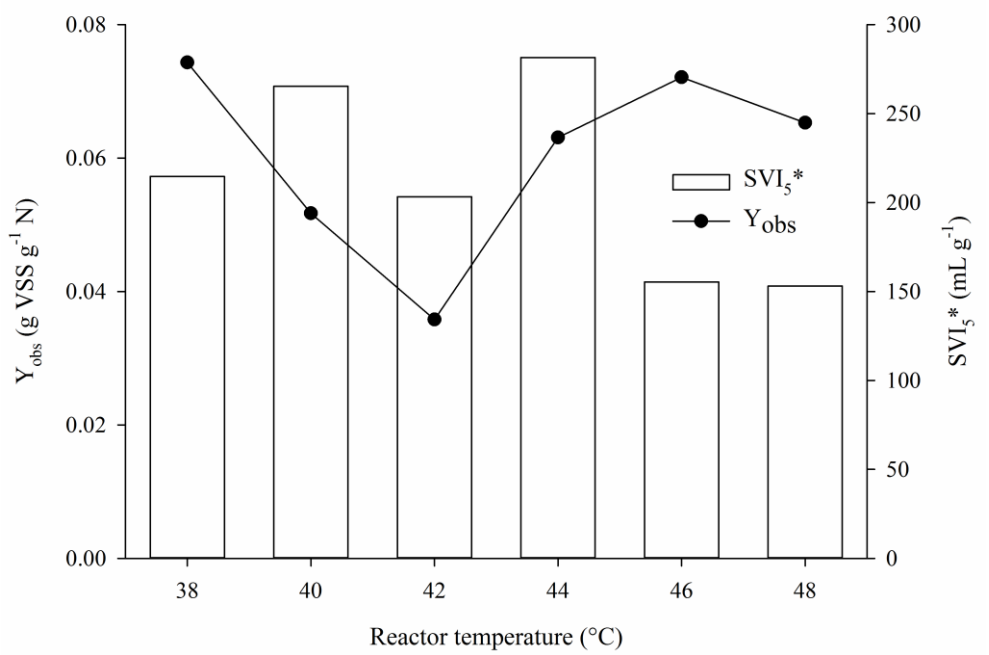

Figure 3.6 Observed sludge yield (Y_{obs}) and sludge volume index (SVI_5*) of the SBR_3 sludge at the different reactor temperatures.

3.2.5. Funtional community analysis

The abundance of selected key groups of nitrifying microorganisms was followed along the temperature increase by means of qPCR. The reactors were inoculated with the same inoculum, comprising a relatively well-balanced amount of AOB versus AOA ($2.1x10^9$ versus $3.5x10^8$ *amoA* gene copies g^{-1} VSS) and *Nitrospira* spp. versus *Nitrobacter* spp. ($6.7x10^9$ versus $4.8x10^{10}$ 16S gene copies g^{-1} VSS). The AOB dominance was preserved in both reactors after

the stabilization period reaching an AOB/AOA ratio of 279 and 7091 in the SBR_3 and MBBR, respectively. The bacterial *amoA* gene abundance kept stable up to 45°C around 10^{10} copies g^{-1} VSS in both reactors, and then gradually decreased (Figure 3.3 **D**). Clear differences in AOA abundances were, however, observed between the different reactors. The MBBR biomass retained significantly less AOA compared with the SBR_3 sludge after the stabilization period (Figure 3.3 **D**, day 16). Moreover, a steep increase in AOA abundance of 3 log units was observed in the SBR_3 at about 44°C, rising from $1.0x10^7$ to $2.9x10^{10}$ copies g^{-1} VSS, while the AOA abundance in the MBBR only slightly increased with 2 log units to $8.6x10^8$ copies g^{-1} VSS at 46°C. This shows a clear shift in dominant ammonium oxidizers in the SBR_3 from AOB to AOA from 45°C on, while this shift never completely occurred in the MBBR. For nitrite oxidation, *Nitrospira* spp. were dominant over *Nitrobacter* spp. in both reactors over the whole experiment (Figure 3.3 **D**). The observed trends in key nitrifier abundances were confirmed by High-throughput Illumina sequencing. The AOB retrieved in the MBBR appeared to be *Nitrosomonas europeae* and the AOA in the SBR_3 (Phy8) belonged to the *Nitrososphaera* genus (Figure 3.8). Interestingly, this AOA (Phy8) only showed a 95% similarity with the original AOA present in the inoculum (Phy56). The closest related known NOB of the dominant *Nitrospira* in both reactors is *Nitrospira japonica J1* with 91% similarity (Figure 3.7).

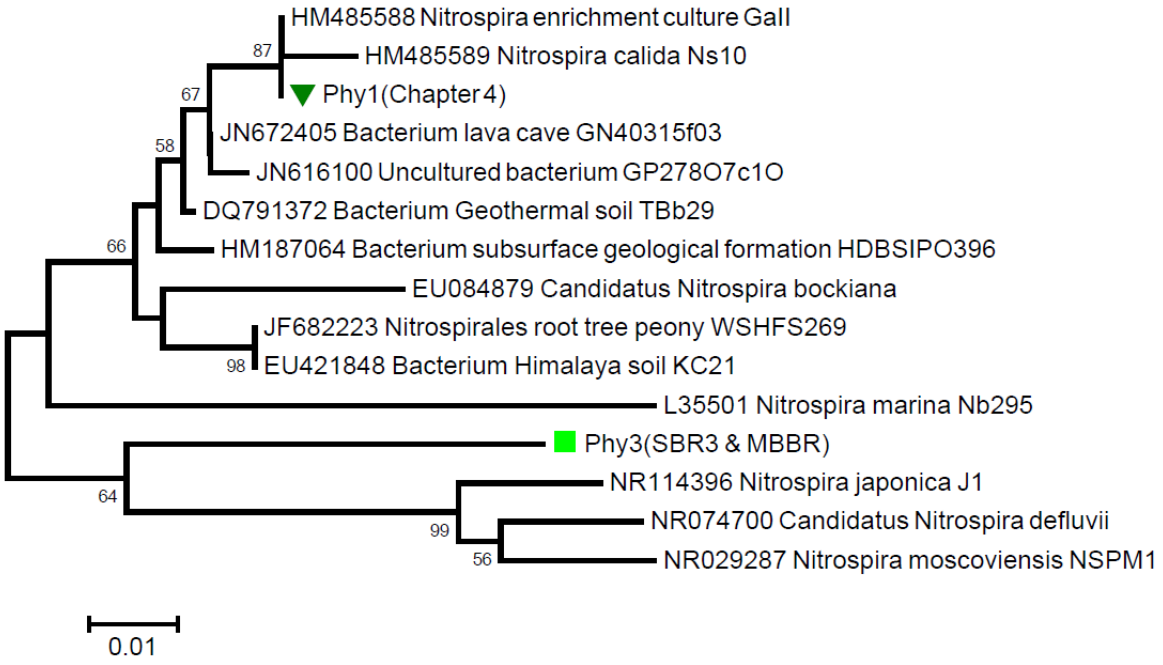

Figure 3.7 Phylogenetic relationships between the most dominant *Nitrospira* 16S rRNA gene sequence in the SBR_3 and MBBR (Phy3) and all described *Nitrospira* cultures or isolates, as well as relevant environmental clone sequences.

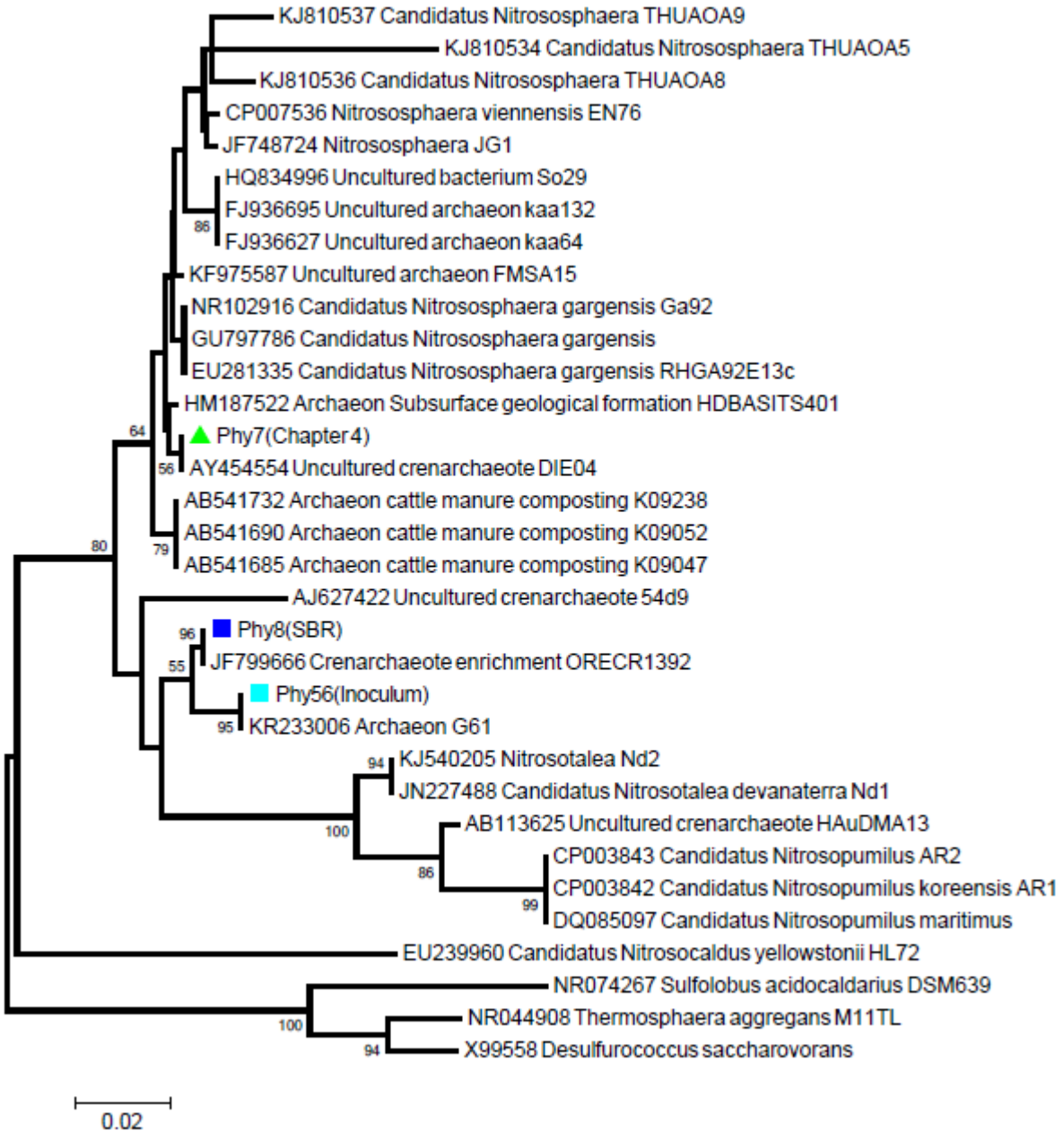

Figure 3.8 Phylogenetic relationships between the archaeal 16S rRNA gene sequences in the SBR_3 (Phy8) and inoculum (Phy56) and all described AOA cultures or isolates, as well as relevant environmental clone sequences.

4. Discussion

4.1. Overall performance

The adaptive capacities of mesophilic nitrifying sludge for different linear temperature increase patterns and different sludge growth modes were explored in this study of which the main results are summarized in Table 3.2. A non-oscillating temperature pattern (SBR_2) appeared to be more effective than an oscillating pattern (SBR_1) for the tested slope of 0.25°C d^{-1} as both the volumetric as specific rates were 2-3 times higher. In general, the 'low-slope' reactors (SBR_3 and MBBR) reached 3 to 30 times higher volumetric rates than the 'high-slope' reactors (SBR_1 and SBR_2), at significantly higher temperatures. Finally, the biofilm based system (MBBR) showed 2.5 lower rates than the parallel the floccular growth system (SBR_3). Overall, within the range of the tested parameters/combinations in this study, the highest temperature with moreover the highest volumetric and specific rates were achieved through the transition of mesophilic nitrifying sludge by a slow, non-oscillating linear temperature increase (SBR_3). The effect of oscillation at a lower slope and oscillation in a MBBR were not investigated in this chapter and could eventually give better results.

Table 3.2 Overview of the volumetric and biomass specific rates achieved at the highest temperature where complete and stable nitrification was observed in the two different reactor experiments. Averages calculated over at least 3 hydraulic retention times (± 3 operation days). n.a.: not applicable, SBR: sequencing batch reactor, MBBR: moving bed biofilm reactor.

	Experiment 1		**Experiment 2**	
Reactor(type)	SBR_1	SBR_2	SBR_3	MBBR
Linear temperature increase	Oscillating	Steady	Steady	
Linear slope (°C d^{-1})	0.25		<40°C: 0.16 >40°C: 0.08	
Oscillating amplitude (°C)	2	n.a.	n.a.	
Highest temperature (°C)	42	42	49	45,5
Ammonium conversion rates*				
Volumetric (mg N L^{-1} d^{-1})	26 ± 5	90 ± 3	794 ± 57	309 ± 30
Specific (mg N g^{-1} VSS d^{-1})	72**	139 ± 18	151 ± 7	67**

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

** Only one biomass measurement available for the specific period

The successful transition of the SBR_3 towards thermophilic temperatures was, remarkably, accompanied with a shift in sludge production trend (Figure 3.6). The decreasing trend sharply reversed at 44°C finally resulting in comparable sludge yields at 38°C and 48°C (0.0687 ± 0.005 g VSS g^{-1} N). Overall, sludge yields were lower than reported values for combined AOB and NOB sludge yield of 0.19-0.21 g VSS g^{-1} N at mesophilic temperatures (Barnes and Bliss, 1983; Henze et al., 2008). In parallel, a clear shift in optimum temperature was observed with the ex-situ activity measurements. These small, fast (few hours), high-throughput activity tests, based on simple spectrophotometrical measurements, could predict the loss of ammonium and nitrite oxidation in the MBBR and SBR_3 (Figure 3.4), respectively. One could thus lower the slope of the imposed temperature slope when the optimum do not seem to evolve with the current temperature and so, steer the temperature increase strategy to achieve thermophilic nitrification.

4.2. Temperature increase pattern

Pre-exposure to a certain stress can result in an increased resilience to a secondary exposure (Philippot et al., 2008; Ryall et al., 2012). In the framework of this study, a pre-exposure to an elevated temperature can e.g. induce the production of heat-shock proteins (HSP) that could possibly protect the biomass during a secondary temperature increase and so, improve the adaptive capabilities. This study however showed that the oscillating temperature pattern did not improve the adaptive capabilities of mesophilic nitrifying sludge towards higher temperatures (Figure 3.1). The tested amplitude of 2°C was possibly too high to observe beneficial effects and thus, smaller oscillating could eventually give better results.

The linear character of the imposed temperature pattern in this study was clearly more successful than stepwise increase patterns reaching only maximum nitrification temperatures of 40 and 42.5°C (Shore et al., 2012)(Chapter 2). This is in line with observations at a lower temperature range (10-20°C) in which the negative effect of a sudden temperature decrease on nitrification was much stronger than a gradual temperature decrease (Hwang and Oleszkiewicz, 2007). A possible benchmark value to compare the temperature slope the nitrifiers in this study experienced is the slope of seasonal temperature fluctuations in natural environments such as tropical soils (0.2°C d^{-1}) (Sierra, 2002) or wastewater treatment facilities

in moderate climates (0.07°C d^{-1}) (Gilbert et al., 2015). Nitrifiers in these environments are namely known to cope with these temperature slopes. The latest slope is perfectly comparable with the slope of 0.08 °C d^{-1} in this study during which the highest nitrification temperature of 49°C was reached (Figure 3.3). Thus, a relatively low slope in temperature increase seems essential to allow transition of nitrifiers to elevated temperatures.

4.3. Sludge growth mode

A floccular growth system (SBR_3) was compared with a biofilm based system (MBBR), as biomass retention of the slow growing autotrophs is essential during the transition process, and could eventually be favored through a biofilm based reactor system. Experiences with thermophilic carbon treatment showed that thermophilic aerobic processes suffer from poor sludge settling properties (Suvilampi and Rintala, 2003), thus, operation of settling based system such as a SBR may be threatened. Remarkably, settling behavior of the SBR_3 sludge in this study did not deteriorate (Figure 3.6), resulting in only minor differences in sludge retention time between both reactors.

The MBBR was initially hypothesized to better cope with the temperature transition, as biofilms show increased resistance to many types of environmental challenges (Gilbert et al., 2002). Recently, Gilbert et al. (2015) showed that nitrate production in a partial nitritation/anammox MBBR was more resilient against a gradual temperature reduction (20°C to 10°C, 0.07 °C d^{-1}), compared with a SBR, though ammonium oxidation declined similarly in both reactor types. Several observations, such as the increased resistance of biofilms towards antibiotics, are mainly explained by the restricted diffusion(Mah and O'Toole, 2001). Recently, other factors, such as slow growth rate, high culture density and heterogeneity, were shown to influence the general stress response in biofilms (Mah and O'Toole, 2011; Ryall et al., 2012), and could eventually favor the adaptive capacities of nitrifiers towards elevated temperatures. This study is however in contrast with this hypothesis, as the ammonium oxidation MBBR failed around 46°C, while it could still be maintained until 49°C in the SBR_3 (Figure 3.3 **B**). The successful transition in the SBR_3 seemed to be related to the observed shift of AOB to AOA dominance that was not achieved in the MBBR (Figure 3.3 **D**). This is in accordance with literature, where most described thermophilic ammonium oxidizers are archaeal (de la Torre

et al., 2008; Hatzenpichler et al., 2008; Lebedeva et al., 2013). The slower growth rate of the nitrifiers in the MBBR, initially supposed to favor the general stress response on a short term (Ryall et al., 2012), probably delayed the essential selection process on a long term. Indeed, an increase in AOA abundance in the biofilm was also observed, though one month later than in the SBR_3 (Figure 3.3 **D**). Furthermore, although both reactors were inoculated with the same AOA/AOB ratio, the relative decrease in AOA during the stabilization period was more pronounced in the biofilm than the flocs resulting in an initially lower AOA abundance in the biofilm. The late start of the increasing trend of AOA in the MBBR suggests that the essential shift could eventually also have been achieved with an even lower slope of temperature increase. Besides the actual abundance of AOA in the biofilm, different, less thermotolerant, AOA species could have been enriched in the biofilm, compared with the floccular sludge. It has to be noted that AOA are not obligate autotrophic ammonia oxidizers (Mußmann et al., 2011). The measured archaeal *amoA* gene abundance in this study thus only gives an indirect proof of the importance of AOA activity in the transition of nitrification to elevated temperatures. The link between AOA abundance and the observed autotrophic ammonia oxidation activity can be confirmed with isotope studies as was performed in Chapter 4.

Observations regarding nitrite oxidation were in line with literature, stating that *Nitrospira* is the most dominant nitrite oxidizer up to 60°C (Lebedeva et al., 2011; Marks et al., 2012; Edwards et al., 2013). In this study, no shifts were observed, and *Nitrospira* was dominant in both reactors over the entire experiment (Figure 3.3 D). However, remarkable differences in free ammonia sensitivity were observed between reactors and over time (Figure 3.5), suggesting that a possible selection on species level occurred during the transition. Overall, nitrite oxidizers were much more sensitive compared with the ammonium oxidizers, finally resulting in the development of a partial nitritation reactor at 48.5°C, opening opportunities for short-cut nitrogen removal processes.

4.4. Practical implications

The results of this study suggest that existing mesophilic nitrifying wastewater plants can be upgraded to thermophilic systems through a slow, non-oscillating linear temperature increase. Excluding the stabilization period, which is non-relevant for existing plants, this could

be achieved in about 140 days. Close monitoring of the transition with the high-throughput activity tests as described in this study, could moreover allow an even faster transition period. It should be emphasized that, beside the temperature increase pattern, the presence of AOA in the mesophilic sludge appeared to be essential for a successful transition. The fact that AOA appear to be distributed in wastewater treatment plants worldwide, even in equal or higher abundance than AOB (Limpiyakorn et al., 2013), opens thus opportunities for thermophilic nitrogen removal.

5. Conclusions

- The oscillating temperature pattern with an amplitude of 2°C and a slope of 0.25°C d^{-1} achieved a low nitrification rate of 26 ± 5 mg N L^{-1} d^{-1} at 42°C and lost all activity at 45°C.
- The moving bed biofilm reactor subjected to a slope of 0.08-0.16°C d^{-1} was able to oxidize ammonium up to 46°C, though, at a low volumetric rate of 32 ± 7 mg N L^{-1} d^{-1}.
- Nitrification rates of up to 800 mg N L^{-1} d^{-1} and 170 mg N g VSS^{-1} d^{-1} were achieved at 49°C through gradual adaptation (0.08 °C d^{-1}) of mesophilic nitrifying sludge in a SBR_3.
- The successful transition from mesophilic to thermophilic ammonium oxidation in the SBR_3 was linked to a dominance shift of archaeal above bacterial *amoA*.
- Ex-situ batch activity measurements can serve as a good tool to monitor the process response to transition, predicting reactor failures, thus enabling steering of the temperature increase pattern.

6. Acknowledgments

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CHAPTER 4:

A ROBUST NITRIFYING COMMUNITY IN A BIOREACTOR AT 50°C OPENS UP THE PATH FOR THERMOPHILIC NITROGEN REMOVAL

This chapter has been redrafted after:

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1. Introduction

Many archaeal *amoA* genes were detected in thermophilic environments (Chapter 1). The difficulty of cultivating the slow growing AOA at elevated temperature under laboratory conditions however resulted in only three enrichments so far (*"Candidatus* Nitrosocaldus yellowstonii"*, "Candidatus* Nitrososphaera gargensis" *and "Candidatus* Nitrosotenius uzonensis"). Moreover, until now, thermophilic AOA and NOB were always separately enriched/studied in batch cultures. No successful partnership of the two groups of thermophilic microorganisms has been described in either batch culture or in bioreactors with the goal of complete nitrification. Beside for *Nitrolancetus hollandicus* (Sorokin et al., 2012), all reported substrate/product inhibitions effect for the described thermophilic nitrogen–converting organisms are relatively high (Hatzenpichler et al., 2008; Lebedeva et al., 2011), making them unsuitable for robust biotechnological applications. Nevertheless, there is a growing interest in the development of thermophilic nitrogen removal processes for the treatment of warm wastewaters implying advantages such as lower sludge production, better settling properties and higher hygienization (Suvilampi and Rintala, 2003).

This study describes the enrichment of autotrophic thermophilic nitrifiers from compost and the successful operation of a thermophilic nitrifying bioreactor with high biotechnological potential. We demonstrate that autotrophic AOA and NOB serve as key players in the microbial community of the thermophilic nitrifying bioreactor. We also provide a phylogenetic, physiological and morphological characterization of this unique nitrifying community.

2. Materials and methods

2.1. Inoculum and batch experiments

Different aerobic compost facilities were sampled during the thermophilic stage (50-70°C): digested organic waste (a), green waste (b), cow manure (c) and a mix of rabbit manure/green waste (d). A 'compost extract' was prepared by shaking 20 g of compost in 200 mL water with glass beads (12 h). The extract was used as inoculum (25 vol%) for enrichment incubations

(50°C) in a buffered medium (pH 7) with final concentrations of 0.929 g KH_2PO_4 L^{-1}, 1.622 g K_2HPO_4 L^{-1} and 0.5 g $NaHCO_3$ L^{-1} with $(NH_4)_2SO_4$ or $NaNO_2$ as the only substrate (20 mg N L^{-1}). All incubations were provided with two different packing materials, Kaldness K1 as well as polyurethane foam, to allow both suspended as biofilm growth.

2.2. Reactor set-up and operation

The compost enrichments showing both NH_4^+ and NO_2^- oxidation (b, d) were transferred to a fixed bed bioreactor. A filling ratio of the carrier material, consisting the Kaldness K1 carriers and polyurethane foam, of about 50% was used to allow for both biofilm as suspended growth. The reactor vessel (2 L, diameter 12 cm) was jacketed, allowing temperature control at 50°C with a circulating thermostatic water bath. The reactor was operated in a sequencing batch feeding/withdrawal mode. The 3-h cycle consisted of a 150-min aerobic reaction period, a 10-min feeding period at the beginning of the cycle, a 15-min settling period, a 5-min decanting period and a 10-min idle period. The bioreactor was fed with a synthetic medium consisting of $(NH_4)_2SO_4$ (10-140 mg N L^{-1}), $NaNO_2$ (0-50 mg N L^{-1}), 9 g $NaHCO_3$ g^{-1} N, KH_2PO_4 (10 mg P L^{-1}), NaCl (1.2 g L^{-1}) and 0.1 mL L^{-1} trace element solution (Kuai and Verstraete, 1998) dissolved in tap water. A flow rate of 3.4 ± 0.2 L d^{-1} resulted in a hydraulic retention time of 14 ± 0.7 h. Any transient NH_4^+/NO_2^- build-up was immediately corrected by adjusting the nitrogen loading, preventing accumulation of free ammonia (FA) or free nitrous acid (FNA). The reactor pH was controlled between pH 6.8 and 7.2 by a dosage of 0.1 M NaOH/HCl. The dissolved oxygen was controlled at 3.6 ± 0.2 mg L^{-1} with air pumps providing aeration through a diffuser stone at a superficial air flow rate of 1.33 m^3 m^{-2} h^{-1}.

2.3. Physiological characterization

Physiological characterization and a range of inhibition tests were performed in ex-situ batch activity measurements in 96-well plates with a working volume of 250 µL, of which 50 µL sludge suspension consisting of homogenized biofilm sludge and suspended sludge. Plates were incubated in a MB100-4A Thermoshaker (Hangzhou Allsheng Instruments, China) at 50°C and 600 rpm, containing a buffer solution with a final concentration of 500 mg P L^{-1} (KH_2PO_4/K_2HPO_4), 500 mg $NaHCO_3$ L^{-1}, 0.1 mL L^{-1} trace element solution (Kuai and Verstraete, 1998) and $(NH_4)_2SO_4$ or $NaNO_2$. Operational parameters in the batch tests varied according to

the investigated parameter. pH, temperature and substrate concentrations were measured in all tests. From these, FA/FNA concentrations were calculated based on their chemical equilibrium (Anthonisen et al., 1976). The effects of the different parameters can only be separated from each other by a combination of different tests as presented in Table 4.1 for ammonium oxidation. A similar strategy was applied for separation of nitrite and FNA effects on nitrite oxidation. All treatments were performed in sextuple, and liquid samples (2 µL) were taken over time for NH_4^+ and NO_2^- analysis. Protein measurements enabled the calculation of specific rates that were converted to volatile suspended solids (VSS) based on the average protein content of the thermophilic sludge (32.7% protein VSS^{-1}).

Table 4.1 Overview of the operational parameters during different ex-situ batch activity measurements enabling the separation of the effect of pH, temperature, ammonium and free ammonia (FA) on ammonium oxidation. = : equal, ≠ : changing.

Investigated parameter	**Operational parameters during batch test**				**Figure**
	Temperature	pH	Ammonium	FA	
FA	=	=	≠	≠	Figure 4.7 **A**
Ammonium	=	=	≠	= (*)	Figure 4.7 **A**
pH	=	≠	≠ (**)	= (*)	Figure 4.8 **A**
Temperature	≠	=	=	= (*)	Figure 4.8 **B**

*: Concentrations below 2.4 mg NH_3-N L^{-1} are considered as not-varying, as Figure 4.7 **A** showed no inhibition of ammonium oxidation up to this concentration
: Figure 4.7 **A showed no influence of ammonium concentration on ammonium oxidation. Ammonium concentration varying but considered as equal as long as corresponding FA is below 2.4 mg NH_3-N L^{-1}.

2.4. High-throughput DNA sequencing and phylogenetic analysis

Biomass samples of the reactor were collected monthly over a six month period (days 245-387), and total DNA was extracted as described in Chapter 3. Prokaryotic biodiversity was analyzed using pair-end high-throughput sequencing (MiSeq Illumina platform) of the regions V5-V6 of the 16S rRNA gene, using the primers 807F and 1050R previously described (Bohorquez et al., 2012). The sequences were analyzed as described before (Camarinha-Silva et al., 2014), obtaining the data presented in Table 4.2. Forward and reverse reads were aligned manually, allowing zero mismatch. Sequencing depth was rarefied to the minimum, obtaining 18191 operational taxonomic units (OTUs) per sample. The vegan, phyloseq and

MASS packages in R were used to plot the rarefaction curves, normalize to the minimum sequencing depth and calculate Pearson's correlation respectively. Phylogenetic analyses were performed with MEGA5 (Tamura et al., 2011) using the neighbor-joining method with Jukes-Cantor correction and pairwise deletion of gaps/missing data. A total of 1000 bootstrap replications were performed to test for branch robustness. Heat map was generated using gplots and RColorBrewer packages.

Table 4.2 Overview of Illumina data before quality filter (raw data), after quality filter and after clustering (cut off 99%).

Sample	1	2	3	4	5	6
Total number of reads (raw data)	42460	22296	30051	35026	33354	26171
Total number of reads after quality filter	35404	18191	22847	28872	26893	21404
Phylotypes after clustering with cut off of 99%	97	103	122	119	102	100

2.5. Electron microscopy

For electron microscopy, biofilm material from three different sampling sites in the bioreactor was fixated and embedded in SPURR as described by Spieck and Lipski (2011) (Spieck and Lipski, 2011). The ultrathin sections were observed using a transmission electron microscope (model JEM 100C or LEO-906E, Zeiss, Jena, Germany).

2.6. Stable isotope probing: membrane lipids

Reactor biomass was incubated (50°C, 100 rpm) in 120 mL gas-tight serum flasks containing 20 mL phosphate buffer (pH 7) with final concentrations of 750 mg P L^{-1} (KH_2PO_4/ K_2HPO_4), 1 g $NaH^{13}CO_3$ L^{-1} and NH_4^+ or NO_2^- as the sole nitrogen source. Liquid samples (2 µL) were taken over time for NH_4^+ and NO_2^- analysis. pH was adjusted through the addition of HCl or $NaH^{13}CO_3$. Biomass from three parallel incubations with NH_4^+ (harvested at day 0, 49 and 85) served for alkyl iodides analysis, while biomass from five parallel incubations with NO_2^- (harvested at day 0, 3, 7, 14 and 21) served for PLFA analysis. The sampling points were determined based on the relative abundance of the AOA/NOB, the oxidation rates and the

sensitivity of the respective biomarker analysis.

Alkyl iodides analysis

Biomass was subjected to acid hydrolysis by refluxing for 3 h with 5% HCl in MeOH. The resulting extract was separated using Al_2O_3 chromatography. Hexane:DCM (9:1) and DCM:methanol (1:1) as eluents, yielding an apolar and polar fraction. An aliquot of the polar fraction was analyzed for tetraether lipids using HPLC/MS (Schouten et al., 2007). The remaining polar fractions were subjected to chemical treatment to release the biphytanyl chains from the tetraether lipids (Lengger et al., 2014). The stable carbon isotopic composition of the released biphytanes was analyzed in replicate using an Agilent 6800 GC coupled to a Thermo Fisher Delta V isotope ratio monitoring mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) (Lengger et al., 2014).

Phospholipid fatty acid analysis

Extraction and derivatization of PLFAs for compound specific ^{13}C analysis was adapted from Huygens et al. 2011 (Huygens et al., 2011). Identification of 11-methyl C16:0 was based on the retention time and comparison with published mass spectra (Lipski et al., 2001) using the mass fragments m/z 185 and m/z 213 resulting from cleavage of the molecule at both sides of the methyl-branch, as these are diagnostic fragments of 11-methyl-branched FAME. Isotopic enrichment was assessed using the m/z 74/(74 + 76) ratio of the methyl acetate ion fragment.

2.7. Chemical analyses

NH_4^+ (Nessler and Berthelot), NO_2^- (IC and Montgomery), NO_3^-, VSS, protein (Lowry), DO and pH were measured as described in previous Chapters.

3. Results

3.1. Thermophilic batch enrichments

Samples from four composting facilities served as inocula for the batch-wise enrichment of thermophilic (50°C) nitrifying communities. The different origin of the organic fractions of the samples resulted in different N-compound distributions in the compost solution. The green waste (a) and rabbit manure/green waste mixture (b) exclusively contained oxidized forms of nitrogen (NO_2^-/NO_3^-), while the digested organic waste (c) and cow manure (d) only contained NH_4^+. This distinction was reflected in the observed thermophilic nitrifying activity. First NH_4^+ and NO_2^- oxidation was observed after approximately 100 days of incubation. Samples (a) and (b) showed both NH_4^+ and NO_2^- oxidation, while samples (c) and (d) only showed NO_2^- oxidation. After one year of incubation and several dilution steps, two highly active NO_2^- oxidizing and two enchained NH_4^+ and NO_2^- oxidizing enrichment communities were obtained.

3.2. Bioreactor performance

The enrichments showing complete nitrification were pooled and served as inoculum for the bioreactor at 50°C. Initial volumetric nitrification rates were low (4.7 ± 2.6 mg N L^{-1} d^{-1}). However, after two months of operation, a clear exponential increase in nitrifying activity was observed in the reactor reaching volumetric NH_4^+ and NO_2^- oxidation rates of 126±7 and 189 ± 17 mg N L^{-1} d^{-1}, respectively (Figure 4.1). After this first stage, due to a technical failure, the community was challenged by a temperature drop to 30°C and a subsequent shock at pH 11 (days 235-238), leading to an initial loss of ammonium oxidation activity. However, the reactor re-stabilized successfully, reaching nitrification rates higher than 200 mg NH_4^+-N L^{-1} d^{-1} (Figure 4.1). Moreover, a low nitrogen loss was observed. Practically all the removed NH_4^+-N was recovered as NO_3^--N (93 ± 4%), confirming that nitrification was the main process involved. The biomass predominantly appeared as an orange to brownish biofilm on the packing material and wall of the reactor vessel.

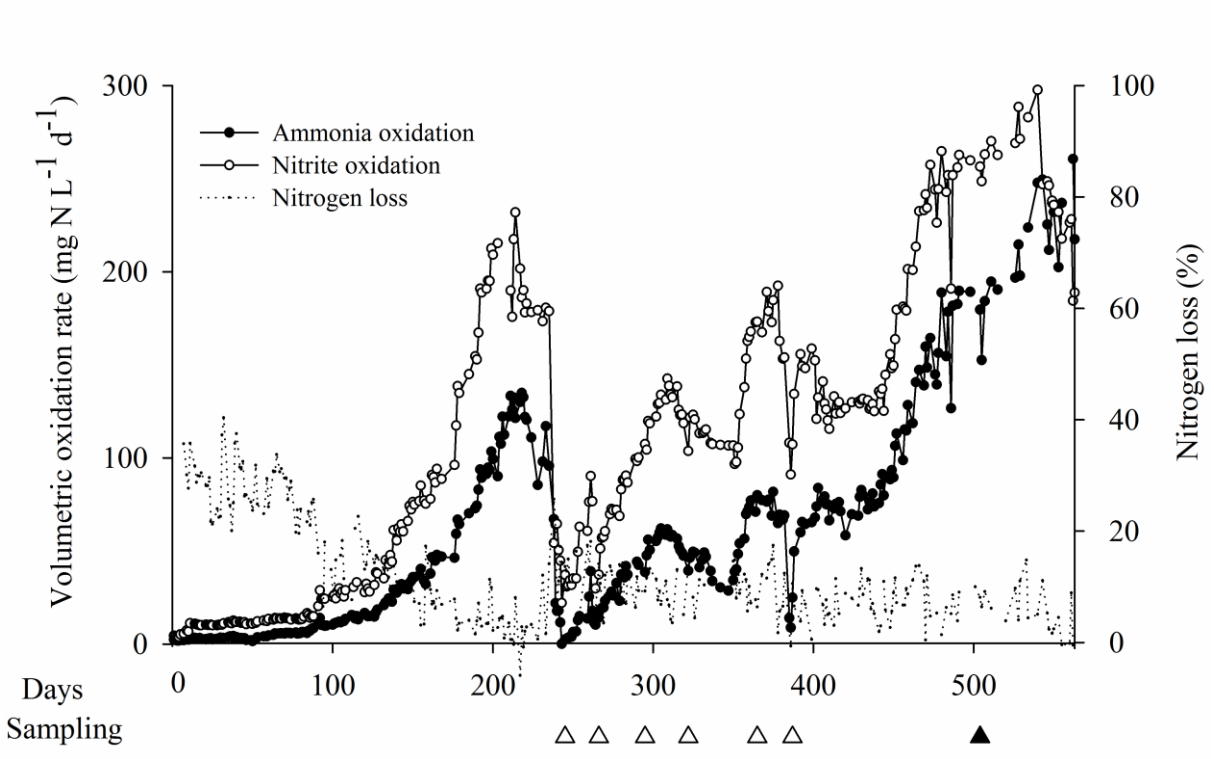

Figure 4.1 Performance (mg N L^{-1} d^{-1}) and nitrogen loss (%), i.e., the amount of removed NH_4^+-N not recovered as NO_2^--N or NO_3^--N, in the thermophilic bioreactor (50°C) inoculated with thermophilic nitrifying batch enrichments from compost samples. The white and black triangles indicate the sampling for high-throughput DNA sequencing and transmission electron microscopy, respectively. Temperature drop (30°C) and a subsequent shock (pH 11) at days 235-238 due to a technical failure.

3.3. Phylogeny and morphology

The thermophilic nitrifying microbial community was analysed over a six month period of the reactor experiment (days 245-387). Illumina sequencing identified one unique sequence (OTU7) of archaea in all samples closely related to the AOA "*Candidatus* Nitrososphaera gargensis" Ga9.2 (99% similarity) (Figure 4.2), while no known AOB could be detected in any of the samples.

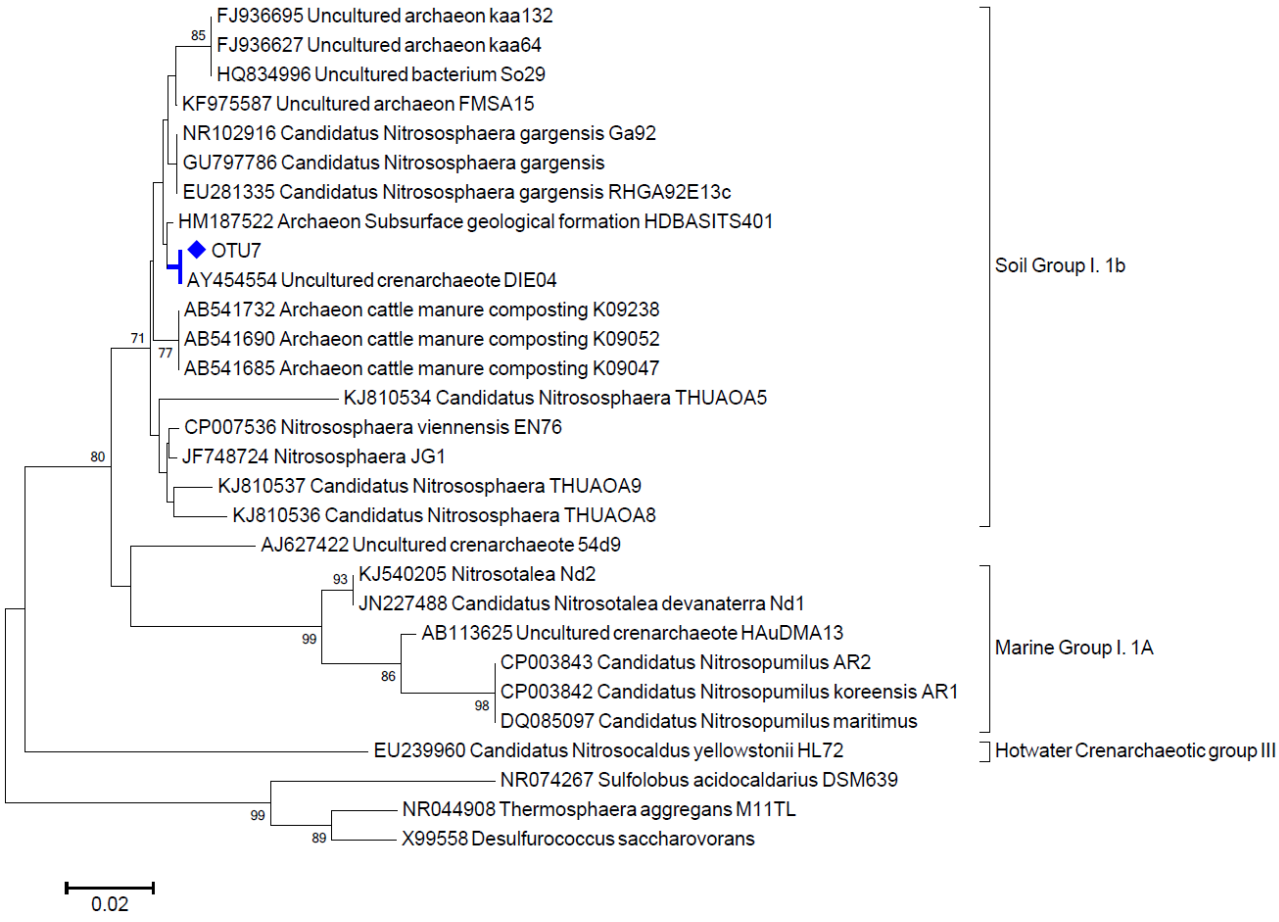

Figure 4.2 Phylogenetic relationships between the archaeal 16S rRNA gene sequence (OTU7) of the thermophilic nitrifying reactor biomass and all described AOA cultures or isolates, as well as relevant environmental clone sequences. OTU7 belongs to the group 1.1b of Thaumarchaeota (formerly Crenarchaeota).

For nitrite oxidation, several different sequences closely related to *Nitrospira* spp. were identified. OTU1, 99% similar to *Nitrospira calida* Ns10 (Figure 4.3), was the most abundant *Nitrospira* in the community in all samples and the only *Nitrospira*-related OTU that strongly increased in abundance over time. A clear enrichment of the different *Nitrospira*

representatives was observed over time. The relative abundance increased gradually from 2% to 22-25% over 6 months of operation. The AOA abundance, however, strongly fluctuated over the different samples from 0.1 to 18%. The unbalanced ratio of AOA/NOB in this community is a result of the influent feeding strategy in which, beside ammonium (20-120 mg N L^{-1}), nitrite was provided along the experiment (± 34 mg N L^{-1}) to prevent limitation in NOB growth in case ammonium oxidation would attenuate (Figure 4.1).

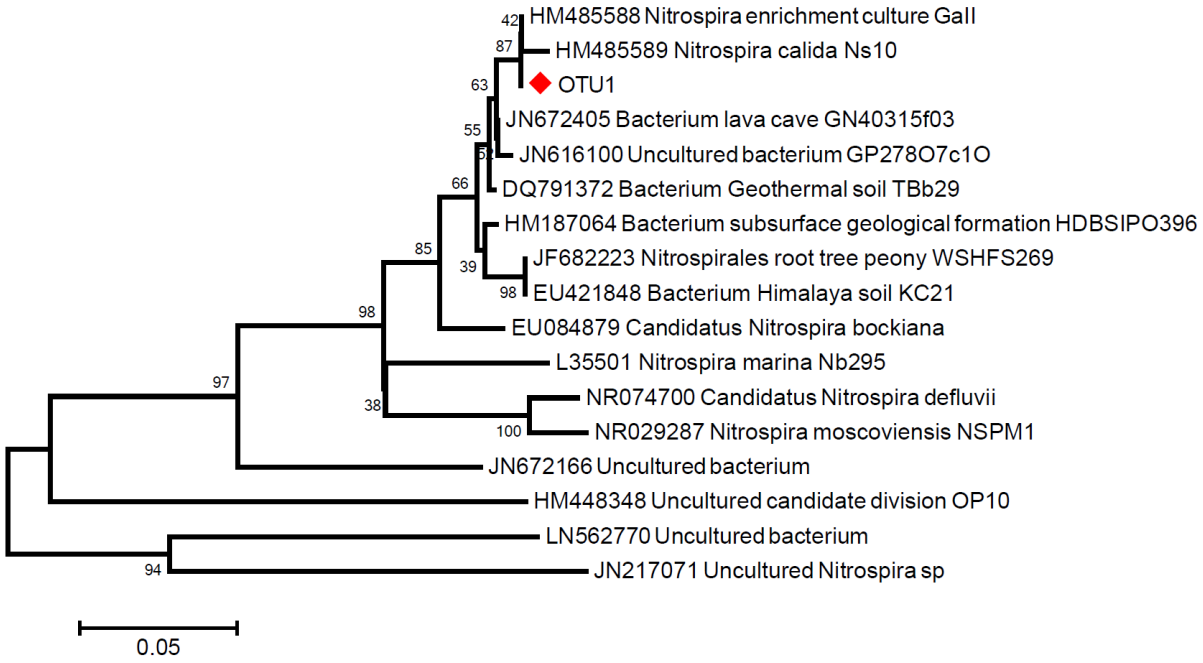

Figure 4.3 Phylogenetic relationships between the *Nitrospira* 16S rRNA gene sequences of the thermophilic nitrifying reactor biomass (OTU1) and all described *Nitrospira* cultures or isolates, as well as relevant environmental clone sequences.

The nitrifying core appeared to have the same preferred satellite populations. Both the abundance of OTU7 and OTU1 were positively correlated (ρ >0.9) with the abundance of OTU146 (most probably *Bacteroidetes* spp.), OTU47 (*Ignavibacterium*), OTU80 (*Planctomycetacea*), OTU87 (unclassified bacteria) and OTU92 (*Betaproteobacteria*) (Figure 4.4

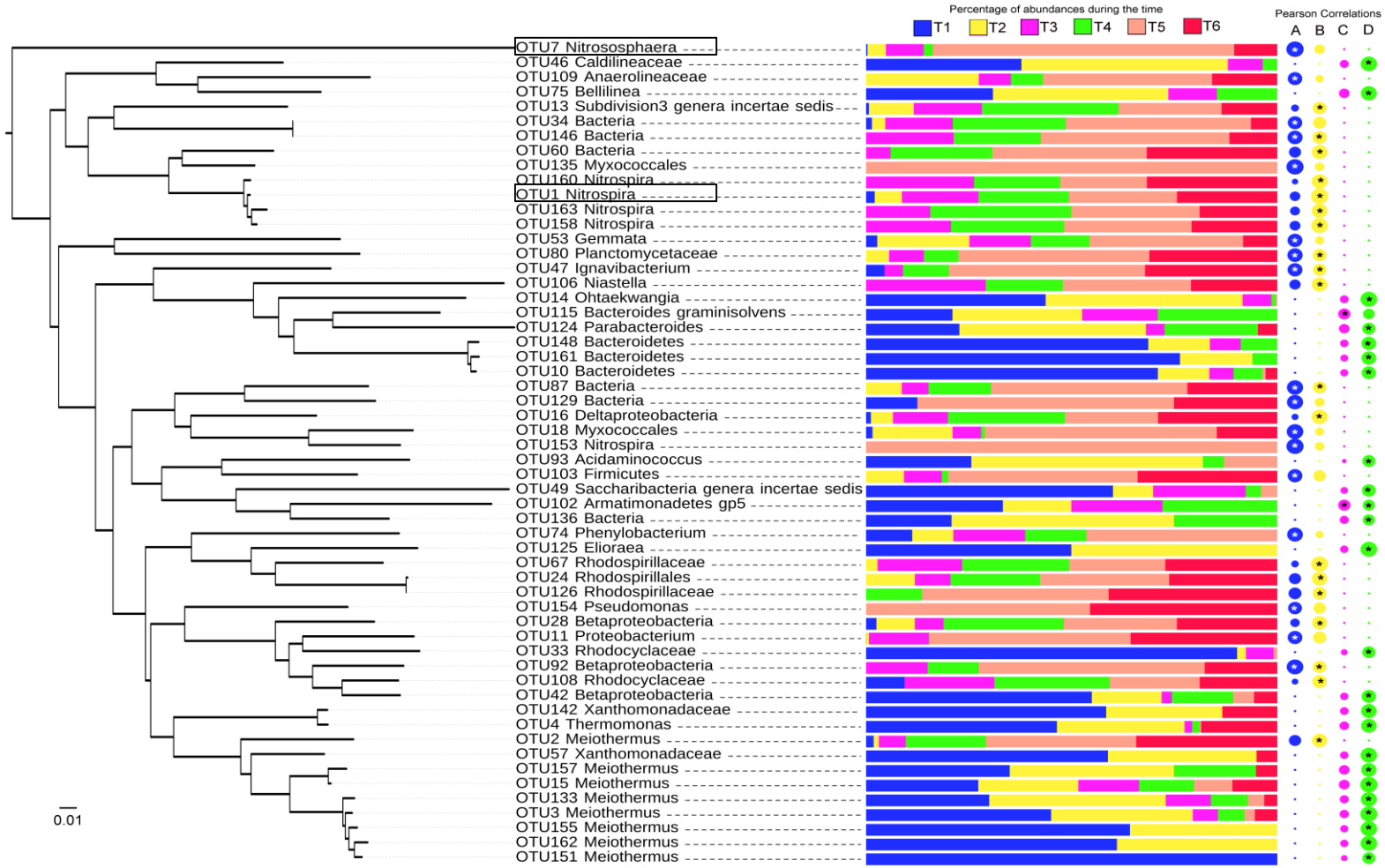

Figure 4.4 Phylogenetic relationships and the relative abundances over time of the OTUs related to bacteria and archaea found in the thermal bioreactor with Pearson correlations higher than 0.9 and lower than -0.9. Column A (blue circles) shows the positive correlations with OTU7, column B (yellow circles) shows the positive correlations with OTU1. Column C (pink circles) shows the negative correlations with OTU7 and column D (green circles) shows the negative correlations with OTU1.

The presence of the described nitrifiers in the biofilm of the thermophilic reactor was morphologically confirmed in all samples through transmission electron microscopy (TEM) (Figure 4.5). Cells of *Nitrospira* spp. were characterized by a spiral-shaped morphology with a pleomorphic cell appearance, a wide periplasmic space and a granular cell interior (Ehrich et al., 1995) and the "*Candidatus* Nitrososphaera gargensis" related AOA were characterised by small, very dense, coccoid-shaped cells (Tourna et al., 2011).

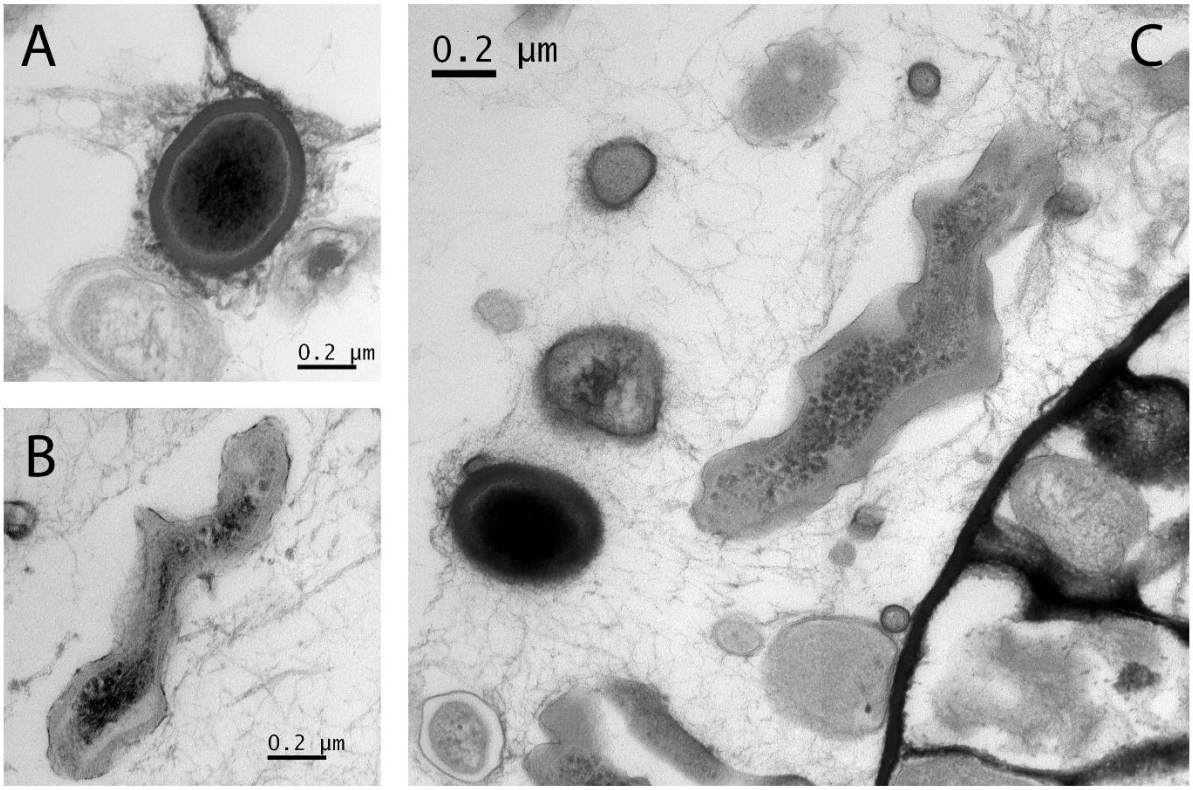

Figure 4.5 Transmission electron micrographs of ultrathin sections of the thermophilic reactor biofilm. (A) single "*Candidatus* Nitrososphaera gargensis"–like AOA cell; (B) a single *Nitrospira* cell; (C) co-occurrence of single AOA and *Nitrospira* cells loosely associated with each other and close to an abundant, but unknown cell type embedded in a dense biofilm structure.

3.4. Carbon incorporation

The autotrophic nature of the AOA and NOB during nitrification was investigated by incorporation of ^{13}C-derived bicarbonate into the characteristic membrane lipids during two sets of incubations, one with NH_4^+ and another with NO_2^-. Isotopic analysis of the biphytane moieties of the characteristic archaeal membrane lipids, glycerol dibiphytanyl glycerol tetraether lipids (GDGTs), was performed for AOA. The GDGTs were dominated by

crenarchaeol, in agreement with culture studies of "*Candidatus* Nitrososphaera gargensis" (Pitcher et al., 2010). The two biphytanes released showed considerable enrichment in ^{13}C compared to the start of the incubation, pointing at AOA autotrophy (Figure 4.6). The activity of NOB was determined by assessing the incorporation of ^{13}C-labeled bicarbonate into 11-methyl C16:0, a specific biomarker for moderately thermophilic *Nitrospira* (Lipski et al., 2001; Spieck and Lipski, 2011). The isotopic label was incorporated in the 11-methyl C16:0 phospholipid fatty acid biomarker after a lag-time of 3 days at the rate of 0.3% d^{-1} during the 21 days of incubation. Interestingly, the NOB ^{13}C enrichment (%) appeared to be linear with the total amount of nitrogen oxidized (Figure 4.6), demonstrating that the autotrophic carbon assimilation by NOB occurred concurrently with the NO_2^- oxidation. For AOA, however, due to the lower sensitivity of the AOA biomarker analysis and lower AOA oxidation rates only two valid data points remained and do not allow to draw conclusions regarding linearity. Furthermore, the partnership between "*Candidatus* Nitrososphaera gargensis" and *Nitrospira calida* was confirmed, as a 26% ^{13}C enrichment was observed for the *Nitrospira* biomarker at the end of the incubation fed with NH_4^+.

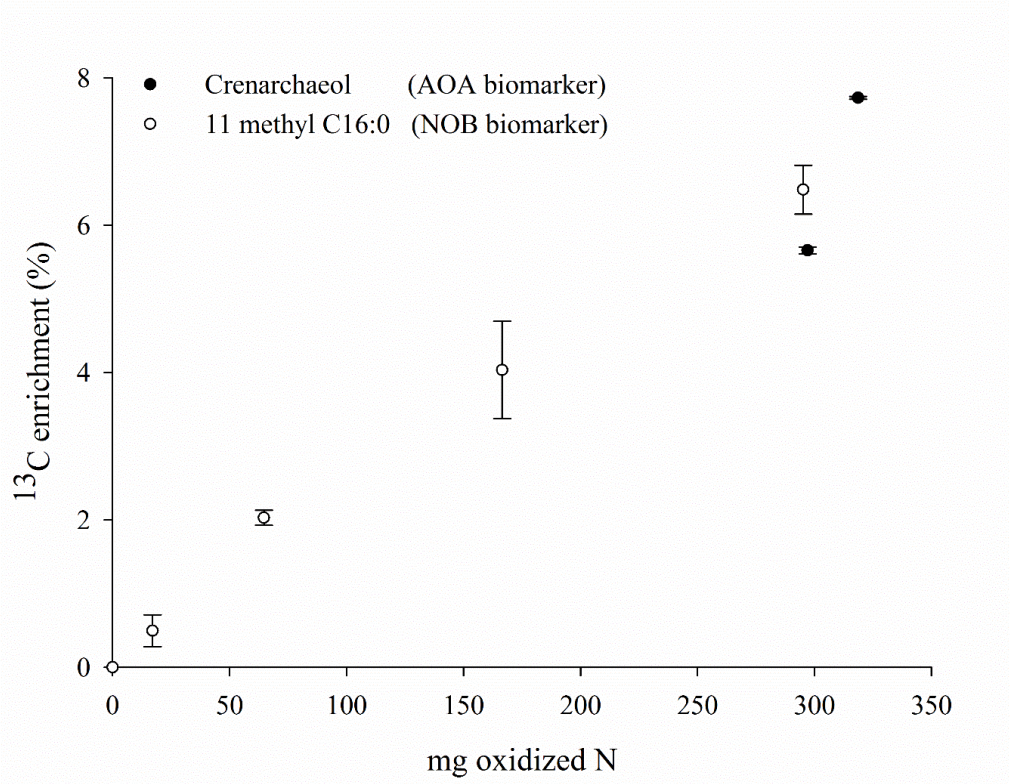

Figure 4.6 Relationship between the absolute amount of nitrogen oxidized and the ^{13}C incorporation in characteristic biomarkers: the biphytane moieties of the glycerol dibiphytanyl glycerol tetraether lipids (GDGTs), more specifically crenarchaeol, as a biomarker for "Candidatus Nitrososphaera gargensis" and the 11 methyl C16:0 as a specific phospholipid

fatty acid biomarker of *Nitrospira* spp. Data points represent the average replicate extractions (n=3), error bars represent the standard error.

3.5. Physiological characterization

The thermophilic biomass showed specific nitrifying rates up to 198 ± 10 and 894 ± 81 mg N g^{-1} VSS d^{-1}, for NH_4^+ and NO_2^- oxidation, respectively. Taken into account an average relative abundance of 10% AOA and 25% NOB and the simplified assumption that total protein was equally distributed among all organisms in the culture, these rates result in a specific AOA and NOB rate of 18 ± 1 and 33 ± 3 µg N mg^{-1} protein h^{-1}, respectively. With respect to the development of biotechnological applications and effective process control strategies, it is important to distinguish the inhibitory effects of NH_4^+ from those of free ammonia (FA) and NO_2^- from those of free nitrous acid (FNA). The thermophilic ammonium and nitrite oxidizers were both sensitive to FA, while insusceptible to NH_4^+. Ammonium oxidation was not inhibited up to 300 mg NH_4^+-N L^{-1} for the batch activity series with low FA, while it was inhibited for the series tested at a higher FA, resulting in an IC_{50} of 7.5 mg NH_3-N L^{-1} (Figure 4.7 **A**). Nitrite oxidation was slightly more sensitive for FA with an IC_{50} of 5.0 mg NH_3-N L^{-1} (Figure 4.7 **B**). Regarding NO_2^-/FNA inhibition, ammonium oxidizers were clearly inhibited by NO_2^- and not by FNA. Both the series with high and low FNA gave the same inhibition response with increasing NO_2^- concentrations (Figure 4.7 **C**). Sensitivity was, however, very low, characterized with an IC_{50} of 2117 mg NO_2^--N L^{-1}. In contrast, the NOB were extremely sensitive to FNA and not to NO_2^- with an IC_{50} of 0.0010 mg HNO_2-N L^{-1} (Figure 4.7 **D**). Lowering FNA while applying the same NO_2^- concentrations namely eliminated the inhibitory effect. Nitrate inhibition of nitrite oxidation was also observed (IC_{50} 360 mg NO_3^--N L^{-1}).

Thermophilic NH_4^+ oxidation showed a pH optimum at pH 7, maintaining >70% of its activity within the tested pH range (pH 6-8) (Figure 4.8 **A**). Although the bioreactor was controlled between pH 6.8-7.2, it showed increasing NO_2^- oxidation at lower pH, given low FNA concentrations (Figure 4.8 **C**). Ammonium oxidation showed a broad temperature optimum (45-55°C), while nitrite oxidation showed a clear optimal activity at the reactor temperature (50°C). The thermophilic NH_4^+ oxidation could be inhibited by the conventional nitrification inhibitor ATU at neutral pH with an $IC_{50/100}$ of 3.5/8.8 mM.

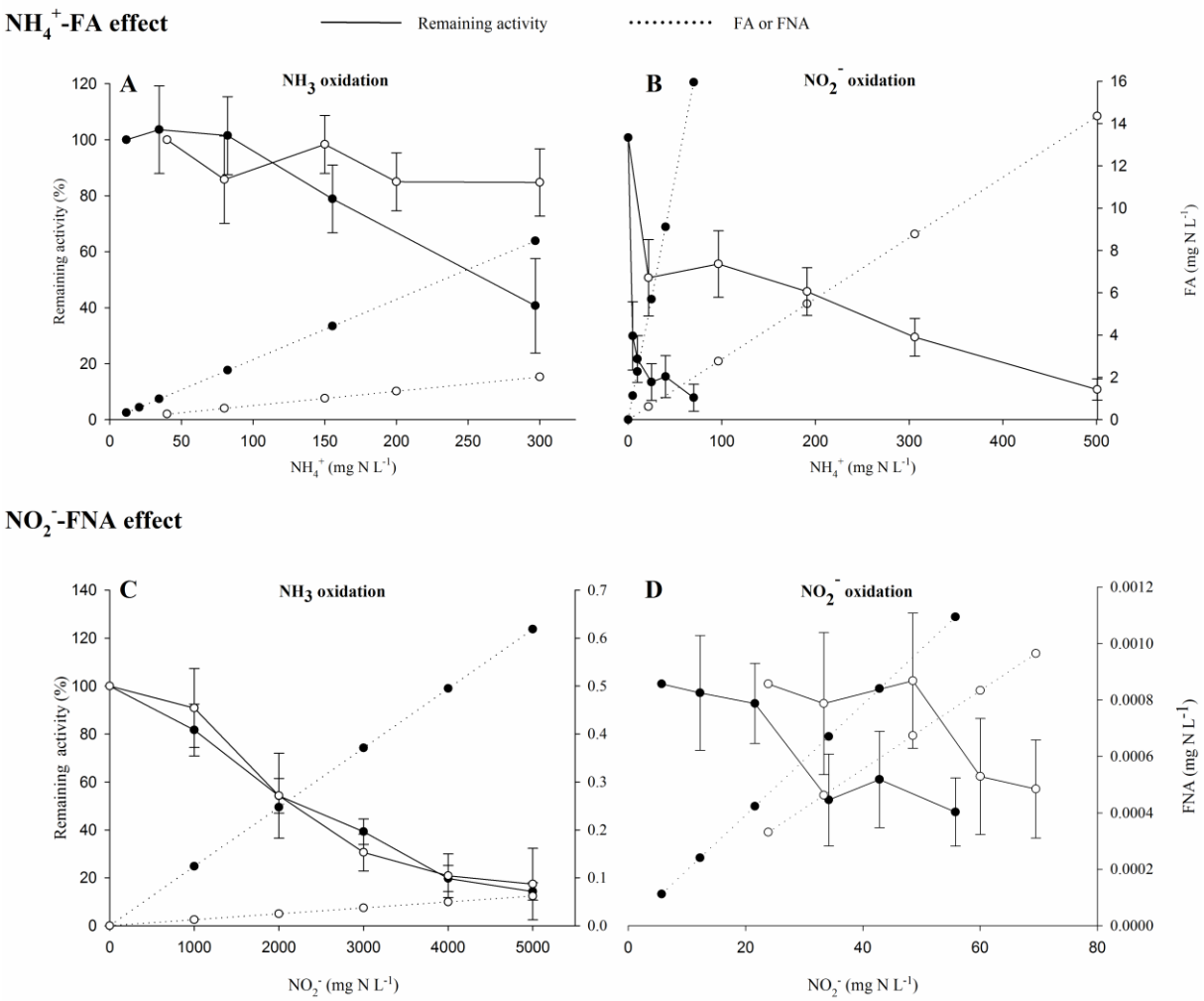

Figure 4.7 Effect of ammonium/FA (free ammonia) and nitrite/FNA (free nitrous acid) on thermophilic ammonia (A and C) and nitrite (B and D) oxidation. Each figure represents two sets of experiments (filled versus empty circles) where he full lines show the resulting inhibition pattern, while the dotted lines show the corresponding FA/FNA of the corresponding test.

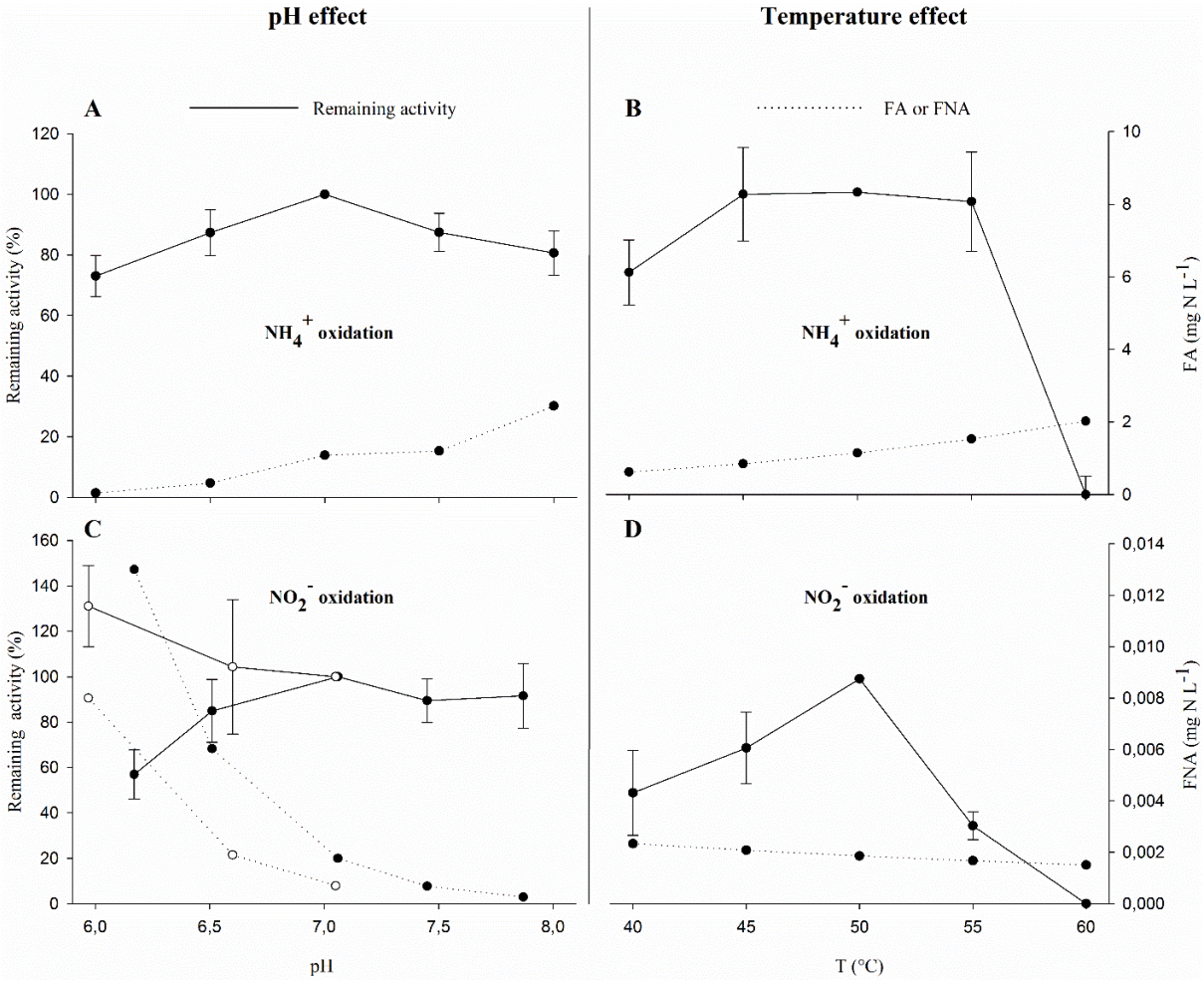

Figure 4.8 Effect of pH and temperature on thermophilic ammonium (A and B) and nitrite (C and D) oxidation. The full lines represent the remaining activity, while the dotted lines show the corresponding FA/FNA of the corresponding test. In Figure C, two sets of experiments are presented, in which the same pH range is tested but at different nitrite and thus FNA concentrations.

4. Discussion

In this study, the enrichment of coupled autotrophic thermophilic ammonium and nitrite oxidizers from compost was achieved followed by the successful operation of a thermophilic nitrifying bioreactor, opening up opportunities for nitrogen removal in warm wastewater.

The thermophilic nitrifying community consisted of an AOA and NOB closely related to "*Candidatus* Nitrososphaera gargensis" and *Nitrospira calida*, respectively, both of which were originally isolated from geothermal springs (Hatzenpichler et al., 2008; Lebedeva et al., 2011).

In contrast to the oligotrophic nature of these geothermal springs, this study enriched nitrifiers from aerobic compost, a nutrient-rich high-temperature anthropogenic environment. Although many archaeal *amoA* genes (Maeda et al., 2011; Zeng et al., 2011) and even "*Candidatus* Nitrososphaera gargensis"–like sequences (Yamamoto et al., 2011; Oishi et al., 2012) were detected during composting processes, so far, no autotrophic thermophilic nitrifiers were enriched from compost. Only a heterotrophic AOB growing at 50°C related to *Bacillus halodurans* was isolated previously from animal waste composting (Shimaya and Hashimoto, 2011). The presence of the described core nitrifiers in the bioreactor was, furthermore, linked with their activity and functionality. Incorporation of ^{13}C labeled bicarbonate was observed into crenarchaeol and 11-methyl C16:0, characteristic membrane lipids for "*Candidatus* Nitrososphaera gargensis" (Pitcher et al., 2010) and *Nitrospira* (Lipski et al., 2001), respectively (Figure 4.6). Although the carbon assimilation confirmed the autotrophic activity of the studied nitrifiers, it does not exclude the presence of other, unknown autotrophic or heterotrophic nitrifiers. An abundant cell type, embedded in a dense biofilm structure could not be identified (Figure 4.5 **C**). Together with the observed delay/heterogeneity of the AOA presence over time, this could suggest that an uncharacterized ammonium oxidizing organism was also present, as was recently observed in reactors with low dissolved oxygen concentrations (Fitzgerald et al., 2015). The linearity of the nitrite oxidation and the ^{13}C enrichment in the stable isotope experiment (Figure 4.6), however, suggest that at least the NOB *Nitrospira calida* was an important thermophilic nitrifier in the biomass community.

The physiological characterization revealed that the specific oxidation rates of both AOA (18 ± 1 µg N mg^{-1} protein h^{-1}) and NOB (33 ± 3 µg N mg^{-1} protein h^{-1}) were in the same order of magnitude as related nitrifiers. In particular, the specific rates for AOA range from 11 to 24 µg N mg^{-1} protein h^{-1} (Kim et al., 2012), while reported rates for *Nitrospira* spp. range between 16 and 42 µg N mg^{-1} protein h^{-1} (Nowka et al., 2015). Interesting differences in substrate/product tolerances were observed. Until now, data concerning NH_4^+/NH_3 inhibition on (thermophilic) AOA has been limited attributing the inhibitory effect to NH_4^+ without excluding FA inhibition. However, with respect to biotechnological applications and the development of effective process control strategies, this distinction can be of great importance and was determined in this study. The "*Candidatus* Nitrososphaera gargensis"–like AOA in the thermophilic nitrifying

bioreactor appeared to be insensitive to NH_4^+, while it was significantly inhibited by FA concentrations of 7.4 mg NH_3-N L^{-1} (Figure 4.7 **A**). At a neutral pH and a temperature of 50°C, this inhibition corresponds to a NH_4^+ concentration of 260 mg NH_4^+-N L^{-1}. This concentration is 6 times higher than the inhibitory NH_4^+ concentration reported for "*Candidatus* Nitrososphaera gargensis" (Hatzenpichler et al., 2008). The higher FA tolerance could be attributed to the fact that the AOA in this study originated from nutrient-rich compost in contrast with oligotrophic geothermal springs. Indeed, the AOA detected in cattle manure compost by Oishi et al. (2012) showed ammonium oxidizing activity at 46°C up to FA concentrations of 18 mg N L^{-1} (10 mM, pH 7.8). The thermophilic NOB in the bioreactor of our study were also sensitive to FA and insensitive to NH_4^+, but the higher sensitivity (IC_{50} of 5.0 mg NH_3-N L^{-1}) could allow a selective NOB inhibition based on FA. Furthermore, the AOA were insensitive to FNA, while the NOB were extremely sensitive to FNA (IC_{50} of 0.0010 mg HNO_2-N L^{-1}) (Figure 4.7 **C, D**). Both the insensitivity of AOA for FNA and the high sensitivity of NOB for FA and FNA suggest that a selective NOB inhibition could be easily established in the described thermophilic nitrifying community, enabling the development of more cost-effective nitrogen removal processes, such as nitritation/denitritation or deammonification.

With respect to the biotechnological applications of thermophilic nitrifiers in wastewater treatment, it is important to establish a robust and stable satellite community around the main functional players. Pearson correlations indicated that both the AOA and NOB in the thermophilic biomass evolved towards the same preferred satellite community (Figure 4.4). Interestingly, these microbial groups were also identified together with closely related AOA and NOB sequences in natural nitrification-driven thermophilic environments (Lin et al., 2012; Marks et al., 2012; Nishizawa et al., 2013). As observed for methanotrophs, for which the methanotrophic activity was stimulated by increased heterotrophic richness of the satellite community (Ho et al., 2014), the co-occurrence of these microbial groups in natural thermophilic environments, as in the bioreactor described in this study, might have a beneficial effect on the nitrifying activity. Furthermore, positive correlations between both key players and the presence of the phyla Bacteroidetes, Firmicutes and Deinococcus-Thermus, among others, can facilitate the development of complete nitrogen removal processes, as these groups were described as the main constituents of a thermophilic denitrifying reactor (Chapter 5).

Until now, the main thermophilic nitrogen removal mechanism was assumed to be ammonia stripping and nitrogen assimilation into biomass. However, as discussed in Chapter 1, not all ammonia can be removed through stripping and depending on the ammonium concentration in the influent and the local market/value of ammonium sulphate, ammonium recovery is not always cost competitive and thus development op thermophilic biotechnology for nitrogen removal is necessary. Besides eliminating cooling requirements, thermophilic nitrogen removal also lowers sludge production and confers better settling properties (Suvilampi and Rintala, 2003). These advantages apply not only to warm wastewaters but also to wastewaters on sites with excess heat available. A few lab-scale studies have explored the potential of thermophilic nitrification for wastewater treatment, but achieved no more than 40-42.5°c (Shore et al., 2012; Courtens et al., 2014a). Thus far, this is the first study describing a thermophilic nitrifying bioreactor at 50°C. Although challenges such as the effect of carbon on the autotrophic/heterotrophic competition and the coupling of nitrification with a reductive nitrogen removal process (denitrification, anammox) have to be addressed to enable implementation, this study paves the way for thermophilic nitrogen removal.

5. Conclusions

- Thermophilic autotrophic ammonium and nitrite oxidizers were batch-wise enriched from compost samples and served as inoculum for a nitrifying bioreactor at 50°C with high biotechnological potential.
- The nitrifying community contained up to 17% ammonium oxidizing archaea (AOA) closely related to "*Candidatus* Nitrososphaera gargensis", and 25% nitrite oxidizing bacteria (NOB) related to *Nitrospira calida*.
- Their autotrophic nitrifying activity was confirmed by incorporation of ^{13}C-derived bicarbonate into the respective characteristic membrane lipids during nitrification.
- The combination of different inhibition tests enabled the separation of the effect of interlinked parameters such as temperature, pH, NH_4^+ and FA. NOB were more sensitive to FA than the AOA and moreover strongly inhibited by FNA while AOA could

only be inhibited by high NO_2^- concentrations, independent of the FNA concentration. These observed difference in product/substrate inhibition opens the path for short-cut nitrogen removal processes.

6. Acknowledgments

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CHAPTER 5:

TRADE-OFF BETWEEN MESOPHILIC AND THERMOPHILIC DENITRIFICATION: RATES VS. SLUDGE PRODUCTION, SETTLEABILITY AND STABILITY

This chapter has been redrafted after:

Courtens, E. N. P., Vlaeminck, S.E., Vilchez Vargas, R., Verliefde, A, Jauregui R., Pieper D. H. and Boon, N. (2014) Trade-off between mesophilic and thermophilic denitrification: rates vs. sludge production, settleability and stability. Water Research, 63, 234-244

1. Introduction

Thermophilic denitrifying micro-organisms are widely spread in natural ecosystems (Chapter 1). However, until now, only one study was focused on the development of a thermophilic denitrifying reactor for wastewater treatment. Laurino and Sineriz (1991) investigated denitrification in a lab-scale upflow sludge blanket (USB) reactor at 55 °C fed with ethanol as carbon and energy source. The USB reactor was inoculated with thermal mud originating from a hot spring and started-up in batch mode for 15 days. After switching to continuous mode, a maximum nitrogen removal rate of 1317 mg N L^{-1} d^{-1} with a nitrate removal efficiency of 78 % was observed, resulting in a maximal specific removal rate of 51 mg N g^{-1} VS d^{-1}.

The current study investigated whether a non-thermophilic inoculum, i.e. mesophilic denitrifying sludge (26 °C), can be used for the start-up of a thermophilic (55 °C) sequential batch reactor (SBR). A parallel mesophilic control SBR (34 °C) was inoculated with the same sludge enabling an extensive comparison between mesophilic and thermophilic denitrification. Both functional aspects such as maximal specific nitrate removal rate, sludge production, sludge settleability and nitrous oxide production and phylogenetic diversity of the microbial community were compared for different substrate complexities ranging from synthetic influent to real waste streams.

2. Materials and methods

2.1. Set-up and operation of the denitrifying reactors

Two parallel sequential batch reactors (SBR) had an effective liquid volume of 2 L and an inner diameter of 12 cm. Operational temperatures were chosen from an application point of view, representing the typical temperatures of mesophilic (34°C) and thermophilic (55°C) digestates, for the mesophilic (control) and thermophilic SBR, respectively. The reactor vessels were jacketed, and the temperature was controlled with a circulating thermostatic water bath. The two-hour cycle consisted of a 90 min reaction period including stirring (60 rpm), followed by a 15 min settling period and a 15 min decanting period. The reactors were inoculated with nitrifying/denitrifying (N/DN) sludge, originated from a landfill leachate wastewater treatment plant with an average temperature of 26.3 ± 3.6°C, at an initial biomass

concentration of 4.0 ± 0.2 g volatile suspended solids (VSS) L^{-1}. During start-up of the reactors, considerable amounts of sludge washed out. After stabilization, sludge was wasted in order to keep the sludge concentration around 2 g VSS L^{-1}. For the mesophilic SBR 1.3 ± 0.7 g VSS was wasted on a daily basis, while practically no sludge was wasted in the thermophilic SBR, resulting in a sludge residence time (SRT) of 2.3 ± 0.5 and 4.9 ± 0.9 days for the mesophilic and thermophilic SBR, respectively. An identical feeding strategy was applied for both reactors, whereby different wastewater matrices and COD types were used ranging from sodium nitrate containing tap water to industrial wastewater (WW) from the fertilizer industry, sodium acetate (NaAc) and diluted molasses. Other than NO_3^- and COD, the influent also contained $(NH_4)_2SO_4$ (0.05 g N g^{-1} NO_3^--N) and KH_2PO_4 (10 mg P L^{-1}) and was acidified throughout the whole experiment with HCl resulting in an influent pH of 2.5 ± 0.4 in order to indirectly control the pH in the reactor. Three main feeding periods were distinguished according to the COD and NO_3^- source: the synthetic period (COD_{NaAc}/N_{NO3}), the real wastestream/synthetic period ($COD_{Molassas}/N_{NO3}$) and the real wastestream (WS) period ($COD_{Molassas}/N_{Fertilizer\ WW}$), respectively, each including different phases, depending on the NO_3^- loading rate (Table 5.1). The transitions between the three feeding periods occurred after at least 5 times the sludge retention time (SRT of 2.3 ± 0.5 and 4.9 ± 0.8 days for the mesophilic and thermophilic SBR) to ensure a 'stable' microbial community and at a same loading rate enabling comparison of the different influents. However, if the transition resulted in nitrite accumulation, the loading was lowered. The synthetic period (COD_{NaAc}/N_{NO3}) consisted of a start-up phase of 14 days (phase I), a high loading phase (phase II) for determination of the maximum volumetric/specific removal rates and a moderately loaded phase (phase III), facilitating the determination of settling properties and comparison between the two systems. The real WS/synthetic feeding period ($COD_{Molassas}/N_{NO3}$) with diluted molasses as carbon source included 2 phases (IV and V), where phase V served as an adaptation phase to the characteristics of the industrial WW from fertilizer industry (Table 5.1). Finally, in the last feeding period, the real WS period ($COD_{Molassas}/N_{Fertilizer\ WW}$), two different batches of industrial WW were used, resulting in two phases (VI and VII). Average volumetric and specific denitrification activities were calculated based on nitrate removal over stable operational periods of minimum 5 days. The removed COD/N, or more specifically $COD/NO_3\text{-}N_{equivalent}$ was calculated taking into account the nitrite production (eq. 1) (Matějů et al., 1992). Per mole COD to denitrify a mole nitrate to nitrogen

gas, about 0.4 is used for the reduction to nitrite, while 0.6 is used for the reduction of nitrite to nitrogen gas.

$$\text{Removed COD/N } (g/g) = \frac{COD_{influent} - COD_{effluent}}{(NO_3^- consumption - NO_2^- production) + 0.4 \times NO_2^- production} \quad (1)$$

Table 5.1 Overview of the different feeding periods, which were identical in both reactors. WW: wastewater, WS: wastestream, * different value in thermophilic reactor

Feeding period	Synthetic			Combi Real WS/Synthetic		Real WS	
COD source	Na-acetate			Molasses		Molasses	
NO_3^- matrix	Tap water			Tap water		Fertilizer WW	
Phase	I	II	III	IV	V	VI	VII
Duration (d)	13	16	17	13	13	14	9
NO_3^- (mg N L^{-1})	100-400	400(*200)	200	150	300	287 ± 4	182 ± 7
NO_3^- loading (mg N L^{-1} d^{-1})	350-1500	1508±65 (*739±27)	765±19	568±29	383±38	475±62	325±18
COD_{in}/N_{in} (g/g)	4.6±0.3	4.3±0.5	4.1±0.3	4.9±0.3	5.0±0.6	5.4±0.4	5.3±0.3

2.2. Denitrification activity batch tests

Parallel with the start-up of the reactors, anoxic batch tests were performed at 34°C and 55°C with the same inoculum in order to clarify the impact of the temperature shock on the denitrifying activity. Serum flasks with a volume of 120 mL were used, containing 80 mL of mixed liquor buffer solution (pH 8) with final concentrations of 0.6 g KH_2PO_4 L^{-1} and 10.5 g K_2HPO_4 L^{-1} and a biomass concentration of 3.6±0.1 g VSS L^{-1}. The serum flasks were closed with rubber stoppers and flushed with N_2 gas. Flushed substrate solution of $NaNO_3$ and NaAc (COD/N = 4) was supplemented by means of needled syringes to a final concentration of 100 mg N L^{-1}. After 72 hours a second spike of substrate ($NaNO_3$ and NaAc) was provided.

Similarly, during the feeding period with real WS ($COD_{Molassas}/N_{Fertilizer\ WW}$) (phase VI), anoxic batch tests were performed with the mesophilic and thermophilic reactor sludge investigating maximal specific rates and nitrous oxide (N_2O) production. The same buffer medium was used with a biomass concentration of 0.4 ± 0.04 g VSS L^{-1}. Flushed substrate solutions of $NaNO_3$ with NaAc (COD/N = 4) and $NaNO_3$ with molasses (COD/N = 6.5) were supplemented by means of needled syringes to a final concentration of 50 mg N L^{-1}. All tests were performed in triplicate on a shaker (100 rpm). Liquid samples were taken over time for nitrite, nitrate and

COD analysis. The last batch tests also included head-space gas sampling and gas pressure measurements for N_2O analysis.

2.3. Chemical analyses

Ammonium (Nessler), total Kjeldahl-N, TSS, VSS, and biochemical oxygen demand (BOD_5) were measured according to standard methods (Greenberg et al., 1992). COD was measured by photometric methods using Nanocolor test tubes (Macherey-Nagel, Germany). Nitrite, nitrate, DO and pH were determined as described in previous chapters. Gas pressure was measured using a tensiometer (Infield 7 with T1Kc sensor head, UMS, München, Germany). N_2O measurements were performed with a Shimadzu GC-14B gas chromatograph (Shimadzu, Kyoto, Japan) with an electron capture detector and two packed columns (1 and 2 m, respectively; Porapack Q, 80/100 mesh). The operating conditions were as follows: carrier gas N_2 (55 mL/min), injector temperature 105°C, column and oven temperature 55°C and detector temperature 250°C. The chromatograph was calibrated using N_2O standard gas (250±13 ppmv or 25.3 ± 1.5 ppmv in He).

2.4. Sludge characteristics

A Mastersizer S (Malvern, Malvern, UK) was used for size distribution measurements of the sludge samples with a glass flask as small volume dispersion unit. The results were calculated using the "polydisperse" analysis model in the Mastersizer software. Biomass settleability (SVI_5^*) and sludge yield (Y) were determined as described in Chapter 3.

2.5. High throughput DNA sequencing of the denitrifying microbial communities

Biomass samples (2 mL) were collected of the inoculum and both reactors over the different time periods and DNA extraction was performed as described in Chapter 3. Biodiversity was analyzed using high throughput sequencing (MiSeq Illumina platform). For that, regions V5-V6 of the 16S rRNA gene was amplified and targeted with adapters and barcodes suitable for Illumina sequencing (Bohorquez et al., 2012; Camarinha-Silva et al., 2014). Samples from both reactors were analyzed obtaining an average of 24844 OTU, clustered into 197 unique taxa, and taxonomically annotated using Silva database (Pruesse et al., 2007). The vegan package in R (version 3.0.2) was used to calculate diversity indexes such as Shannon, Pielou (diversity

function) and Bray-Curtis dissimilarity indexes (vegdist function). Each set of reads was normalized to the minimum sequencing depth using phyloseq package.

3. Results and discussion

3.1. Start-up of thermophilic denitrification

The mesophilic and thermophilic SBR showed a similar start-up in terms of specific nitrogen removal activity (Figure 5.1, phase I). As the inoculum was derived from a mesophilic N/DN plant (26°C), this suggests direct adaptation to the higher mesophilic (34°C) but, more importantly also to the thermophilic temperatures (55°C). In contrast to the study of Laurino and Siñeriz (1991), in which specific thermal mud was used for the start-up of a thermophilic denitrifying reactor via 15 days of batch mode operation, this study showed that a more efficient start-up could be reached via continuous operation, even without specific thermophilic inocula. The continuous start-up probably facilitated the enrichment of thermophilic bacteria by preventing the accumulation of decay- and by-products while making space for 'new' organisms.

Parallel with the start-up of the reactors, anoxic batch tests with the inoculum were performed at 34°C and 55°C in order to clarify the temperature shock impact on the denitrifying activity. Although no difference could be observed in the nitrate consumption at 34°C and 55°C in the batch tests, a significant nitrite build-up occurred at 55°C and resulted in a 54% lower total specific nitrogen removal (Figure 5.2). Similarly, during the start-up of the reactors, the thermophilic SBR showed nitrite build-up while the mesophilic SBR did not (Figure 5.1). After 13 days start-up however, this nitrite build-up decreased and disappeared. Interestingly, while the direct activity measurements of the anoxic batch tests showed a 54% lower total specific nitrogen removal at 55°C compared to 34°C, after 72 hours, the specific nitrogen removal at 55°C was 52% higher than at 34°C. At 55°C, the decay and lysis of bacteria probably predominated in the direct activity measurements resulting in a lower nitrogen removal. Indeed, the VSS content clearly decreased over time both in the batch tests (Figure 5.2) as well as in the thermophilic SBR (Table 5.2). This was also observed during the start-up

of a thermophilic aerobic wastewater treatment process inoculated with mesophilic sludge, initially measuring an increase of COD as a result of cell lysis, where COD removal was only observed after some hours (Suvilampi and Rintala, 2003).

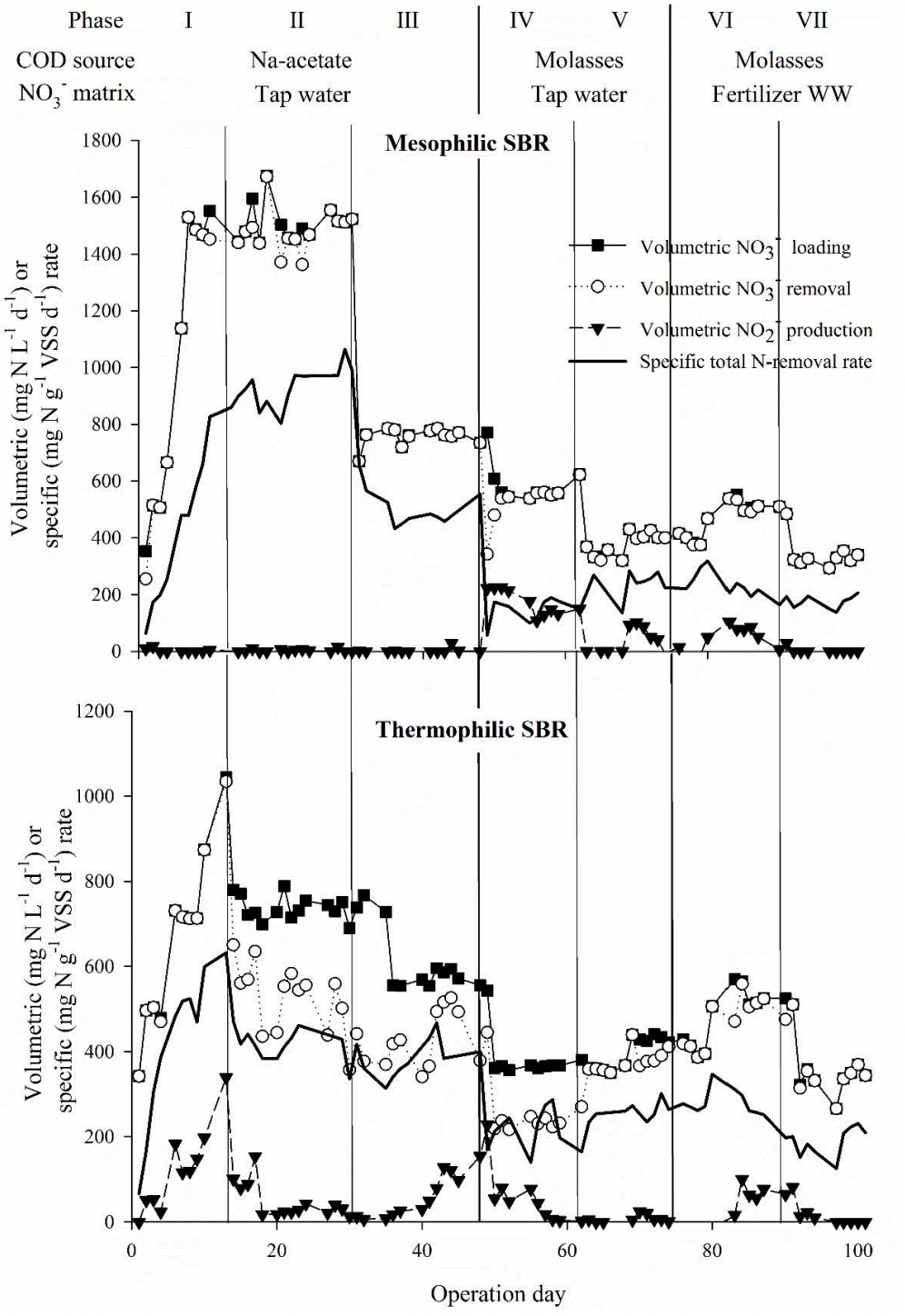

Figure 5.1 Reactor performance of the mesophilic (top) and thermophilic (bottom) denitrifying SBR along the different feeding periods represented by the volumetric NO_3^- loading/removal and NO_2^- production (mg N L^{-1} d^{-1}) and total specific nitrogen removal rates (mg N g^{-1} VSS d^{-1}).

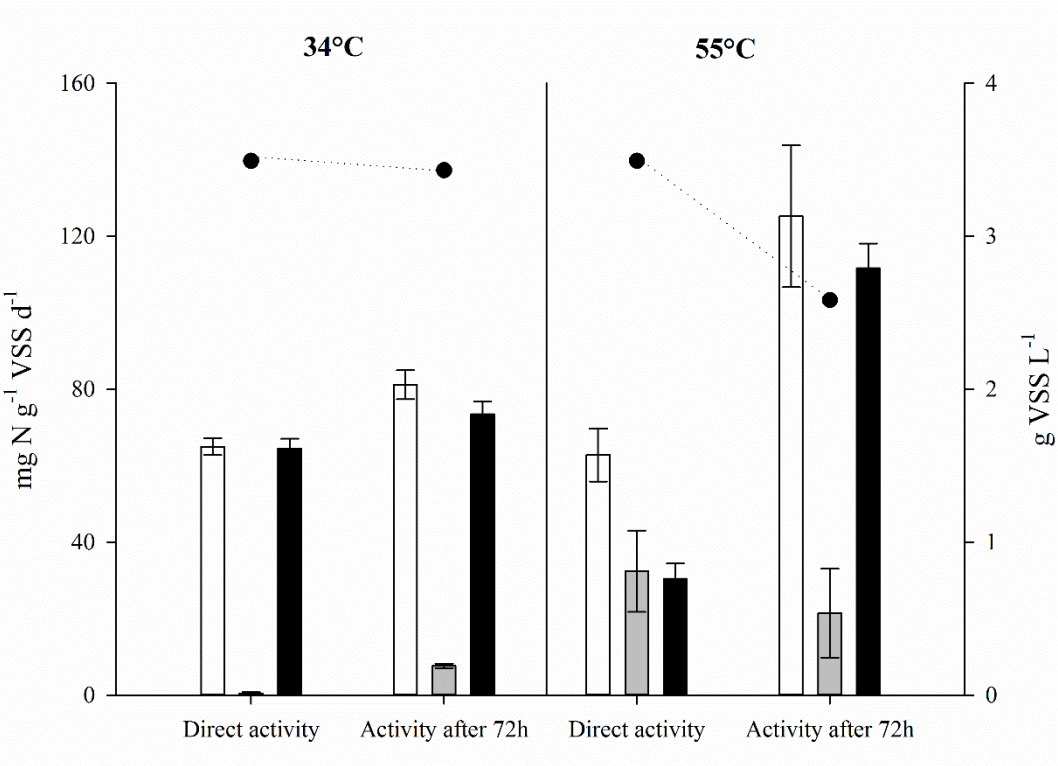

Figure 5.2 Effect of a temperature shock on the denitrifying activity of the mesophilic inoculum (26°C), immediately after heating and after 72 hours at the shocked temperature. Nitrate removal (white), nitrite production (grey), total nitrogen removal (black) and VSS concentration (black dots)

Table 5.2 Reactor performance parameters for the mesophilic and thermophilic SBR during the three different feeding periods; n(Synthetic)=46, n(Real WS/Synthetic)=26, n(Real WS)=23.

	Feeding period	**Mesophilic SBR**	**Thermophilic SBR**
pH	Synthetic	8.9 ± 0.1	8.7 ± 0.2
	Real WS/Synthetic	7.6 ± 0.5	7.9 ± 0.3
	Real WS	7.9 ± 0.1	8.1 ± 0.1
Removed COD/N (g COD g^{-1} N)	Synthetic	3.6 ± 0.3	2.8 ± 0.3
	Real WS/Synthetic	4.5 ± 0.8	4.5 ± 1.1
	Real WS	5.0 ± 0.6	4.9 ± 1.1
$VSS_{sludge}/TSS_{sludge}$	Synthetic	0.68 ± 0.06	Decreasing from 0.72 to 0.28
	Real WS/Synthetic	0.84 ± 0.04	0.29 ± 0.06
	Real WS	0.87 ± 0.04	0.46 ± 0.07
Mean floc size (µm)	Inoculum	63	63
	Real WS/Synthetic	242	72
	Real WS	381	87

Similarly, Lopez-Vazquez et al. (2013) examined the immediate denitrification activity of industrial N/DN sludge (34°C) at different temperatures (35-55°C). Curiously, denitrification activity could only be detected up to 50°C, while at 55°C no significant activity was measured. It is unclear whether this observation suggests that not all mesophilic inocula could be used for direct start-up of thermophilic denitrifying reactors above 50°C or, as these batch activity tests were performed on a short term (hours), whether the decay and lysis of the bacteria possibly predominated in this stage resulting in no net nitrogen removal.

3.2. Denitrification performance

After the start-up, in phase II of the reactor experiment, the loading was kept high in order to determine the maximum specific nitrogen removal rates (Figure 5.1, phase II). The mesophilic sludge reached about twice the specific nitrogen removal rates of the thermophilic sludge, i.e. 922 ± 21 and 435 ± 21 mg N g^{-1} VSS d^{-1}, respectively. Nevertheless, the observed specific thermophilic rates were about a factor 8.5 higher than the rates described by Laurino and Siñeriz (1991) who reached about 51 mg N g^{-1} VS d^{-1} in a UASB reactor at 55°C. The large difference in specific removal rate between these two thermophilic reactors can potentially be attributed to the reactor configuration, resulting in different sludge aggregation states and sludge densities. UASB reactors are known to form dense granular sludge with lower specific activities compared with floccular sludge (Jin et al., 2012). As the thermophilic SBR in this study had a relatively low biomass content (1.2 ± 0.5 g VSS L^{-1}) compared with the UASB reactor described by Laurino and Siñeriz (1991) (25.8 g VS L^{-1}), the volumetric nitrogen removal rates were lower reaching 496 ± 100 mg N L^{-1} d^{-1} (SBR) versus 855 ± 215 mg N L^{-1} d^{-1} (UASB). Typical specific mesophilic denitrification rates range from about 50 to 1000 mg N g^{-1} VSS d^{-1} (Henze et al., 2008). The obtained thermophilic rates in this study were in the range of typical mesophilic denitrification whereas the observed maximal mesophilic nitrogen removal rates could be considered as relatively high. These high removal rates led to extensive N_2 production, which resulted in floating sludge, and were consequently not feasible for practical SBR operation. Therefore, from phase III on, the nitrate loading of the mesophilic SBR was decreased to the loading of the thermophilic SBR (Table 5.1), facilitating the settling and, as such, a comparison between the two systems was possible (see subsection 3.3).

The synthetic feeding period consisting of tap water provided with $NaNO_3$ and NaAc was followed by a semi-synthetic feeding period where the COD source was replaced by diluted molasses (Table 5.1, phase IV). Molasses mainly consist of sugars with sucrose as the main constituent accounting for 32.5% (m/m) (Hamlin et al., 2008). Both reactors suffered from this abrupt change in COD source as an immediate decrease in total nitrogen removal of 89% and 59% was observed for the mesophilic and thermophilic SBR, respectively (Figure 5.1, phase IV). As several studies already showed that molasses can be used as a good carbon source for denitrification (Ueda et al., 2006; Hamlin et al., 2008) and highly diluted (500x) molasses were used in this study, it is unlikely that some inhibitory compounds in the complex molasses broth could have been the cause of the nitrite accumulation. Partial inhibition of the denitritation resulting in nitrite accumulation can also be a result of high oxygen levels or increased pH (Glass and Silverstein, 1998; Oh and Silverstein, 1999). The DO level in both reactors was always < 0.05 mg O_2 L^{-1} and the pH actually decreased (± pH 8.8 to ± pH 7.8) after the COD change (Table 5.2). This indicates that the change in COD source itself caused the nitrite accumulation.

The higher resilience of the thermophilic denitrifying sludge towards changes in COD source was also reflected in the results of the anoxic batch activity tests (Figure 5.3), performed during the last feeding period (Real WS, phase IV). The denitrifying activity of the mesophilic and thermophilic sludge acclimated to molasses was determined for both molasses as well as for acetate. Although no substantial difference between acetate and molasses could be observed for the thermophilic sludge, the total nitrogen removal of the mesophilic sludge with acetate as COD source was about 7.6 times lower than with molasses (Figure 5.3). Similarly, Cherchi et al. (2009) showed that acetate-acclimated mesophilic biomass could use only acetate efficiently, while marginal denitrification rates were obtained with other carbon sources such as glucose and methanol. The disability of specific carbon-acclimated biomass to instantly use other carbon sources can be linked to a highly specialized, non-diverse community. The higher microbial diversity/evenness in the thermophilic denitrifying sludge at the end of the synthetic feeding period with acetate (see subsection 3.4) could thus potentially explain the higher resilience to the abrupt COD change.

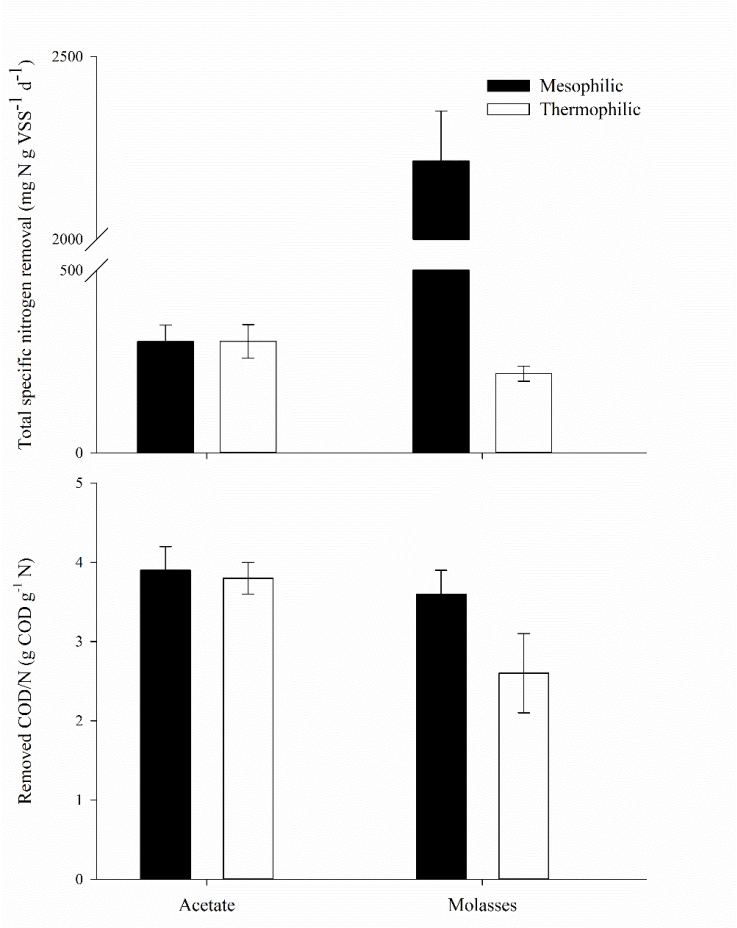

Figure 5.3 Effect of carbon source (acetate vs. molasses) on the denitrifying activity of mesophilic (34°C) and thermophilic (55°C) sludge acclimated to molasses and fertilizer wastewater (phase VI) presented by the total specific nitrogen removal rates (top) and the removed COD/N (bottom).

Beside this higher resilience of the thermophilic SBR towards changes in COD source, also a lower COD requirement, and thus higher denitrification efficiency was observed. During the synthetic feeding period with acetate (phases I-III) a ratio of 3.6 ± 0.3 g $COD_{removed}$/g $N_{removed}$ was measured in the mesophilic SBR while only a ratio of 2.8 ± 0.3 was observed in the thermophilic SBR (Table 5.2). The influence of temperature on the consumed COD/N ratio was already observed in a lower temperature range where at 10°C and 25°C, a ratio of about 6 and 4 was measured, respectively (Peng et al., 2007). As stated by Peng et al. (2007), a high degree of endogenous respiration, typical for thermophilic conditions, results in a considerably lower apparent growth yield and a thereby lower consumed COD/N ratio. Indeed, the thermophilic sludge showed a lower sludge production (See subsection 3.3, Figure 5.4). Additionally, the lower consumed COD/N ratio can also be attributed to more internally available COD as a

result of a higher cell decay/lysis at elevated temperatures. After the COD change however, no differences in removed COD/N ratio could be detected anymore (Table 5.2). In this phase, the effect of carbon source possibly dominated over the temperature effect. Once acclimated to the molasses, the anoxic batch activity tests show that the thermophilic sludge again had a lower removed COD/N ratio (Figure 5.3).

In the third phase, also the NO_3^- matrix was changed from tap water to NO_3^- rich industrial WW from fertilizer industry (phase VI, 287 ± 4 mg NO_3^- L^{-1}), possibly containing additional inhibitory compounds. Similar with the change in COD source in phase IV, a transient nitrite build-up was observed in both reactors (Figure 5.1). As nitrite build-up is a known factor triggering N_2O emissions in nitrification but also in denitrification reactors (Kampschreur et al., 2009), N_2O emission was investigated in parallel batch tests during phase VI. High nitrite concentrations namely lead to a lower denitrification rate and accumulation of NO and N_2O (Schulthess et al., 1995). Although a nitrite concentration of 21 ± 5 and 27 ± 6 mg NO_2^--N L^{-1} was measured in the mesophilic and thermophilic microcosms in this study, negligible N_2O emission (≤ 0.05% N of reduced NO_3^--N) was measured in all treatments. Finally, both reactors were able to treat the industrial wastewater using the by-product from sugar refinery (molasses) as carbon source (Figure 5.1, phase VI and VII).

3.3. Sludge characteristics

Different sludge characteristics of the mesophilic and thermophilic denitrifying biomass were monitored over the three feeding periods. The particle size of the mesophilic sludge flocs was clearly higher than the thermophilic sludge flocs reaching a volume mean diameter of 381 µm compared to 87 µm (Table 5.2, Real WS). This trend was also observed in a study that compared mesophilic (20-35°C) and thermophilic (55°C) activated sludge processes (Suvilampi et al., 2005). The floc sizes in our study were completely in the range of the size distribution of the cited study where 150-500 µm flocs were dominant in the mesophilic activated sludge, whereas 50-150 µm flocs were dominant in the thermophilic activated sludge. The higher complexity of the influent matrix clearly increased the particle size of the mesophilic denitrifying sludge. It was shown that activated sludge fed with glucose produces more extracellular polymeric substances (EPS) than sludge that was fed on acetate (Li and Yang,

2007). As EPS are known to determine floc structure/formation and molasses mainly consist of sugars, this can eventually explain the observed increase in mesophilic floc size with a change in COD composition. However, for the thermophilic denitrifying sludge, only a slight increase could be observed along the different feeding periods (Table 5.2). This could possibly be justified by the fact that the properties of specific EPS functional groups rather than the quantity of bound EPS determine the difference in bioflocculation behavior between thermophilic and mesophilic sludge (Liao et al., 2011).

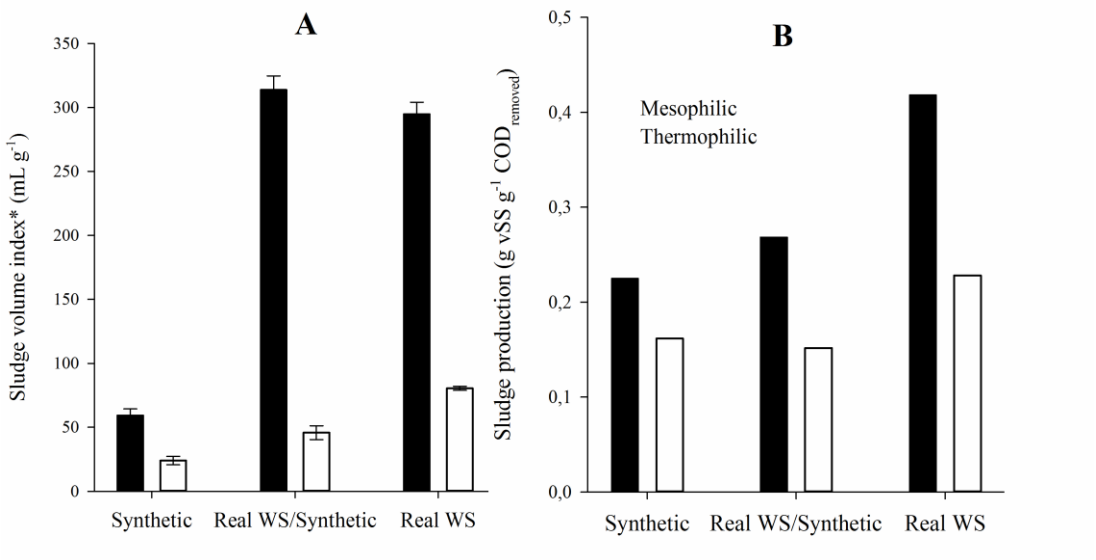

Figure 5.4 Impact of mesophilic (34°C) vs. thermophilic (55°C) temperature on sludge characteristics. **(**A) Sludge volume index* (n=3) and (B) sludge production for the mesophilic and thermophilic denitrifying biomass for the different feeding periods.

Although the thermophilic denitrifying sludge consisted of much smaller particles, it showed better settling properties reaching a 3.6 times lower sludge volume index (SVI*) in the last phase (Figure 5.4 **A**, Real WS). This is surprising as thermophilic aerobic processes are generally known to suffer from poor settling properties as a result of poor floc formation under thermophilic conditions (Suvilampi and Rintala, 2003). The better settling of the thermophilic sludge could have been the result of a higher inorganic content (54% versus 13% in the thermophilic and mesophilic sludge, respectively, Table 5.2) and hence a higher specific density. Indeed, several studies already showed a good inverse correlation between both VSS/TSS ratio and density and VSS/TSS ratio and settling velocity (Schuler and Jang, 2007; Vlyssides et al., 2008). This can lead to implementation of shorter settling times and thus higher volumetric loadings in SBR or smaller settlers in continuous systems. This high inorganic

content was also observed in the thermophilic granular denitrifying sludge described by Laurino and Siñeriz (1991) accounting for 43%. Beside the large effect of temperature on SVI*, the settleability of both sludge types clearly declined with increasing complexity of the influent matrix (Figure 5.4 **A**). Similarly, Li and Yang (2007) demonstrated that acetate-fed activated sludge had more EPS and consequently performed better in terms of bioflocculation and sludge sedimentation than glucose-fed sludge.

Thermophiles have faster growth rates than mesophiles, however, due to even higher maintenance energy and decay rates, this results in lower net microbial growth (Lapara and Alleman, 1999). The thermophilic SBR showed about 50% less sludge production in the last phases (Figure 5.4 **B**, real WS), thereby strongly reducing operational costs. The sludge production values in this study (Figure 5.4 **B**) were in the range of reported sludge production values under thermophilic aerobic conditions ranging between 0.05 and 0.3 kg SS kg^{-1} $COD_{removed}$ (Suvilampi and Rintala, 2003). Similar with the SVI* measurements, sludge production increased with increasing complexity of the influent matrix (Figure 5.4 **B**). This was also the case in the study conducted by Hamlin et al. (2008) who compared sludge production for different carbon sources. In the cited study, the order of sludge production from lowest to highest appeared to be methanol < acetic acid < starch < molasses.

3.4. Diversity, evenness and dynamics of the microbial community

Phylogenetic studies comparing mesophilic and thermophilic aerobic wastewater treatment systems demonstrated that elevated reactor temperatures resulted in a lower microbial diversity and more selective microbial communities limited to a narrow range of COD types (Tripathi and Allen, 1999; LaPara et al., 2000). In this study however, illumina sequencing indicated that the diversity of the thermophilic DN sludge was nearly always comparable or even higher than the mesophilic DN sludge (Figure 5.5 **A**). Species richness were comparable in both sludge types ranging between 91-112 species and 100-112 species for the mesophilic and thermophilic sludge, respectively. Nevertheless, the microbial community evenness showed the same trend as the microbial diversity (Figure 5.5 **A**). Remarkably, at the end of the synthetic period, both the microbial diversity and evenness were about 2.3 times higher in the thermophilic sludge compared with the mesophilic sludge (Figure 5.5 **A**). As discussed above,

this can possibly explain the higher resilience of the thermophilic SBR towards the abrupt change in COD source from phase IV on (Figure 5.1), confirming that the initial community evenness favours functionality under selective stress (Wittebolle et al., 2009).

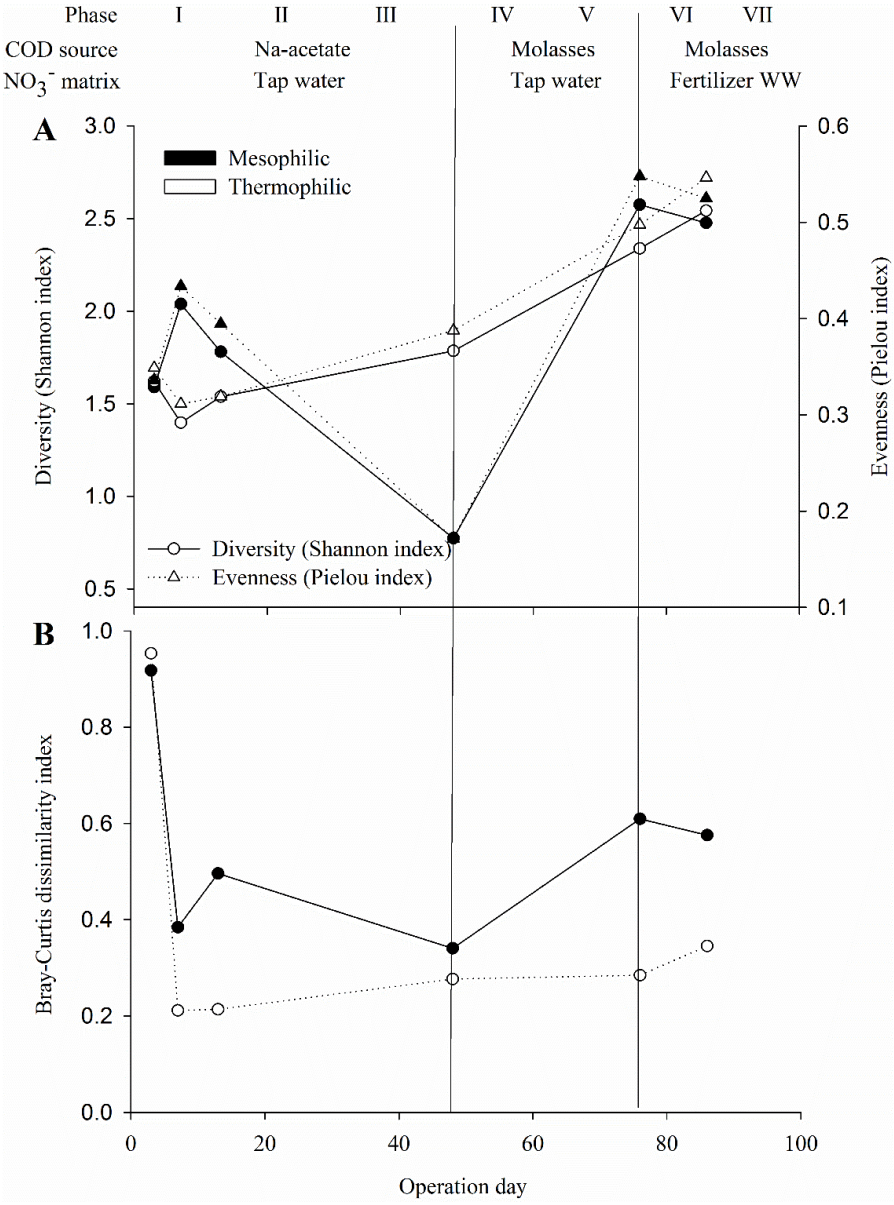

Figure 5.5 A. Diversity (Shannon index) and evenness (Pielou index) **B.** microbial community dynamics represented by the Bray-Curtis dissimilarity indexes for the mesophilic and thermophilic sludge along the operational periods. The Bray-Curtis dissimilarity indexes were calculated compared with the previous sampling point (first sample compared with inoculum).

Beside a higher (or equally) diverse microbial community, the thermophilic sludge also showed a much more stable microbial community over the different feeding periods. The Bray-Curtis dissimilarity indexes (BC), a statistic used to qualify the compositional dissimilarity between two different samples (0< BC< 1), where 0 means the two samples have the same

composition (sharing all the species), while 1 means the two sites do not share any species, of the thermophilic sludge was namely always lower than the mesophilic sludge (Figure 5.5 **B**). As each sample is compared to the previous sample in time, this shows that the thermophilic sludge composition is less dynamic and thus more stable than the mesophilic sludge. In particular for the transition from acetate to molasses, a large difference in dynamics was observed, resulting in a Bray-Curtis dissimilarity index of 0.61 and 0.28 for the mesophilic and thermophilic sludge, respectively. Furthermore, the Bray-Curtis dissimilarity indexes of the thermophilic sludge are completely in the range of normal temporal variations (13-41%) observed in steady-state activated sludge lab reactors (Hai et al., 2014) indicating that a quit stable microbial community was reached in all the feeding periods. The higher microbial community stability is possibly due to a lower competition at extreme conditions as a result of less competitors, because less micro-organisms are able to grow. However, no difference in species richness was observed in this study, rejecting this hypothesis. Dion (2008) stated it differently: "*Competition between individuals may play a larger role in nonextreme environments, whereas environmental pressures would be determinant under extreme conditions.*" The lower competition at extreme conditions would thus not result from lower microbes able to grow, but to the higher environmental pressure.

Although the most abundant genera for both the mesophilic and thermophilic denitrifying sludge remained present after switching from acetate to molasses as carbon source (Figure 5.6), some new genera were detected. This indicates that adaptation of denitrifying communities to changes in carbon sources may involve both the regulation of enzyme expression in existing populations and the enrichment of new populations. Both the mesophilic and thermophilic community strongly increased in diversity and evenness with increased COD complexity (Figure 5.5 **A**). Similarly, Lee and Welander (1996) observed that crude syrup (90% sucrose), compared with acetic acid, selected for a more heterogeneous and metabolically versatile microbiota. The change in NO_3^--matrix from tap water to fertilizer WW further increased the diversity and evenness of the thermophilic sludge, while it slightly decreased in the mesophilic sludge (Figure 5.5 **A**).

Overall, during the real WS feeding period the mesophilic sludge was dominated by *Bacteriodetes* (54%) consisting of 36% *Porphyromonadaceae* (*Paludibacter*) and 18%

Saprospiraceae (Figure 5.6). It has to be noted that the results are based on read abundance and can differ from the actual abundance as different bacterial groups can have different 16S rRNA gene copy numbers (Fogel et al., 1999). Nevertheless, these results offer a good estimation of the dominant bacterial groups. Although the thermophilic sludge obtained by Laurino and Siñeriz (1991) was almost exclusively composed of bacteria belonging to the *Firmicutes (Bacillus)*, only 14% of the thermophilic sludge in this study consisted of these spore-forming bacteria. Other than *Firmicutes* (14%), the most abundant phyla were *Deinococcus-Thermus* (30%, *Thermus*), *Proteobacteria* (24%, *Hydrogenophilus*) and *Bacteroidetes* (9%, *Bacteriodes*) *(*Figure 5.6)**.**

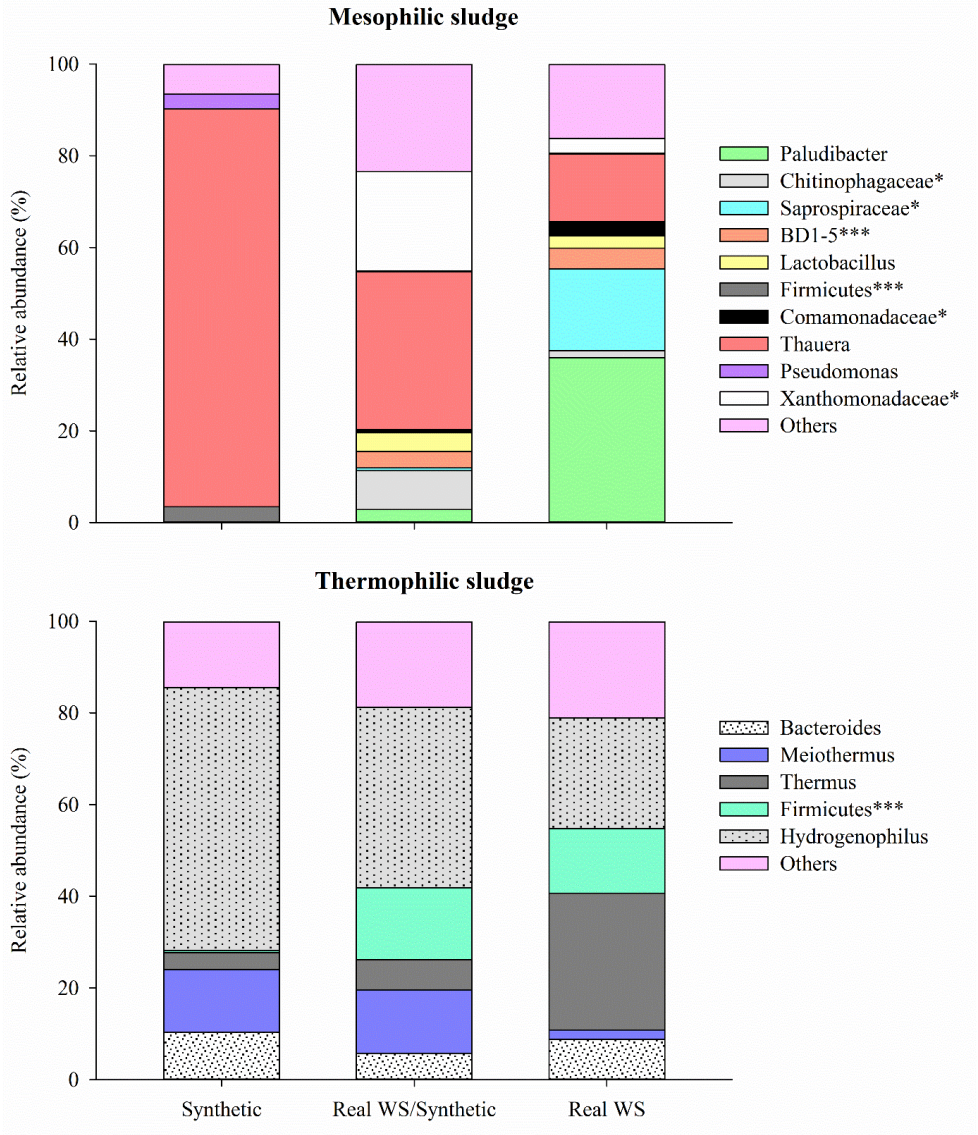

Figure 5.6 Distribution of the most abundant genera (*family, **order or ***phylum) in the mesophilic and thermophilic denitrifying sludge at the end of each feeding phase (based on read abundance, taxa representing more than 3% of the communities).

4. Conclusions

- Denitrifying activity of mesophilic sludge (26°C) was maintained at 55°C resulting in a start-up of a thermophilic denitrifying SBR in less than one week, hereby providing the potential for a rapid conversion of existing mesophilic systems with cooling to thermophilic systems
- The thermophilic denitrification showed a 73% lower sludge volume index so that shorter settling times and thus higher volumetric loadings in SBR or smaller settlers in continuous systems can be implemented.
- Other than the elimination of cooling, thermophilic denitrification could lower operational costs as a 45% lower sludge production was observed compared with mesophilic denitrification.
- Both the mesophilic (34°C) and thermophilic (55°C) SBR were able to treat nitrate-rich industrial wastewater using a by-product from sugar refinery (molasses) as carbon source, whereby the thermophilic sludge showed a higher resilience towards a change in COD type from acetate to molasses.
- The microbial diversity and evenness of the thermophilic denitrifying sludge was always comparable or even higher than the mesophilic sludge and moreover, showed a more stable community over time.

5. Acknowledgments

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CHAPTER 6:

GENERAL DISCUSSION

In this doctoral work, lab-scale reactor experiments based on synthetic feed demonstrated that thermophilic nitrogen removal through nitrification and denitrification is biotechnologically feasible. The (initial) oxidation of ammonium to nitrate, nitrification, clearly seemed to be the limiting step for the development of thermophilic nitrogen removal and was therefore extensively studied in 3 chapters (Chapters 2-4). The interdisciplinary approach linking thermophilic N-cycling microbiology and engineering resulted in the successful operation of a thermophilic nitrifying bioreactor. To the best of the author's knowledge, this has never been described before and could moreover be achieved through two fundamentally different strategies (Chapter 3 & 4). In this general discussion, the different investigated strategies will be compared and evaluated from a biotechnological but also from an ecological perspective, in order to enlarge the understanding of the adaptation-selection process. The main identified microbial players will therefore be analysed in depth, especially in relation with the niche differentiation for thermophilic nitrogen removal. Finally, the potential for practical implementation was examined through a detailed cost calculation and discussion of the future challenges.

1. Essential ingredients for successful thermophilic nitrification

1.1. Selective pressure

As cited by the Dutch botanist and microbiologist Baas Becking in 1934 '**Everything is everywhere, but, the environment selects**', any selective pressure (within the biochemical limits of life) should result in the development of a stable microbial community. In relation with biotechnology and especially practical implementation or retrofitting, one may want this selection process as efficient as possible. An overview of the 5 investigated temperature selection pressure strategies to achieve thermophilic nitrification and their results are presented in Table 6.1. The two fundamentally different selection categories, one imposing a gradual increasing temperature selection pressure (Chapter 2 & 3) while the other an instant temperature selection (Chapter 4), could both successfully achieve thermophilic nitrification (± 50°C). From a practical point of view, however, the gradual temperature increase (Chapter 3) seems the best option as higher volumetric rates were achieved in half the time (150 versus more than 300 days).

Table 6.1 Overview of the different investigated temperature increase strategies applied to achieve thermophilic nitrification in this thesis. The main results concerning reactor performance and microbial community are presented at the highest temperature where complete and stable nitrification was achieved. Ch.: Chapter, A: amplitude, Os: oscillating, vol.: volumetric.

Ch	T pattern	Slope (°C d^{-1})	Tmax (°C)	Δt (d)	Synthetic Influent (mg N L^{-1})	Nitrification rate		Nitrogen oxidizing community			
						Vol. (mg N L^{-1} d^{-1})	Specific (mg N g^{-1} VSS d^{-1})	AOB	AOA	*Nitrobacter*	*Nitrospira*
2	Step	2.5	42.5	90	20-50	184 ± 9	184	+	-	-	+
			*47.5	*180	*0-50/100	*366 ± 9	*302				
3	Os	0.25 (A: 2°C)	42	35	10-250	26 ± 5	72	+	+	+	+
3	Linear	0.25	42	35	10-250	90 ± 3	139	+	+	+	+
3	Linear	0.08	49	150	400-1000	794 ± 57	155	+ *Nitrosomonas* genus	+++ *Nitrosophaera* genus	+	++
4	Instant	-	50	>300	10-140	229 ± 13	198	-	+++ *Nitrosophaera gargensis*	-	+++ *Nitrospira calida*

*different value for nitrite oxidation as nitrite was separately dosed in this case.

Within all the experiments imposing a gradual temperature increase in this thesis and literature, a clear negative correlation is observed between the maximum nitrification temperature and the applied slope of temperature increase (Table 6.1). In other words, the smaller the temperature increase per day, the higher the nitrification temperatures that were achieved. This suggests that the transition from mesophilic to thermophilic nitrification only succeeds if enough time is provided. Although different regulatory adaptations can help microbes to survive and grow at elevated temperatures (Chapter 1), the transition process thus seemed to be a rather slow process, indicating that selection, rather than regulatory adaptation, occurred**.** When transferring a microbial community to a new environment, or a certain stress is imposed, there might be a direct response through regulatory adaptation, however with a limit in the level of fitness (Ryall et al., 2012) . On a longer term, increased fitness is achieved by selection, such as mutational adaptations on a population level as presented in Figure 6.1 or by replacement/out-competition of certain dominant species by others on a community level.

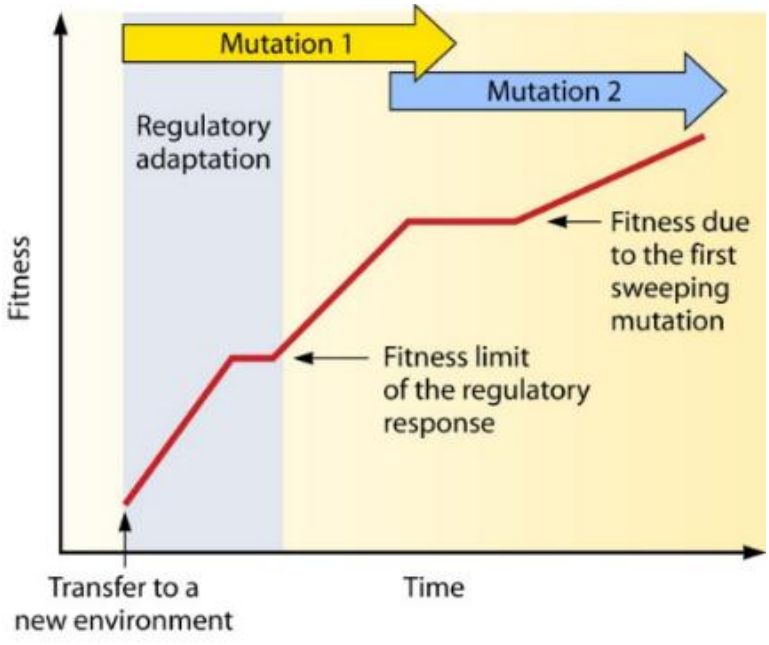

Figure 6.1 Fitness increase through regulation and mutational processes in a population, relative to the pre-stressed state, upon a transition to a new environment (Ryall et al., 2012).

It is very complex to exactly determine which adaptation/selection processes occurred within the sludge, i.e. a mixed microbial community. Further research with pure cultures or synthetic microbial communities and the use of meta-/transcript-genomics techniques could possibly elucidate that. In Chapter 4, most likely a selection on community level occurred, as a high temperature was directly imposed. Yet, the gradual temperature increase in Chapter 3 probably allowed that also selection on a population level (i.e. within a certain species) and regulatory adaptations took place. Although these processes could have helped out the transition process from mesophilic to thermophilic nitrification in this case, after all, the clear shift observed with qPCR from AOB to AOA again indicates towards a selection on community level. As the selection on community level seemed to be dominant in the two successful strategies, it is clear that the initial microbial community, i.e. the inoculum, plays a big role in the efficiency of the selection process and will be discussed in the next section.

1.2. Main microbial players

The two successful strategies started with a different inoculum and a different selection procedure was applied. However in both cases, a very similar nitrogen oxidizing community was achieved, consisting of *Nitrososphaera*-related AOA and *Nitrospira*-related NOB (Table 6.1). The imposed final reactor conditions, achieved by different manners, thus selected for very specific micro-organisms.

1.2.1. Inoculum and influent

Although upon Baas Becking '**everything is everywhere**', logically, the efficiency of the selection process is correlated with the initial presence/abundance of those main microbial players in the initial microbial community, i.e. inoculum. The mesophilic nitrifying sludge inoculum in Chapter 3 contained a relatively high abundance of archaeal *amoA* and 16S *Nitrospira copies*, 1 x 10^9 and 3 x 10^{11} copies g^{-1} DW , respectively. This is in accordance with the fact that *Nitrospira* is the most prevailing NOB genus in wastewater treatment plants (WWTP) (Daims et al., 2006) and that AOA appear to be distributed in WWTP worldwide (Limpiyakorn et al., 2013). In contrast, in natural thermophilic environments where thermophilic nitrifiers are known to be present, their abundance is generally quite low (e.g. 10^2 – 10^4 copies g^{-1} hydrothermal sediment (Wang et al., 2009a)). In the semi-engineered

compost system, serving as an inoculum in Chapter 4, the archaeal *amoA* abundance is typically fluctuating in course of the composting process, ranging from 0 to 10^7copies g^{-1} DW compost (Yamamoto et al., 2010). Regrettably, NOB in the composting process have not been characterized well (Maeda et al., 2011). The time variation in which both thermophilic nitrification systems were achieved in this PhD thesis (Table 6.1) can thus probably, beside the difference in selection pressure and selection processes occurring, partly be explained by the difference in initial abundance of AOA and *Nitrospira* species.

Although mainly synthetic feed was used in this thesis, the microbial community of the influent can also influence the microbial community of the bioreactor. In case of warm wastewaters however, influent microbial diversity will probably be low.

1.2.2. K-strategists ?

The natural thermophilic environments in which thermophilic nitrifiers were detected are generally known to be oligotrophic. Reported ammonium concentrations in hot springs and hydrothermal plumes range from 7 to 100 µM and 0.2-3 µM, respectively, while nitrite is generally not detected (Lebedeva et al., 2005; de la Torre et al., 2008; Baker et al., 2012). It is thus logic that AOA and *Nitrospira*, known as K-strategists characterized by a high substrate affinity and a low growth rate (Ward et al., 2011), are mainly retrieved in these environments (Chapter 1, Table 1.4).

In contrast with the thermophilic oligotrophic natural environments, feeding operation in this thesis always ensured non-limiting ammonium conditions. In fact, although the feeding operation was equal over time, the real substrate for ammonium oxidation, ammonia (NH_3), increased with increasing temperature (equilibrium $NH_4^+ \leftrightarrow NH_3 + H^+$). Curiously, also here a selection was made towards these K-strategist with high substrate affinity, although both r-strategist AOB and *Nitrobacter* were initially significantly present in the inoculum (Chapter 3). Moreover, as decay rates increase with increasing temperature, one could expect a selection for fast growing micro-organisms in order to compensate for the high decay. Microbial diversity in general is known to decrease over 40°C (Sharp et al., 2014), suggesting that in accordance with the essence of the r/K selection theory, in which organisms strive to maximize their fitness for survival in either uncrowded (r) or crowded (K) environments, elevated

temperatures would make room for r-strategists (Andrews and Harris, 1986). These contradictory aspects suggest that the general accepted r/K theory may not be the appropriate ecological explanation for the specific niche differentiation of AOA and *Nitrospira* in thermophilic environments. Nevertheless, the conception of the ecological strategy has previously been invoked by different researchers, resulting in a third less-known type of ecological strategy, the L-strategy. Literally stated, this strategy is implemented by micro-organisms that are well adapted to the adverse/extreme environment or in other words, micro-organisms that can maintain their high population densities under unfavorable conditions (Golovlev, 2001). Typical examples of L-strategists are spore-forming bacteria or non-spore-forming bacteria that may occur in the so-called viable but nonculturable state (VBNC).

AOA versus AOB

First characterized archaea thrived in high-saline, high-temperature, acid and strict anoxia conditions and were therefore originally labeled as extremophiles (Woese et al., 1978). The subsequent discovery that archaea are abundant throughout world's oceans and many other non-extreme environments in the biosphere however rejected this ecological perspective (Delong, 1992; Chaban et al., 2006). The unifying ecological principle accepted today is that adaptation to **chronic energy stress** is the crucial factor that distinguishes the archaea from the bacteria (Valentine, 2007) and explains the out competition of AOB by AOA in this thesis. An overview of the cellular energy losses is presented in Figure 6.2. At elevated temperatures, micro-organisms face two main energetic challenges: high rates of biochemical breakdown but more importantly, an increased membrane permeability. The high permeability of ions (especially protons) menaces the proton motive force (PMF) and thus energy transduction in the cytoplasmic membrane. Maximum growth temperature of micro-organisms thus mainly depend on the degree of homeoproton permeability adaptation, i.e. the limit of ion permeability (van de Vossenberg et al., 1995). The primary biochemical basis for this adaptation is the membrane composition. The typical ether lipids in archaea (Table 1.3, Chapter 1) strongly reduce the ion permeability due to a higher stability (restriction in hydrocarbon chain mobility) and a lower membrane dipole potential compared with the ester bound fatty acid chains in bacteria. Furthermore, it was recently shown that AOA use the most

energy efficient aerobic pathway for carbon dioxide fixation, offering advantage to AOA in this high energy-requiring thermophilic environment (Könneke et al., 2014).

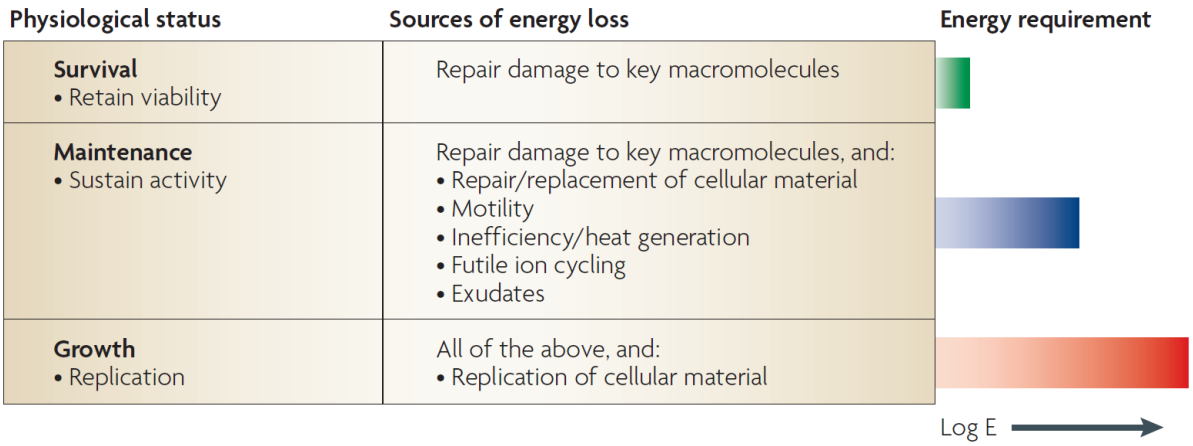

Figure 6.2 The energy requirements for conditions of survival, maintenance and growth. (Valentine, 2007)

Nitrospira versus *Nitrobacter*

The energetic challenges described above apply for any microbial cell. Besides AOA for ammonium oxidation, it is arguable that nitrite oxidizing archaea (NOA) may also be present in the environment and be selectively enriched at elevated temperatures. However, until now, no NOA were discovered yet, although different researchers cite the possibility (Ward et al., 2007; You et al., 2009). The upper temperature limit for known nitrite oxidizers is lower than for the isolated ammonium oxidizers. A maximum temperature of 60-65°C was reported for *Nitrospira*, while the AOA *"Candidatus* Nitrosocaldus yellowstonii*"* grows up to 74°C (Chapter 1). As furthermore nitrite is generally not accumulating in natural environments, some unknown micro-organisms may fill this gap unless nitrite is chemically oxidized (Udert et al., 2005). Besides NOA, other unknown bacterial lineages could also be capable to oxidize nitrite at elevated temperatures. Recently, a new member of the phylum Chloroflexi was described with nitrite oxidizing activity up to 63°C (Sorokin et al., 2012).

The permeability of the ester bound bacterial cell membranes can be adjusted to a certain extent by the nature of the fatty acids, partly explaining the out competition of *Nitrobacter* by *Nitrospira* in this thesis (Chapter 3). Based on literature, a clear difference in degree of saturation can namely be observed between both genera. The fatty acid profile of *Nitrobacter*

is characterized by the unsaturated vaccenic acid as the main compound (C18:1 cis 11, 80-92%), while the saturated palmitic acid (C16:0) is the dominant fatty acid (50%) in enrichment cultures of *Nitrospira* growing at 42-47°C (Lipski et al., 2001).

Another difference between both genera that can, beside the membrane permeability, possibly explain the selection of *Nitrospira* at elevated temperatures is the location of the nitrite oxidoreductase enzyme (NXR). The cytoplasmic NXR of *Nitrobacter* depends on nitrate/nitrite transport across the cytoplasmic membrane (Ward et al., 2011). In contrast, the Nitrospira NXR is located in the periplasm, eliminating the need of energy-requiring nitrate/nitrite transport and hereby offering advantage for *Nitrospira* concerning the high energetic challenges at elevated temperatures.

1.2.3. Satellite community

The nitrification reactors in this thesis were operated with ammoniacal synthetic wastewater, without the presence of organic carbon. However, in successful thermophilic reactor in Chapter 4, the core autotrophic nitrifying community only comprised 25-40% of the total microbial community, even after two years of selective enrichment. Illumina sequencing revealed that the large satellite community mainly consisted of heterotrophs. Indeed, it is known that autotrophic nitrifiers reduce inorganic carbon to form biomass and excrete organic carbon (i.e. soluble microbial product, SMP) that supports heterotrophic growth (Ni et al., 2011). Yet, the fraction of heterotrophic growth in the autotrophic nitrification reactors of this work are remarkably higher (60-75%) than observations reported in literature. Heterotrophic fractions of 15%, 30% and 30-62% were reported for nitrifying suspended sludge, granules and biofilm, respectively (Ni et al., 2011; Gilbert et al., 2014a). The specificities of the interactions of the heterotrophs with the nitrifiers are largely unknown and especially in the thermophilic case, the 'chicken-egg' paradox appears. Is the increased heterotrophic fraction a consequence of the thermophilic operation resulting in higher decay rates and thus more SMP, or can thermophilic nitrification only be achieved with the help of this large heterotrophic community? Heterotophs namely produce organic compounds that can stimulate nitrifiers (Rittmann et al., 1994). Especially the results in Chapter 3, in which a very small non-nitrifying bacterial fraction was observed in the 'early-crashing' MBBR (5%) in contrast with the successful SBR (75%), particularly suggest that the heterotrophic community

plays an essential role in the development of thermophilic nitrification. More insights into this fundamental matter would allow the development of indirect control strategies to ensure the stability of the sensible nitrifying community.

1.3. Culture history

Beside the genotype, and thus the presence of the main microbial players in the inoculum, the immediate culture history of a population/community is an inevitable determinant of the overall effect of an environmental transition (Ryall et al., 2012). Factors such as growth rate, growth phase, culture density and growth state namely have a major influence on microbial gene expression/regulation and thus the immediate regulatory adaptation. This was discussed in Chapter 4 where, beside the fact that the MBBR contained a lower initial abundance of AOA in the biofilm compared with the SBR, the different response could also be influenced by the different growth state (suspended vs biofilm) of the micro-organisms of concern.

Another aspect of 'microbial history' that affects the regulatory response is prior exposure to a stress. This was clearly demonstrated in Chapter 2 in which an initial salt stress increased the tolerance towards a subsequent temperature shock resulting in more efficient temperature transitions and higher nitrification temperatures. Preliminary tests showed that the reverse phenomenon could eventually also be applied i.e. using temperature shocks to improve the tolerance of nitrifying sludge towards higher salinities. Overnight incubation of nitrifying sludge at 40°C abolished the subsequent inhibitory effect of 10 g NaCl L^{-1} on ammonium oxidation at a subsequent incubation at 30°C (Figure 6.3). Further research is needed to find the appropriate incubation time and temperature to enlarge this effect towards higher salinities and eventually also nitrite oxidation. If so, this temperature-induced halophilic nitrogen removal can offer a solution for industries coping with highly saline waste streams (e.g. concentrated urine, mustard tuber wastewater) or wastewater with high fluctuations in salinity (e.g. fish canning industry).

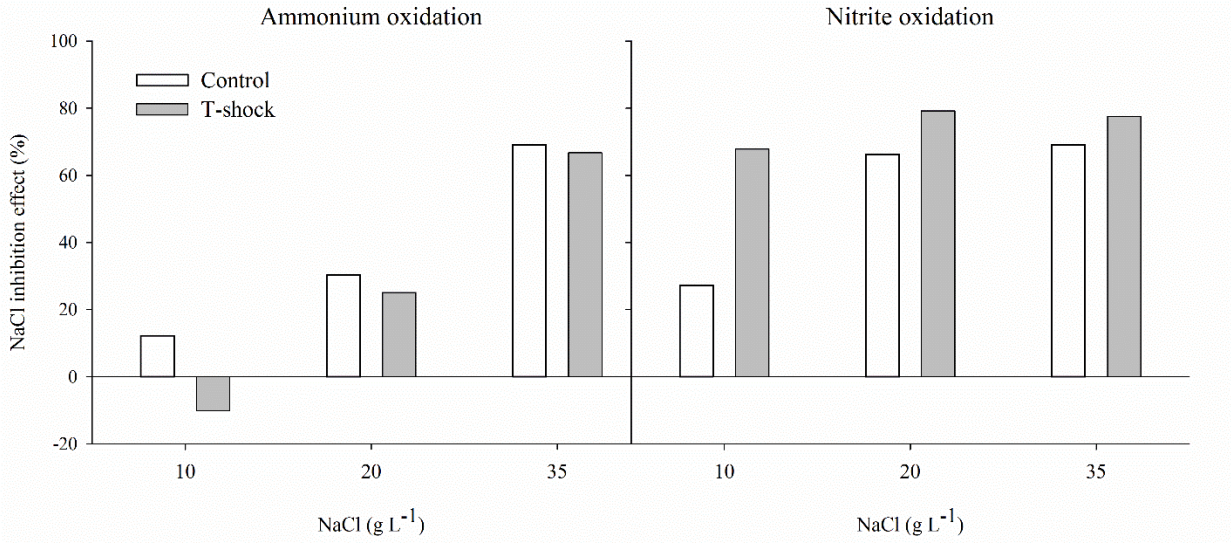

Figure 6.3 Salt inhibition effect on ammonium (left) and nitrite (right) oxidation at 30°C after a prior overnight incubation without salt addition at 40°C.

2. Thermophilic denitrification

In contrast with nitrification, thermophilic denitrification was achieved without any issues. The big difference in efficiency to achieve the thermophilic conditions can basically be explained by two main factors.

From a thermodynamic point of view, it is clear that heterotrophic denitrification yields much **more energy** than autotrophic nitrification. More Gibbs free energy is available through the denitrification reactions (Chapter 1) and less Gibbs energy is required for biomass formation from organic carbon compared to carbon dioxide (Heijnen et al., 2009). As a consequence, shorter doubling times and higher sludge yields are obtained resulting in more mutations per unit of time and thus a faster selection on population level. The higher growth rates would also allow a more rapid replacement of dominant species by other and thus an accelerated selection on community level. Finally, beside the increased growth rates, the selection on a community level is also facilitated by the **large diversity** among denitrifiers and the fact that heterotrophic bacteria are predominant in wastewater treatment plants.

3. Potential for practical implementation

This thesis delivered the proof of concept that thermophilic nitrogen removal through nitrification/denitrification is possible in lab-scale reactors based on synthetic feed. It is however crucial for further developments to determine whether this process is favorable from an economical perspective and which future challenges will have to be tackled enabling practical implementation.

3.1 Economic perspective

A detailed cost calculation was performed in accordance with Courtens et al. (2014b) focusing on the nitrogen removal step (N/DN), comparing mesophilic (30°C) versus thermophilic (50°C) treatment. The calculations were carried out based on three industrial case studies (Table 6.2) nowadays cooling their wastewater enabling conventional mesophilic treatment.

Table 6.2 Wastewater characteristics and current installation (focus on reactor basin, sludge settling/thickening and cooling device) of the three cases used in the cost calculation. AD: anaerobic digestion. MBR: membrane bioreactor. * anaerobically pretreated

Case		**1**	**2**	**3**
Industry		Manure treatment	Potato processing	Waste to energy
Wastewater				
Origin		Liquid fraction pig manure	'Frites' production *	Supernatant thermophilic AD
Temperature	°C	>40	45	45
Flowrate	$m^3\ d^{-1}$	342	1944	288
COD	$mg\ L^{-1}$	20000	1335	11000
N	$mg\ L^{-1}$	5000	249	250
COD/N		4	5.4	44
Current installation		SBR	CSTR + thickener	MBR
Reactor volume	m^3	16000	9153	2500
Settling/thickening	m^3	-	1853	-
Cooling device				
- Type		Surface aerator	Cooling tower	Cooling tower
- Motor	kW	2*15	2*30	6

Following assumptions were made:

- All **civil engineering work** was: accounted for at current prices, including installation; and calculated at the detail level of, for instance, concrete, rebar, shutters, pumps and valves for the aeration basin and sludge thickener. The costs for detailed engineering were separately added as 20% of the equipment capital expenditure (CAPEX). All capital costs were amortized at 3.5% over 10 years.
- Due to contradictory observations in this thesis compared with literature concerning specific rates of mesophilic vs. thermophilic conditions, for the economic calculations the same specific rates (and VSS concentrations) were assumed. Also equal VSS concentrations were assumed, resulting in equal **reactor volumes**. As the reduced sludge production was in line with literature, **sludge thickener and dewatering systems** were designed (55%) smaller in the thermophilic scenario based on the parameter below.
- The assimilation due to **heterotrophic growth** was calculated taken into account an observed yield factor of 0.27 and 0.12 kg COD in biomass per kg COD removed, for mesophilic and thermophilic treatment at a sludge retention time of 15 days. These yields were derived from a maximum yield factor of 0.47 kg VSS per kg COD with a decay coefficient of 0.096 d^{-1} (30°C) at mesophilic temperatures (van Haandel and van der Lubbe, 2007), and a maximum yield factor of 0.51 kg VSS per kg COD with a decay coefficient of 0.33 d^{-1} (50°C) for thermophilic treatment (Vogelaar et al., 2003). 1.42 g COD g^{-1} VSS was assumed. The nitrogen content of the biomass was assumed to be 5%, with a value of 1.33 g COD g^{-1} VSS this results in a value of 0.067 g N g^{-1} $COD_{removed}$. For autotrophic conversions sludge production was neglected. The sludge dewatering centrifuge was assumed to concentrate incoming sludge to 20% DW (0.7 kg VSS kg^{-1} TSS), implying an operational cost of 150€ ton^{-1} DW and further disposed at 320 € ton^{-1} DW resulting in a total sludge treatment and disposal cost of 470 € ton^{-1} DW (Paul et al., 2006) (Figure 6.4).
- The **oxygen demand** for nitrification/denitrification could be calculated from the stoichiometry in Chapter 1 (Table 1.1) and was 4.34 kg O_2 kg^{-1} N removed. Oxygen savings by denitrification were calculated based on nitrogen oxygen equivalents (NOE) of 2.86 O_2/NO_3. Furthermore assuming an oxygen demand for COD removal of 0.73 - 0.88 kg O_2 kg^{-1} COD removed based on the observed yield (mesophilic-thermophilic), an actual electrical oxygen transfer efficiency of 1.5 kg O_2 kWh^{-1} and an electricity price of 0.1 € kWh^{-}

[1] (van Haandel and van der Lubbe, 2007), the resulting operational costs (OPEX) for aeration were calculated (Figure 6.4).

- **Mixing and pumping** energy cost were considered equal in both scenarios and calculated based on the assumption that about 30% of the total electrical consumption is for non-aeration purposes (Zessner et al., 2010).
- The **cost for cooling** was calculated based on the installed power of the cooling device assuming operation at 80% capacity.
- **Effluent norms** were set on 25 mg COD L^{-1} and 20 mg N L^{-1}.

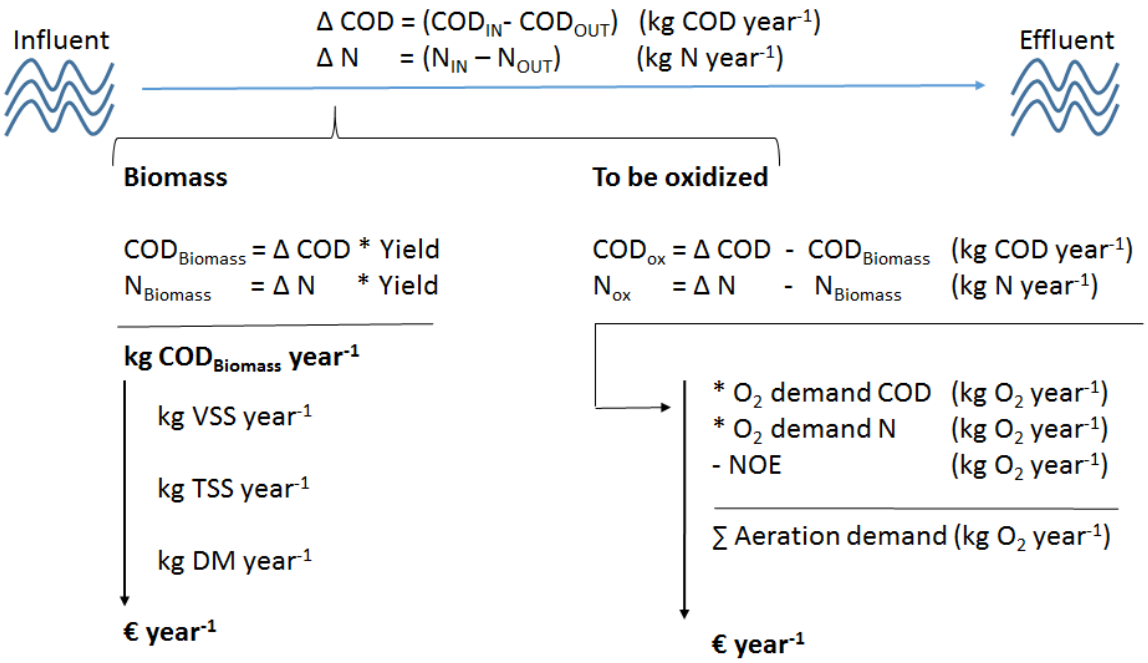

Figure 6.4 Illustration of OPEX calculation for sludge production and aeration demand.

Table 6.3 Capital and operational costs (CAPEX and OPEX) for nitrogen removal from three types of industrial wastewaters through mesophilic and thermophilic nitrification/denitrification. TM: treatment, DP: disposal. The 'Δ' column represents the relative advantage of thermophilic versus mesophilic treatment for the corresponding section (%). *newly built

	1 – Manure treatment					**2 – Potato processing**					**3 – Waste to energy**				
	Mesophilic		Thermophilic		Δ	Mesophilic		Thermophilic		Δ	Mesophilic		Thermophilic		Δ
	10^3 €	10^3 € y^{-1}	10^3 €	10^3 € y^{-1}	%	10^3 €	10^3 € y^{-1}	10^3 €	10^3 € y^{-1}	%	10^3 €	10^3 € y^{-1}	10^3 €	10^3 € y^{-1}	%
CAPEX															
Reactor basin*	4 129		4 129			2 363		2 363			664		664		
Settler & thickener	-		-			1 280		577		55	-		-		
Centrifuge (dewatering)	1 168		521		55	431		193		55	538		240		55
Cooling system	17		-		100	136		-		100	13		-		100
Carbon dosage system	29		-		100	-		-			-		-		
Electric installation	70		70			70		70			70		70		
Control & automation	59		59			59		59			59		59		
Detailed engineering	1 094		956			868		652			269		207		
Sum CAPEX	**6 563**	**789**	**5 735**	**690**	**13**	**5 206**	**574**	**3 914**	**447**	**22**	**1 613**	**194**	**1 240**	**149**	**23**
OPEX															
Cooling wastewater		13		-	100		39		-	100		4		-	100
Aeration		169		212	-25		51		67	-31		42		61	-48
Mixing/pumping		73		73			22		22			18		18	
Carbon addition		11		-	100		-		-			-		-	
Sludge TM & DP		345		154	55		128		57	55		159		71	55
Sum OPEX		**611**		**439**	**28**		**240**		**146**	**39**		**222**		**150**	**32**
CAPEX + OPEX		**1 400**		**1 128**	**19**		**814**		**593**	**27**		**416**		**299**	**28**

Although the development of thermophilic nitrogen removal in this thesis initially emerged from the idea to treat warm wastewaters without cooling requirements, the economic benefit of eliminating the cooling step seem marginal. It can save no more than 3% on capital expenditures and 2% on operational costs, except for the potato processing plant (case 2) where high flowrates have to be cooled and operational savings get up to 16% (Table 6.3). Cooling of warm wastewaters with heat exchangers and cooling towers is thus theoretically feasible. The cost calculation however shows that implementation of thermophilic nitrogen treatment can potentially involve total costs savings of 19 to 28% (CAPEX + OPEX), as highlighted in green in Table 6.3.

The major share in the economical savings (70-99%) is related with the reduced sludge production at elevated temperatures. Although thermophiles have faster growth rates than mesophiles, a lower net microbial growth is observed due to even higher maintenance energy and decay rates (Chapter 5, (Lapara and Alleman, 1999)). This is directly translated in a reduction of the sludge disposal costs (OPEX) but also implies that smaller settlers/thickeners can be build resulting in a reduction of capital costs, but also a more efficient utilization of valuable industrial surface space. A lower observed sludge yield and thus a lower flow of carbon to sludge however means that more carbon has to be oxidized to carbon dioxide resulting in a higher cost for aeration (25-48%), especially for streams with high COD/N ratios (case 3). Nonetheless, in all cases, the cost savings from reduced sludge production greatly compensate the increase need of aeration. It has to be noted that the imposed SRT also has an influence on the observed sludge yield, as presented in the equation below, and thus the respective savings.

$$Y_{observed} = \frac{Y_{max}}{(1 + SRT \times decay\ coefficient)}$$

As thermophiles also have higher decay coefficients, imposing higher SRT could enlarge the benefits of thermophilic treatment even more. The costs for addition of an extra carbon source to achieve full denitrification in waste streams with low COD/N ratios as in case 1 could moreover be lowered/avoided as thermophilic denitrification showed a lower COD requirement compared with mesophilic denitrification (Chapter 5).

Overall, thermophilic nitrogen removal can treat a wide range of nitrogen-rich waste streams whereas the exploitable advantages not only depend on the kind of waste stream/loading, but also on the degree of dependence on additional processing units such as for hygienisation, digestion, and rest heat recovery. As the main economic benefit of thermophilic nitrogen removal seems related with the reduced sludge production, the opportunities are not only applicable to warm wastewaters. Many industries have an excess of rest heat on-site that could easily be recovered to warm up a mesophilic waste stream to temperatures above 45°C. Indeed, the capital cost for a plate heat exchanger would not be higher than 0.4% of the total CAPEX as the prices, depending on the flowrate, range from 4 000 (cases 1 & 3) to 14 000 € (case 2) (E-Ster Bvba). An overview of possible application domains and their heat source/pattern are presented in Table 6.4.

Table 6.4 Overview of possible application domains divided based on the heat source origin and pattern.

Heat source	Temperature profile	Examples
Water	Fixed	Thermophilic digestate; Industrial wastewater (e.g. steel industry, fertilizer industry)
	Varying	Domestic wastewater (seasonal e.g. Saudi Arabia); Industrial wastewater (batch units, shutdowns/start-ups)
Metabolic/ operational	Fixed	Autothermal thermophilic aerobic digestion (ATAD)
	Varying	Industrial wastewater (e.g. manure)
External	Fixed	Any other streams with rest heat available

3.2 Future challenges

This thesis delivered the proof of concept that thermophilic nitrogen removal through nitrification/denitrification is possible in lab-scale reactors based on synthetic feed. First of all, the robustness of this process should be proven on a long term and real wastestream, especially in terms of fluctuations of influent wastewater flow and content. Furthermore, in order to complete the curve of the known 'text book' Figure 1.4 (page 14) and allow modelling of nitrification at elevated temperatures, determination of kinetic parameters is needed.

3.2.1. Carbon

Thermophilic nitrification and denitrification were studied separately in this thesis. As both processes are usually separated in space in conventional systems (Chapter 1), one would theoretically not expect too much troubles to link both processes. The feasibility of thermophilic nitrification was however demonstrated based on ammoniacal synthetic wastewater, though, without any carbon source (Chapters 2-4). Depending on the COD/N ratio, the presence of carbon can influence the **competition between autotrophs and heterotrophs** and thus possibly endanger the thermophilic nitrification performance. Aerobic heterotrophs namely compete with nitrifiers for oxygen and space in the sludge floc/biofilm. The COD/N ratio affects this competition, in which an increasing ratio implies a decreasing nitrifying activity as a result of increasing heterotrophic activity as shown for mesophilic conditions in Figure 6.5. Although the reduced nitrifying activity at elevated COD/N is generally not a problem to maintain nitrification at mesophilic temperatures, especially in the thermophilic case where a big heterotrophic fraction was observed in the reactors fed without carbon, actual thermophilic growth kinetics for both aerobic/anoxic heterotrophs and nitrifiers should be determined in order to evaluated whether the presence of carbon could form a problem.

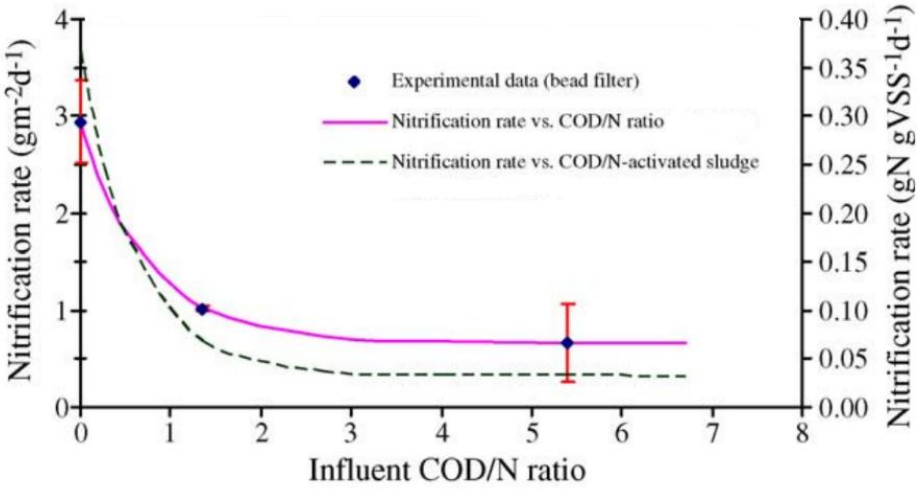

Figure 6.5 Relationship between nitrification rate and influent COD/N ratio (Adapted from Chen et al. (2006))

Increasing activity of thermophilic aerobic heterotrophic bacteria due to elevated carbon content will not only affect the autotroph-heterotroph competition, but could also deteriorate

sludge characteristics. Although both nitrification and denitrification experiments in this thesis showed an enhanced settling behavior with increasing temperatures (Chapter 4 – 5), the common opinion is that thermophilic aerobic carbon processes suffer from poor sludge settling (Suvilampi and Rintala, 2003).

3.2.2. Oxygen

Increasing temperatures result in a lower oxygen solubility: the oxygen saturation concentration (C_S) at 25°C is 8.3 mg O_2 L^{-1}, while 5.6 mg O_2 L^{-1} at 50°C. This leads to a smaller driving force (C_S - C) and hence to a lower oxygen transfer rate (OTR).

$$OTR = K_{LA} \times (C_S - C)$$

At the other hand, the diffusion rate of oxygen increases with increasing temperatures, while the liquid viscosity and surface tension decrease, hereby increasing the oxygen transfer coefficient (K_{LA}). Overall, Vogelaar et al. (2000) showed that the decreasing C_S was completely offset by the increased K_{LA} within the temperature range of 20-55°C, suggesting that no big troubles concerning aeration can be expected at thermophilic temperatures.

The membranes of the conventional fine bubble membrane diffuser systems have maximum operating temperatures. In case of thermophilic aeration other, more expensive, membranes will have to be used. However, other alternative exist such as fine bubble ABS tube diffusers with a tolerance up to 100°C, stainless steel coarse bubble diffusers or surface aerators.

3.2.3. Short-cut nitrogen removal

The cost calculation clearly showed that implementation of thermophilic nitrification/denitrification could economically make sense. More cost savings could however be achieved with the development of thermophilic short-cut nitrogen removal processes such as nitritation/denitritation and partial nitritation/anammox. Beside a reduction in the aeration demand, these processes also reduce the carbon requirements (Chapter 1). The cost associated with chemical purchase and the associated biomass production is hereby lowered, but also additional influent carbon could become available for gas production and energy generation through anaerobic digestion, important from a resource recovery perspective.

A crucial factor to enable the development of short-cut nitrogen processes is the selective out-competition of NOB. This thesis shows potential for selective NOB inhibition as thermophilic NOB not only seem more sensitive for FA than thermophilic AOA, but also show an inhibition for FNA, while AOA are insensitive (Chapter 3). The SBR in Chapter 4 confirmed this difference in FA sensitivity, but also showed that NOB surrender before the AOA during the gradual imposed temperature increase easily resulting in partial nitritation. Although different studies showed the occurrence of anammox in several natural thermophilic environments (Chapter 1), the enrichment of thermophilic anammox bacteria will be the biggest challenge. The low growth rate of the anammox bacteria will even lower at thermophilic conditions, requiring a lot of patience and perseverance for enrichment.

4. Conclusions

Biological nitrogen removal (BNR) is a well-established technology within the wastewater treatment industry. Although thermophilic nitrogen cycling microorganisms were already enriched/detected in multiple thermophilic environments, all full- and lab-scale realizations are however mesophilic. The unexplored transposition capability of thermophilic nitrogen converting microbes into useful, biotechnological communities was explored in this thesis.

The interdisciplinary approach linking thermophilic N-cycling microbiology and engineering resulted in the successful operation of a thermophilic (50°C) nitrifying bioreactor with comparable specific rates with literature. This was never described before and could moreover be achieved through two fundamentally different strategies. On the one hand, a thermophilic nitrification reactor at 49°C was achieved by subjecting an established mesophilic nitrifying community to a slow (0.08°C d^{-1}), non-oscillating linear temperature increase (Chapter 3). It was furthermore shown that salt amendment can be used as a tool for a more efficient temperature transitions of mesophilic sludge (Chapter 2). On the other hand, a thermophilic nitrification reactor at 50°C was achieved through selective enrichment from compost samples (Chapter 4). Both strategies showed the importance of ammonium oxidizing archaea (AOA) and *Nitrospira* as key players for succesfull thermophilic nitrification. Finally, also the biotechnological potential of thermophilic denitrification was demonstrated as a thermophilic SBR could immediately be started-up with mesophilic activated sludge (26 °C) and showed,

despite the lower specific rates, a 73% lower sludge volume index, a 45% lower sludge production and a higher resilience towards a change in carbon source compared with the mesophilic SBR.

Overall this PhD research expanded the microbial ecology knowledge of thermophilic nitrogen cycling microbes from natural thermophilic environments to engineered systems. For the first time, coupled thermophilic ammonia- and nitrite-oxidizers were enriched, moreover from a nutrient-rich inoculum, and resulted in a thermophilic nitrification bioreactor opening opportunities for thermophilic nitrogen removal biotechnology. Regarding to practice, this means that warm wastewaters could eventually treated without cooling. Significant total cost savings (19-28%) could be reached compared with mesophilic treatment so that even mesophilic waters could profit from the savings in case rest heat is available.

Abstract

Nitrogen is a major wastewater component in our global society, and its treatment prevents environmental deterioration. Biological nitrogen removing (BNR) biotechnology is mostly the rational solution, with thousands of full-scale realizations established, which are however all mesophilic. In contrast, for carbon treatment, thermophilic biotechnology has been established for over 30 years. Experiences with thermophilic activated sludge for instance pointed out several advantages**,** such as a higher stability, higher rates, a lower sludge production and a better hygienization.

Thermophilic nitrogen cycling microorganisms such as ammonium oxidizing archaea (AOA), nitrite oxidizing bacteria (NOB) and even anammox bacteria were separately isolated/enriched or detected in multiple thermophilic environments. At the beginning of this research these microbes represented a hidden treasure of natural resources, with an unexplored transposition capability into useful, biotechnological communities. The goal of this research was therefore to develop thermophilic biological nitrogen removal processes.

The initial oxidation of ammonium to nitrate, nitrification, clearly seemed to be the limiting step for the development of thermophilic nitrogen removal and was therefore extensively studied in 3 chapters (**Chapters 2, 3 and 4**). The interdisciplinary approach linking thermophilic N-cycling microbiology and engineering resulted in the successful operation of a thermophilic nitrifying bioreactor. This was never described before and could moreover be achieved through two fundamentally different strategies (**Chapter 3 and 4**).

The **first strategy** involved the transition of an established mesophilic nitrifying community to elevated temperatures (**Chapter 2 and 3**). A first research chapter (**Chapter 2)** demonstrated that salt amendment can be used as a tool for a more efficient temperature transitions and could eventually reach higher nitrification temperatures. Batch activity tests showed an increased activity of aerobic ammonium oxidizing bacteria (AOB) of 20-21% at 40 and 45°C by the addition of 5 g NaCl L^{-1}. For NOB, the activity remained unaltered at 40°C, yet decreased by 83% at 45°C. In a subsequent long-term continuous reactor test, temperature was increased from 34 to 40, 42.5, 45, 47.5 and 50°C. The AOB activity showed 65 and 37% higher

immediate resistance in the salt reactor (7.5 g NaCl L^{-1}) for the first two temperature transitions, and lost activity from 45°C onwards. NOB activity, in contrast to the batch tests, was 37 and 21% more resistance in the salt reactor for the first two transitions. Despite the beneficial effect of salt addition, overall, no ammonium oxidation above 42.5°C was achieved with this step-wise temperature increase pattern. Therefore, in the second research chapter **(Chapter 3),** the adaptive capacities of mesophilic nitrifying sludge were evaluated for linear temperature increase patterns (non-oscillating vs. oscillating and slopes of 0.25 vs. 0.08 °C d^{-1}). Furthermore, the effect of sludge growth mode (suspended vs. attached growth) was investigated by comparing a sequencing batch reactor (SBR) with a moving bed biofilm reactor (MBBR)**.** The oscillating temperature pattern (0.25 °C d^{-1}) and the moving bed biofilm reactor (0.08 °C d^{-1}) could not reach nitrification at temperatures higher than 46°C. However, nitrification rates up to 800 mg N L^{-1} d^{-1} and 150 mg N g^{-1} VSS d^{-1} were achieved at a temperature as high as 49°C by imposing the slowest linear temperature increase to suspended sludge. Microbial community analysis revealed that this successful transition related with the dominance shift of AOA above AOB, while *Nitrospira* dominance over *Nitrobacter* was constant. This observation was accompanied with an increase in sludge yield and a shift in maximal optimum temperature, determined with ex-situ temperature sensitivity measurements, predicting an upcoming reactor failure at higher temperature. Overall, the results of this research chapter suggest that existing mesophilic nitrifying wastewater treatment plants can be upgraded to thermophilic systems through a slow, non-oscillating linear temperature increase, steered by ex-situ temperature sensitivity measurements.

The **second strategy** used in this thesis to develop thermophilic nitrification is the selective enrichment of thermophilic nitrifiers from natural thermophilic environments. In **Chapter 4**, samples from composting facilities were used as inoculum for the batch-wise enrichment of thermophilic nitrifying communities. Subsequently, the enrichments were transferred to a bioreactor fed with synthetic influent to obtain a stable, high-rate nitrifying process. The community contained up to 17% AOA closely related to "*Candidatus* Nitrososphaera gargensis", and 25% NOB related to *Nitrospira calida*. Incorporation of ^{13}C-derived bicarbonate into the respective characteristic membrane lipids during nitrification supported their activity as autotrophs. Specific activities up to 198 ± 10 and 894 ± 81 mg N g^{-1} VSS d^{-1} for

AOA and NOB were measured, and interesting difference in substrate/product inhibitions were observed that open a way for short-cut nitrogen removal processes.

The potential of thermophilic denitrification (55°C) was explored in the last research chapter (**Chapter 5**), in which it was extensively compared to mesophilic denitrification (34°C). Remarkably, the thermophilic SBR could immediately be started up with mesophilic activated sludge (26 °C), obtaining nitrogen removal rates higher than 500 mg N g^{-1} VSS d^{-1} in less than one week. Although the parallel mesophilic SBR showed twice as high specific nitrogen removal rates, the maximum thermophilic denitrifying activity in this study was nearly 10 times higher than the activities reported thus far. The thermophilic SBR moreover had a 73% lower sludge volume index, a 45% lower sludge production and a higher resilience towards a change in carbon source compared with the mesophilic SBR. The higher resilience was potentially related to a higher microbial diversity and evenness of the thermophilic community. Overall, this **Chapter 5** showed the capability of mesophilic denitrifiers to maintain their activity after a large temperature increase suggesting that existing mesophilic process systems could thus efficiently be converted to thermophilic systems.

Finally, a detailed cost calculation in the general discussion (**Chapter 6**) revealed that, although the development of thermophilic BNR in this thesis initially emerged from the idea to treat warm wastewaters without cooling requirements, the economic benefit of eliminating the cooling step seem marginal. Thermophilic versus mesophilic nitrogen removal treatment could save up to 19 to 28% of the total costs, in which the major share in the economical savings is related with the reduced sludge production at elevated temperatures.

Generally, this work showed that both thermophilic nitrification and denitrification are biotechnologically possible. The upgrade of existing wastewater treatment plants to thermophilic BNR could be possible, hereby implying significant economic advantages. The effect of carbon on thermophilic nitrification and the coupling of both processes should however further be investigated to allow full-scale application.

Samenvatting

Reactieve stikstofcomponenten zijn belangrijke afvalwatercomponenten die, indien geloosd in het milieu, aanleiding kunnen geven tot ernstige globale ecologische problemen. In de meeste gevallen is biologische stikstofverwijdering via de conventionele nitrificatie/ denitrificatie de meest rationele oplossing. Hoewel dit een goed bestudeerd proces is, met duizenden volle schaal installaties, blijven toepassingen boven de 40°C een grote uitdaging. Nochtans zou de implementatie van thermofiele biologische stikstofverwijdering door het elimineren van de koelingsbehoefte van belang kunnen zijn voor de behandeling van warme afvalwateren. Studies met betrekking tot thermofiele koolstofverwijdering toonden bovendien reeds aan dat aerobe thermofiele processen ook stabieler zijn, hogere omzettingssnelheden bereiken zodat kleinere reactoren kunnen worden gebruikt, minder slib produceren en een hogere graad van hygiënisatie bereiken.

Thermofiele stikstof omzettende micro-organismen zoals ammonium oxiderende archaea (AOA) en nitriet oxiderende bacteriën (NOB) werden tot nog toe al gedetecteerd of aangerijkt uit natuurlijke thermofiele omgevingen, maar hun potentieel voor toepassing in afvalwaterbehandeling werd nog niet onderzocht. Aan de start van deze thesis vormden deze thermofiele stikstofcycli microben dus een verborgen schat aan natuurlijke hulpbronnen, met een onontgonnen vermogen in nuttige, biotechnologische consortia. Het doel van deze thesis was daarom de ontwikkeling van thermofiele biologische stikstofverwijdering processen.

De initiële oxidatie van ammonium tot nitraat of nitrificatie bleek snel de limiterende stap te zijn voor de ontwikkeling van thermofiele stikstofverwijdering. Dit werd daarom uitgebreid onderzocht in drie hoofstukken (**Hoofdstuk 2, 3 en 4)**. De interdisciplinaire microbiologische/ingenieurs-benadering bracht een thermofiele nitrificatie bioreactor tot stand. Dit werd nog nooit beschreven en kon bovendien worden bewerkstelligd via twee fundamenteel verschillende strategieën.

De **eerste strategie** omvat de transitie van een gevestigde mesofiele stikstofverwijderende gemeenschap naar thermofiele temperaturen (**Hoofdstuk 2 en 3**). **Hoofdstuk 2** toonde aan dat zoutstress de thermo-elasticiteit van mesofiel nitrificerend slib verbetert. Een

kortstondige zout toevoeging kan gebruikt worden als een middel om efficiëntere temperatuurtransities en eventueel hogeren temperaturen te bereiken. In batch experimenten werd als gevolg van een 5 g L^{-1} NaCl zoutstress een 20-21% hogere activiteit van ammonium oxiderende bacteriën (AOB) waargenomen bij 40°C en 45°C. Dit fenomeen werd bevestigd in een continu reactor experiment waarbij een controle reactor werd vergeleken met een zout reactor (7.5 g L^{-1} NaCl) tijdens een stapsgewijze temperatuursverhoging van 34°C tot 50°C. De AOB in de zout reactor vertoonde een 65 en 37% hogere weerstand voor de eerste twee temperatuur stappen van 34°C naar 40°C en 42.5°C. De NOB, dat in de batchtesten negatief beïnvloed werden door het zout, vertoonden in de zout reactor wel een verhoogde weerstand van 37 en 21% voor dezelfde temperatuur sprongen en bereikte temperaturen tot 47.5°C. Niettegenstaande het positief effect van het zout, was 42.5°C de hoogste temperatuur met volledige nitrificatie via deze stapsgewijze temperatuur opdrijving. Daarom werden, in **Hoofdstuk 3**, de adaptieve capaciteiten van mesofiel nitrificerend slib geëvalueerd voor een lineaire temperatuur opdrijving waarbij het effect van temperatuur-oscillatie, temperatuur helling (0.25 vs. 0.08 °C d^{-1}) en slib groei modus (gesuspendeerd vs. biofilm) werd onderzocht. Tijdens de oscillerende temperatuur opdrijving (0.25°C d^{-1}) en de biofilm gebaseerde reactor werd geen nitrificatie activiteit gemeten boven de 46°C. Nitrificatie snelheden tot 800 mg N L^{-1} d^{-1} en 150 mg N g^{-1} VSS d^{-1} werden echter bereikt op 49°C in de reactor met gesuspendeerd slib waarbij een lineaire helling van 0.08 °C d^{-1} werd opgelegd. Microbiële gemeenschaps-analyse toonde aan dat deze succesvolle transitie gelinkt was met een dominantie shift van AOA over AOB. Parallelle ex-situ temperatuur gevoeligheidstesten voorspelden bovendien elk reactor falen. De resultaten van dit hoofdstuk suggereren dat bestaande mesofiele nitrificerende systemen kunnen omgezet worden naar thermofiele systemen via een trage, niet-oscillerende lineaire temperatuur opdrijving waarbij de transitie kan worden gestuurd via ex-situ temperatuur gevoeligheidstesten.

De **tweede strategie** in dit doctoraatsonderzoek om thermofiele nitrificatie te bewerkstelligen was de selectieve aanrijking van thermofiele nitrificeerders uit natuurlijke thermofiele omgevingen. In **Hoofdstuk 4** dienden stalen van aërobe compost faciliteiten als inoculum voor de bathgewijze aanrijking van thermofiele nitrificerende gemeenschappen (50°C). Vervolgens werden de aanrijkingen overgebracht in een sequentiële bacth reactor (SBR) gevoed met

synthetisch N-houdend influent, waarbij een stabiel systeem werd bereikt met hoge nitrificatie snelheden. De thermofiele nitrificerende gemeenschap bestond uit 17% ongecultiveerde AOA gerelateerd met "Candidatus Nitrososphaera gargensis" (99%) en 25% NOB gerelateerd met *Nitrospira calida (98%)*. Incorporatie van ^{13}C gemerkt bicarbonaat in de respectievelijke karakteristieke membraan lipiden tijdens nitrificatie ondersteunden hun activiteit als autotrofen. De biomassa vertoonde hoge specifieke snelheden tot 198 ± 10 en 894 ± 81 mg N g^{-1} VSS dag^{-1} voor AOA en NOB waarbij interessante verschillen in substraat/product inhibities werden opgemerkt dat de weg openen naar 'short-cut' stikstofverwijderingsprocessen zoals partiële nitritatie/anammox.

Het potentieel van thermofiele denitrificatie (55°C) werd onderzocht in **Hoofdstuk 5**, waarbij het uitgebreid werd vergeleken met mesofiele denitrificatie (34°C). De thermofiele SBR kon meteen worden opgestart met mesofiel slib (26°C) en bereikte stikstofverwijderingssnelheden hoger dan 500 mg N L^{-1} d^{-1} in minder dan één week. Hoewel de mesofiele SBR dubbel zo hoge specifieke snelheden vertoonde, was de maximale thermofiele denitrificerende activiteit 10 keer hoger dan gerapporteerde snelheden. De thermofiele SBR had bovendien een 73% lagere slib volume index, produceerde 45% minder slib, en was robuuster ten op zichte van abrupte veranderingen in influent samenstelling. Moleculaire analyses toonden aan dat de thermofiele gemeenschap even divers, maar veel stabieler was, wat zijn hogere robuustheid naar veranderingen in operationele parameters kan verklaren.

Hoewel de ontwikkeling van thermofiele stikstofverwijdering initieel voortkwam uit het idee om warme afvalwateren te behandelen zonder koeling toonde de gedetailleerde kostenberekening in **Hoofdstuk 6** aan dat het economisch voordeel van het verwijderen van de koelingsbehoefte klein is. Het grootste economische voordeel is gerelateerd met de verlaagd slibproductie. Thermofiele stikstofbehandeling kan, in vergelijking met mesofiele behandeling, een totale kostenbesparing van 19 tot 28% teweegbrengen.

Samengevat toonde dit doctoraatsonderzoek aan dat zowel thermofiele nitrificatie als denitrificatie biotechnologisch mogelijk zijn. Bestaande mesofiele installaties zouden omgevormd kunnen worden tot thermofiele systemen met significante kostenbesparingen. Het effect van koolstof op thermofiele nitrificatie en de koppeling van de afzonderlijke

processen moet echter verder onderzocht worden om volle schaal toepassingen mogelijk te maken.

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CURRICULUM VITAE

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Education

2011-present Ghent University, LabMET: **PhD candidate**

- Funding: Research Foundation - Flanders
- Supervisor: Prof. dr. ir. Nico Boon and Prof. dr. ir. Siegfried Vlaeminck

2006-2011 Ghent University, Faculty of Bioscience engineering: **Master in Bioscience Engineering, option Chemistry and Bioprocess technology (MSc),** greatest distinction

- Erasmus stay of 5 months at Swedish University of Agricultural Sciences (SLU) and Uppsala Universitet, Uppsala (Sweden)
- Master thesis: 'Possibilities of the OLAND technology in gas treatment.
- Promotors: Prof. dr. ir. Willy Verstraete and Prof. dr. ir. Nico Boon

2001-2006 Koninklijk Atheneum II, Stene (Belgium): **Science-mathematics (8 hr)**

International peer reviewed publications (A1)

Courtens, E. N.P., Spieck, E., Vilchez-Vargas, R., Bodé, S., Boeckx, S., Schouten, S., Jauregui, R., Pieper, D.H., Vlaeminck, S.E. * and Boon, N. * A robust nitrifying community in a bioreactor at 50°C opens up the path for thermophilic nitrogen removal. *equally contributed. *Submitted*

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De Clippeleir, H., Vlaeminck, S.E**.,**, De Wilde F., Jordaens R., **Courtens E**. **N.P.**, Boeckx P., Verstraete W. and Boon N. Mainstream sewage treatment with partial nitritation–anammox: potential role of NO production. WEF/IWA Nutrient Removal and Recovery Conference : Trends in Resource Recovery and Use. Vancouver, 28 – 31 July 2013

De Clippeleir, H., **Courtens, E.N.P.**, Vlaeminck, S.E**.,** Boon N. & Verstraete, W. Efficient total nitrogen removal in an ammonia gas biofilter through high-rate OLAND. IWA Specialist Conference: Ecotechnologies for Wastewater Treatment. Santiago de Compostela, 25 – 27 June 2012.

Conferences and symposia

Oral presentations

Courtens, E.N.P., Boon, N., Verliefde A. & Vlaeminck, S.E. Some like it hot: technological characterization of thermophilic vs. mesophilic denitrification. IWA Specialist Conference – Global challenges: Sustainable wastewater treatment and resource recovery, Kathmandu (Nepal), 26-30 october 2014

Courtens, E.N.P., Vlaeminck, S.E., De Schryver, P. & Boon, N .Pushing the limits of mesophilic nitrification: salt shocking enhances thermotolerance. IWA Specialist Conference – Global challenges: Sustainable wastewater treatment and resource recovery, Kathmandu (Nepal), 26-30 october 2014

Courtens, E.N.P., Boon, N., Vilchez-Vargas, R. & Vlaeminck, S.E. A nitrifying community under continuous cultivation at 50°C: phylogenetic and physiological characterization. 19th European Nitrogen Cycle Meeting. Ghent, 10-12 September 2014.

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Vlaeminck, S.E.***, Courtens, E.N.P.*** & Boon, N. Denitrification is 'hot': demonstration of potential advantages of thermophilic vs. mesophilic denitrifying sludge. IWA Conference: Activated sludge – 100 years and counting, Essen,12-14 June 2014. ***equally contributed. poster price award**

Kerckhof, F.M., **Courtens, E**. **N.P.**, Geirnaert, A., Hoefman, S., Ho, A., Vilchez Vargasa, R., Pieper, D., Sandoval, R., Vlaeminck, S.E., Van de Wiele, T., Heylen, K., Vandamme, P. & Boon, N. Cryopreserving functionality and diversity of mixed microbial cultures. BCCM 30th Anniversary Meeting & BSM Annual Symposium. Brussels, 26-27 november 2013

De Clippeleir, H., Vlaeminck, S.E., **Courtens, E**. **N.P.**, Verstraete, W. & Boon, N. Oxygen-limited autotrophic nitrification/denitrification maximizes net energy gain in technology schemes with anaerobic digestion. Tweede Vlaamse Vergistings FORUM. Gent, 19 September 2012.

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Courtens, E. N.P., De Clippeleir, H., Berckmoes, K., Verstraete, W., Boon, N. & Vlaeminck, S.E. Simple strategies to control the oxygen budget of an OLAND rotating biological contactor. 17th Symposium on Applied Biological Sciences. Leuven, 10 February 2012.

Zekker, I.*, Vlaeminck, S.E.*, Bagchi, S., **Courtens, E**. **N.P.**, De Clippeleir, H., Kerckhof, F.-M. & Boon, N. Selecting nitrifying inocula on different ammonium concentrations. 17th Symposium on Applied Biological Sciences. Leuven, 10 February 2012.

Courtens, E.N.P., De Clippeleir, H., Vlaeminck, S.E. & Boon, N. OLAND als innovatieve behandeling van ammoniakhoudende gasstromen. 'ie-prijzen' Koninklijke Vlaamse Ingenieursvereniging (KVIV). Antwerpen, 1 December 2011. **Awarded with excellent poster price**

Courtens, E. **N.P**, De Clippeleir, H., Vlaeminck, S.E**.,** Boon, N. & Verstraete, W. Simple strategies to control the oxygen budget of an OLAND rotating biological contactor. First international symposium on Microbial resource management in biotechnology: Concepts & applications'. Ghent, 30 June – 1 July 2011

Awards

B-IWA Research paper Award 2015 for the paper :

> **Courtens, E. N. P.,** Vlaeminck, S.E., Vilchez Vargas, R., Verliefde, A, Jauregui R., Pieper D. H. and Boon, N. (2014) Trade-off between mesophilic and thermophilic denitrification: rates vs. sludge production, settleability and stability. Water Research, 63, 234-244 .

Ernest du bois prize from the 'Koning Boudewijnstichting' for a PhD on the theme of water and its availability on a global level. Financial support of 20 000€ for the project : **Ontwikkeling van thermofiele biotechnologie voor stikstofverwijdering: de sleutel naar goedkopere en duurzamere afvalwaterbehandeling**

Most ingenious Flemish master thesis (IE-net, 2011)

International study leaves and workshops

28th July – 1st August 2014 : Research stay at Biocenter Klein Flottbek, University of Hamburg, Germany

14th -28th June 2014 : Intensive training programme - Arctic Microbiology: education and training in field work and analysis, University of Akureyri, Akureyri, Iceland

2nd September 2013, Global Scientist Cycle (N-Cycle) organized by Tokyo University of Agriculture & Engineering, Tokyo, Japan

Professional activities during PhD research

Tutoring and evaluation of tasks of 'Environmental technology: biotechnological processes'

Practical exercises coordinator 'Microbial Ecology & Environmental Sanitation'

External services for water treatment industry

International

- 2012: Advanced Waste Water Solutions bv (Pure blue): incapsulation of partial nitritation/anammox sludge for pilot-scale OLAND-RBC in chemical fertilizer industry
- 2015: Suez environnement: qPCR and DGGE analysis of Cleargreen biomass samples

National (Belgium)

- 2013: Hydrio bvba : Wastewater treatment feasibility study (biological nitrogen removal from fire-fighting wastewater)

Internal services for LabMET and Faculty of Bioscience engineering

Organisation of 'Wetenschapsweek'

Organisation of 'Opendeurdag' of the Faculty of Bioscience engineering

Responsible several lab rooms and ion chromatograph

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