TU Delft

Delft University of Technology

Stratification of nitrifier guilds in granular sludge in relation to nitritation

Soler-Jofra, Aina; Wang, Ru; Kleerebezem, Robbert; van Loosdrecht, Mark C.M.; Pérez, Julio

DOI
10.1016/j.watres.2018.10.064

Publication date
2019

Document Version
Final published version

Published in
Water Research

Citation (APA)
Soler-Jofra, A., Wang, R., Kleerebezem, R., van Loosdrecht, M. C. M., & Pérez, J. (2019). Stratification of nitrifier guilds in granular sludge in relation to nitritation. *Water Research*, *148*, 479-491. https://doi.org/10.1016/j.watres.2018.10.064

Important note
To cite this publication, please use the final published version (if applicable).
Please check the document version above.

Copyright
Other than for strictly personal use, it is not permitted to download, forward or distribute the text or part of it, without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license such as Creative Commons.

Takedown policy
Please contact us and provide details if you believe this document breaches copyrights.
We will remove access to the work immediately and investigate your claim.

This work is downloaded from Delft University of Technology.
For technical reasons the number of authors shown on this cover page is limited to a maximum of 10.

Water Research 148 (2019) 479–491

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Stratification of nitrifier guilds in granular sludge in relation to nitritation

Aina Soler-Jofra [a, 1], Ru Wang [a, b, 1], Robbert Kleerebezem [a], Mark C.M. van Loosdrecht [a], Julio Pérez [a, c, *]

[a] Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, Van der Maasweg 9, 2629, HZ, Delft, the Netherlands
[b] Department of Environment Engineering, College of Environmental & Resource Science, Zhejiang University, Yuhangtang Road 866, 310058, Hangzhou, China
[c] Department of Chemical, Biological and Environmental Engineering, Universitat Autonoma de Barcelona, 08193, Cerdanyola del Valles, Spain

ARTICLE INFO

Article history:
Received 29 June 2018
Received in revised form
19 October 2018
Accepted 22 October 2018
Available online 26 October 2018

Keywords:
Partial nitrification
Temperature
Nitrate-oxidizing bacteria repression
Residual ammonium concentration
Biofilm

ABSTRACT

A lab-scale partial nitritation granular sludge air-lift reactor was operated in continuous mode treating low strength synthetic medium (influent ca. 50 mg-N-NH_4^+/L). Granules were initially stratified with AOB in the external shell and NOB in the inner core at 20 °C. Once temperature was decreased progressively from 20 °C to 15 °C, nitrate production was initially observed during several weeks. However, by maintaining relatively high ammonium concentrations in the liquid (ca. 28 mg-N-NH_4^+/L), effluent nitrate concentrations in the reactor decreased in time and process performance was recovered. Batch tests were performed in the reactor at different conditions. To understand the experimental results an existing one-dimensional biofilm model was used to simulate batch tests and theoretically assess the impact of stratification, dissolved oxygen (DO) and short-term effects of temperature on time course concentrations of ammonium, nitrite and nitrate. This theoretical assessment served to develop an experimental methodology for the evaluation of in-situ batch tests in the partial nitritation reactor. These batch tests proved to be a powerful tool to easily monitor the extent of stratification of nitrifier guilds in granular sludge and to determine the required bulk ammonium concentration to minimize nitrite oxidation. When nitrifier guilds were stratified in the granular sludge, a higher bulk ammonium concentration was required to efficiently repress NOB at lower temperature (ca. 19 versus 7 mg-N-NH_4^+/L at 15 and 20 °C, respectively).
© 2018 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Due to the economic and environmental advantages compared to the conventional nitrification/denitrification processes, partial nitritation/anammox (PN/A) technology has been under development in the past decades reaching around 100 full-scale installations in side-stream conditions by 2014 (Lackner et al., 2014). However, the implementation of PN/A technology for domestic wastewater treatment remains a challenge because of the low wastewater temperatures in winter and high C:N ratios, among others (Cao et al., 2017). One stage PN/A process encountered problems in long-term operation as nitrite-oxidizing bacteria (NOB) tend to proliferate and nitrate accumulates, which in turn results in a decrease of the overall nitrogen removal efficiency through the anammox (AMX) process (De Clippeleir et al., 2013; Winkler et al., 2011).

For two-stage processes, stable PN has been demonstrated feasible at temperatures from 20 to 10 °C and low C:N ratios or without organic carbon (Isanta et al., 2015; Poot et al., 2016; Reino et al., 2016), as well as adequate anammox performance at these conditions (Lotti et al., 2014; Reino et al., 2018). Main advantages of a two-stage PN/A removal processes are: (i) control strategies for NOB repression can be applied separately in the PN reactor; and (ii) the separation of PN/A and anammox processes avoids competition between AMX and NOB for nitrite. Two-stage processes have been recently highlighted as a possible solution for attaining stable PN avoiding NOB proliferation, even at the unfavourable mainstream municipal wastewater treatment conditions (Hendrickx et al., 2012; Ma et al., 2011; Pérez et al., 2015).

At temperatures below 20 °C the maximum specific growth rate

* Corresponding author. Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, Van der Maasweg 9, 2629, HZ Delft, the Netherlands.
E-mail address: J.O.PerezCanestro@tudelft.nl (J. Pérez).
[1] Authors contributed equally to the work.

https://doi.org/10.1016/j.watres.2018.10.064
0043-1354/© 2018 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

(μ_{max}) of NOB is higher than that of ammonium-oxidizing bacteria (AOB) (Hellinga et al., 1998; Hunik et al., 1994), so it seems much more challenging to repress NOB at these conditions. Currently, strategies to control NOB proliferation are based on the general assumption that NOB have lower affinity for oxygen than AOB (Liu and Wang, 2013; Pérez et al., 2009). Thus, operation at low dissolved oxygen (DO) concentration has been commonly adopted as a strategy to accumulate nitrite (Blackburne et al., 2008; Sliekers et al., 2002; Third et al., 2001; Winkler et al., 2011). However, NOB oxygen affinity reported in literature varies among different genera (Nowka et al., 2015; Regmi et al., 2014; Reino et al., 2016). On top of that, a recent study highlighted that colony size of nitrifying organisms in the sludge flocs or biofilm determines the apparent oxygen affinity (Picioreanu et al., 2016). Thus, the intrinsic oxygen affinity, which is strain dependent, will overall play a minor role (Picioreanu et al., 2016).

Another strategy that has been highlighted for successful NOB repression is to control the ratio of oxygen to ammonium concentration (Bartrolí et al., 2010; Isanta et al., 2015; Reino et al., 2016). Short term effects of the bulk DO/ammonium ratio on the nitrate production were studied previously in Poot et al. (2016). The study was carried out with the same granular sludge PN reactor used in the present study. Stratification of nitrifier guilds in the granular biofilm with AOB-dominated outer shell was reported at 20 °C, with a minimum ammonium effluent concentration of 2–5 mg-N-NH_4^+/L for a dissolved oxygen concentration lower than 4 mg-O_2/L (Poot et al., 2016). Therefore, for a given DO concentration, if enough ammonium was available, the external AOB layer would completely exhaust oxygen; whereas NOB, occupying inner layers could not oxidise nitrite because of oxygen limitation. However, when residual ammonium concentration was too low (below 2-5 mg-N-NH_4^+/L), the growth rate of AOB slowed down resulting in less oxygen consumption by AOB and more oxygen was available for NOB, which proliferated (Poot et al., 2016). Previous studies focused on the influence of residual ammonium on NOB repression in long term operation (Isanta et al., 2015; Reino et al., 2016). Poot et al., 2016 focused on the short term effect of residual ammonium at 20 °C, where the advantage on growth rate of AOB compared to NOB is rather small (Hellinga et al., 1998; Hunik et al., 1994).

Here, the residual ammonium concentration required for efficient NOB repression was investigated at 15 °C. At this temperature NOB were shown to exhibit a higher maximum specific growth rate than AOB (Hellinga et al., 1998; Hunik et al., 1994). The question remains if at 15 °C the stratification of nitrifier guilds in the granular sludge persists despite the disadvantage of AOB in terms of maximum specific growth rate.

The aim of this study was to further understand what are the key operating parameters and their specific roles in order to maintain stable PN. Thus, numerical simulations were run with a biofilm model to assess how stratification, DO and temperature impact the stability of partial nitritation in a granular sludge airlift reactor. An *ad-hoc* methodology involving in-situ batch tests was developed (based on the simulations) to investigate how NOB repression is affected by (i) ammonium concentration, (ii) temperature in the range 15–20 °C, (iii) DO and (iv) stratification of nitrifier guilds in the granular sludge. FISH analysis of pottered and cryosectioned granules was used to correlate population dynamics and spatial distribution of AOB and NOB in the granular sludge with the operation and batch tests results.

2. Materials and methods

2.1. Reactor continuous operation

An airlift reactor with 1.5L working volume performing partial nitrification was operated from day 0–153. Inoculum, start-up and part of the operational period at 20 °C were described in a previous paper (Poot et al., 2016). To clarify that the reactor had been operating stable at 20 °C, phase V of the already published data (Poot et al., 2016) was included as days −23 to −1 (Fig. 1). A break is included to indicate that the reactor was stopped and moved to a new building. The influent flow rate was controlled manually to keep an ammonium to oxygen ratio in the effluent adequate for partial nitritation, as described previously (Bartrolí et al., 2010; Isanta et al., 2015; Pérez et al., 2015). DO and pH were followed online but not controlled. Air flow rate was regulated (Fig. 1C) with a mass flow controller (2 L/min capacity, BROOKS, the Netherlands). Temperature was controlled and regulated between 20 and 15 °C with an external jacket. The reactor pH was rather constant at 7.7 ± 0.05 throughout the continuous operation period.

2.2. Wastewater

Synthetic wastewater was used with the same composition as described previously (Poot et al., 2016), containing (per litre of tap water) 0.73 g K_2HPO_4, 0.104 g KH_2PO_4, 1.26 g $NaHCO_3$, 0.236 g $(NH_4)_2SO_4$, 0.25 mL Fe^{2+} solution and 0.12 mL trace elements solution. The Fe^{2+} solution consisted of (per litre demineralized water) 6.37 g EDTA and 9.14 g $FeSO_4 \cdot 7H_2O$, and the pH was adjusted to 2.5 with HCl. The trace elements solution contained (per litre Milli-Q water) 19.11 g EDTA, 0.43 g $ZnSO_4 \cdot 7H_2O$, 0.24 g $CoCl_2 \cdot 6H_2O$, 1.0 g $MnCl_2 \cdot 4H_2O$, 0.25 g $CuSO_4 \cdot 5H_2O$, 0.22 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.20 g $NiCl_2 \cdot 6H_2O$, 0.09 g $HNaSeO_3$, 0.014 g H_3BO_3 and 0.054 g $Na_2WO_4 \cdot 2H_2O$. The pH of the trace elements solution was adjusted to 6 to avoid precipitation with solid NaOH.

2.3. Modelling study

A previously developed mathematical model (Pérez et al., 2014) was slightly modified in the present study to theoretically evaluate the key mechanisms impacting NOB repression in a granular biofilm performing nitritation. The model was used to simulate batch tests. The simulations aimed to characterize the time course concentrations of ammonium, nitrite, nitrate and dissolved oxygen (DO) during a batch test, when imposing either stratified or mixed nitrifier guilds in the granular sludge.

Nitrate, nitrite, ammonium and oxygen were considered as soluble compounds. Particulate species were AOB, NOB and inert biomass. An average granule diameter of 1 mm and a biomass concentration of 1gVSS/L were used in the modelling study. AQUASIM v.2.1d was used as simulation platform. The model description, equations and stoichiometric matrix can be found in Supplementary Information (Tables S1-S3).

To study the impact of the stratification of nitrifier guilds, the distribution of particulate species in the biofilm was imposed to represent two opposite scenarios: i) Stratification of nitrifier guilds, with AOB dominating the external shell and NOB in the inner layers (as previously reported by Poot et al., 2016, see also Fig. 2A), ii) Mixed nitrifier guilds, with AOB and NOB coexisting in the same biofilm layers (Fig. 2B). The chosen biomass profiles for the stratification of nitrifier guilds aimed to investigate how stratification of nitrifier guilds impacts the rates of ammonium oxidation and nitrate production during a batch test.

For the simulation of a batch test, the initial ammonium and nitrite concentrations were assumed equal to 25 mg-N/L. A constant aeration was set to obtain an initial DO concentration of 3 mg-O_2/L (or 2 mg-O_2/L in one of the simulations). Temperature was set to either 20 or 15 °C depending on the simulation (Fig. 3), which affects maximum specific growth rates and decay rates (see Equations S1-S4).

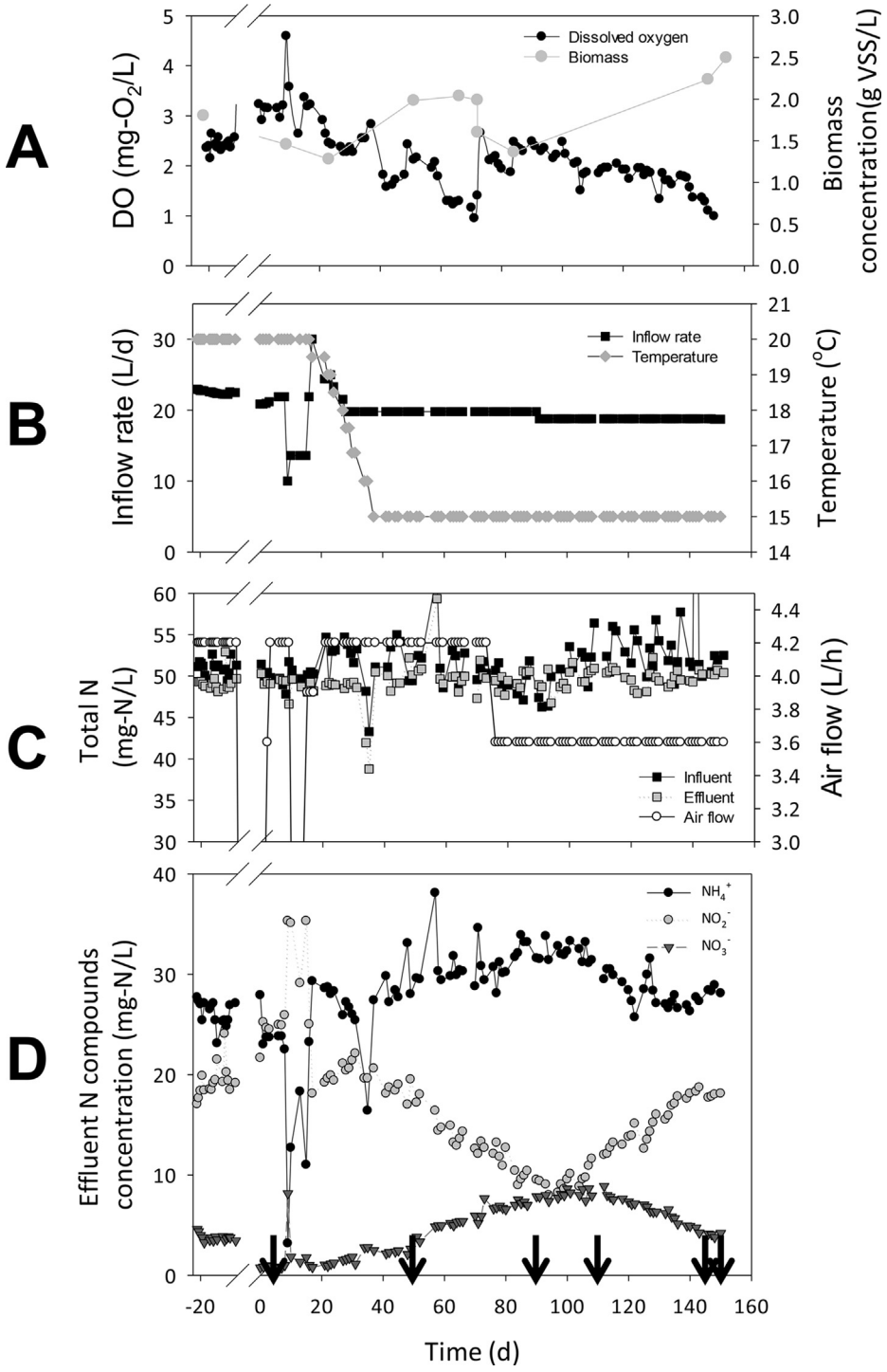

Fig. 1. Main results obtained from continuous reactor operation. Negative time values refer to previous operation at 20 °C already published in Poot et al. (2016). Black arrows indicate days when batch tests were performed. A) Dissolved oxygen concentration and biomass concentration B) Reactor inflow rate and temperature, C) Total nitrogen in the influent and effluent and air flow rate, D) Nitrogen (N) compounds concentration in the effluent. Break in y axis corresponds to time where the reactor was stopped due to moving of the laboratory to a new building and recover of regular operation.

2.3.1. Effect of kinetics & stoichiometry

Since the process is operated under oxygen limitation, the key parameter to be taken into account to assess competition between AOB and NOB is the maximum oxygen respiration rate ($q_{O2max} = Y_{O2} \cdot \mu_{max}$). This lumps the effect of maximum specific growth rate (μ_{max}, affected by temperature) with stoichiometry for oxygen (oxygen yield, see Table 1). To assess the effect of the kinetics and stoichiometry assumed for AOB and NOB in the simulations, additional simulations were run in three scenarios: (i) same fitness, with NOB having the same kinetics than that of AOB (i.e. same maximum specific growth rate, same half saturation coefficients) and same oxygen yield, therefore $q_{O2max,AOB} = q_{O2max,NOB}$; (ii) same kinetics (i.e. same maximum specific growth rate, same half saturation coefficients); (iii) same maximum

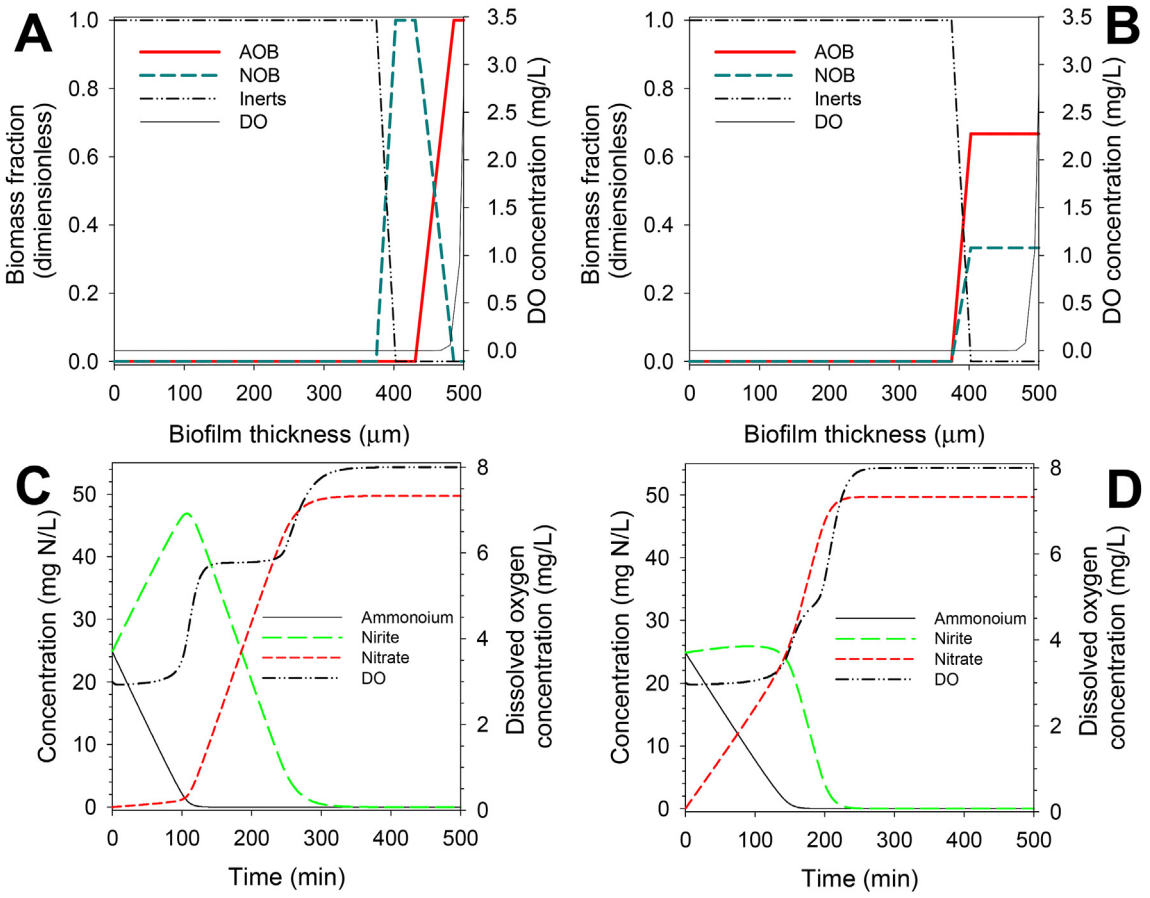

Fig. 2. Imposed biomass distribution for modelling purposes in the granule for **A**: stratified nitrifier guilds; and **B**: mixed nitrifier guilds. Time course concentrations of ammonium, nitrite, nitrate and dissolved oxygen (DO) as calculated with the model for a batch test at 20 °C in case of **C**: stratified nitrifier guilds; and **D**: mixed nitrifier guilds. Initial bulk DO concentration 3 mg-O_2/L.

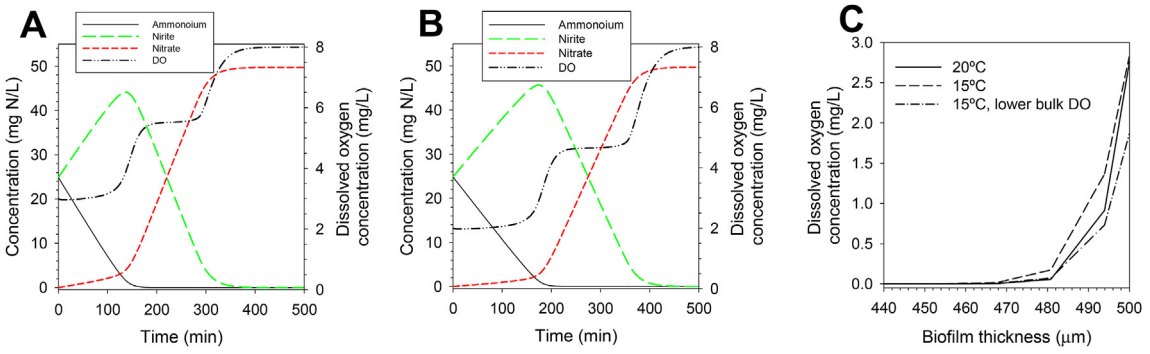

Fig. 3. Time course concentrations of ammonium, nitrite, nitrate and dissolved oxygen (DO) as calculated with the model for a batch test in case of stratified nitrifier guilds at 15 °C for **A**: initial bulk DO = 3 mg-O_2/L and **B**: initial bulk DO = 2 mg-O_2/L. **C**: Comparison of DO profiles in the biofilm during the batch test (after 15 min) at different temperatures and DO concentrations.

specific growth rate, where the maximum specific growth rate of NOB was set equal to that of AOB (Fig. S1). See details of the parameters used for each scenario in Table 1.

2.4. In-situ batch tests

Along with following the reactor performance during continuous operation, in-situ batch tests were performed in the PN granular air-lift reactor. Batch tests aimed to assess the impact of variable ammonium, nitrite and nitrate on the ammonium and nitrite oxidation rates. The experiments were carried out at different DO concentrations and temperatures (as done previously in the simulations) to assess the impact of these variables on NOB repression and the stratification of the nitrifier guilds in the granular sludge.

Continuous operation was stopped by switching off the feeding pump temporarily while the batch experiment was performed. During the batch test, samples were withdrawn at different times from the top section of the reactor, filtered and analysed as described in section 2.5. During the tests DO and pH were not

Table 1
Kinetic parameters (15 °C and pH 7.7). Parameters used in each scenario to test the effect of kinetics in the batch tests. Parameters are set equal to the standard kinetics case if not stated otherwise. Half saturation coefficients have been considered constant for the range of temperatures tested in the simulations.

Symbol	Definition	Value	Unit	References
		Standard kinetics		
	Ammonia oxidizing bacteria (AOB)			
$\mu_{max,AOB}$	Maximum specific growth rate	0.48	d^{-1}	Jubany et al., 2008
b_{AOB}	Decay rate	0.024	d^{-1}	Volcke et al., 2010
Y_{AOB}	Growth yield	0.18	g-COD g^{-1}-N	Jubany et al., 2008
$Y_{O2,NOB}$	Oxygen yield = (3.43-Y_{AOB})/Y_{AOB}	18	g-COD g^{-1} -N	Jubany et al., 2008
$K_{O2,AOB}$	Half saturation coefficient for oxygen	0.2	mg-O_2 L^{-1}	Manser et al., 2005
$K_{S,NH4+}$	Half saturation coefficient for TAN	01.1	mg-N L^{-1}	Wiesmann, 1994
	Nitrite-oxidizing bacteria (NOB)			
$\mu_{max,NOB}$	Maximum specific growth rate	0.56	d^{-1}	Jubany et al., 2008
b_{NOB}	Decay rate	0.028	d^{-1}	Volcke et al., 2010
Y_{NOB}	Growth yield	0.08	g-COD g^{-1}-N	Jubany et al., 2008
$Y_{O2,NOB}$	Oxygen yield = (1.14-Y_{NOB})/Y_{NOB}	13	g-COD g^{-1}-N	Jubany et al., 2008
$K_{O2,NOB}$	Half saturation coefficient for oxygen	0.4	mg O_2 L^{-1}	Proportionally higher than $K_{O2,AOB}$ as in Guisasola et al. (2005)
$K_{S,\ NO2-}$	Half saturation coefficient for TNN	0.5	mg-N L^{-1}	Wiesmann, 1994
		Same fitness		
	Nitrite-oxidizing bacteria (NOB)			
$\mu_{max,NOB}$	Maximum specific growth rate	0.48	d^{-1}	
b_{NOB}	Decay rate	0.024	d^{-1}	
Y_{NOB}	Growth yield	0.06	g-COD g^{-1}-N	
$Y_{O2,NOB}$	Oxygen yield = (1.14-Y_{NOB})/Y_{NOB}	18	g-COD g^{-1}-N	
$K_{O2,NOB}$	Half saturation coefficient for oxygen	0.2	mg-O_2 L^{-1}	
		Same kinetics		
	Nitrite-oxidizing bacteria (NOB)			
$\mu_{max,NOB}$	Maximum specific growth rate	0.48	d^{-1}	
b_{NOB}	Decay rate	0.024	d^{-1}	
$K_{O2,NOB}$	Half saturation coefficient for oxygen	0.2	mg-O_2 L^{-1}	
$K_{S,NO2-}$	Half saturation coefficient for TNN	1.1	mg-N L^{-1}	
		Same maximum specific growth rate		
	Nitrite-oxidizing bacteria (NOB)			
$\mu_{max,NOB}$	Maximum specific growth rate	0.48	d^{-1}	

automatically controlled but measured on-line. When exploring the effects of DO, air flow rate was manually adjusted to achieve the targeted values if required. This manipulation of the air flow rate was carried out for batch tests on days 92, 111 and 153.

2.5. Physicochemical analysis

Samples were withdrawn from the reactor and filtered for daily measurements. Offline analysis for ammonium, nitrite and nitrate concentrations were carried out using Hach Lange cuvette kits. Dry weight (TSS), which refers to the total solid mass per volume of mixture in reactor, and volatile suspended solids (VSS), which was assessed by subtracting the ash content from TSS, were determined according to standard methods (APHA, 2012).

2.6. Procedure for in-situ batch test evaluation

Here we propose a method to analyse the batch tests experimental data, specifically developed to investigate how NOB repression is affected by ammonium concentration, temperature, DO and stratification of nitrifier guilds in the granular sludge. The first step in the procedure is to calculate the rates of ammonium oxidation (ammonium oxidation rate, R_{NH4}) and nitrate production (nitrate production rate, R_{NO3}) during the continuous reactor operation (see Equation (1)), just before starting the in-situ batch test. The initial rates during the batch test, were calculated based on linear regression minimizing the sum of squared errors using the first two sampled measurements and the averaged initial concentration measured during continuous operation. Additionally, a maximum nitrate production rate (maximum R_{NO3}) can be calculated, also by linear regression, when ammonium is fully depleted with the last three sampled measurements.

Using the calculated R_{NO3} during continuous operation and the maximum R_{NO3} when ammonium is depleted, two straight lines can be drawn (using Equation (2)) starting from the initial and final nitrate concentrations, respectively. On the graph, the measured points deviating from those lines can be easily identified (see Fig. S2 for an example). The slope obtained with the R_{NO3} during continuous operation indicates from which point onwards, NOB repression is not as efficient as it was during the continuous operation (see red solid line in Fig. 4). The slope obtained with the maximum R_{NO3}, indicates at which time point R_{NO3} is maximum (see dashed green line in Fig. 4). For a better comparison of rates, the specific nitrate production rate (q_{NO3}) was used (Equation (3)) in Table 2.

During the batch test, three periods are to be highlighted (see light grey, white and dark grey areas, respectively in Fig. 4). Assuming a well stratified granule with AOB in the outer core and NOB in the inner layers, the following periods can be described:

- **Nitrate production during oxygen limitation** (due to ammonia oxidation). Not enough oxygen is available for NOB in the inner layers because the ammonium concentration is enough for AOB

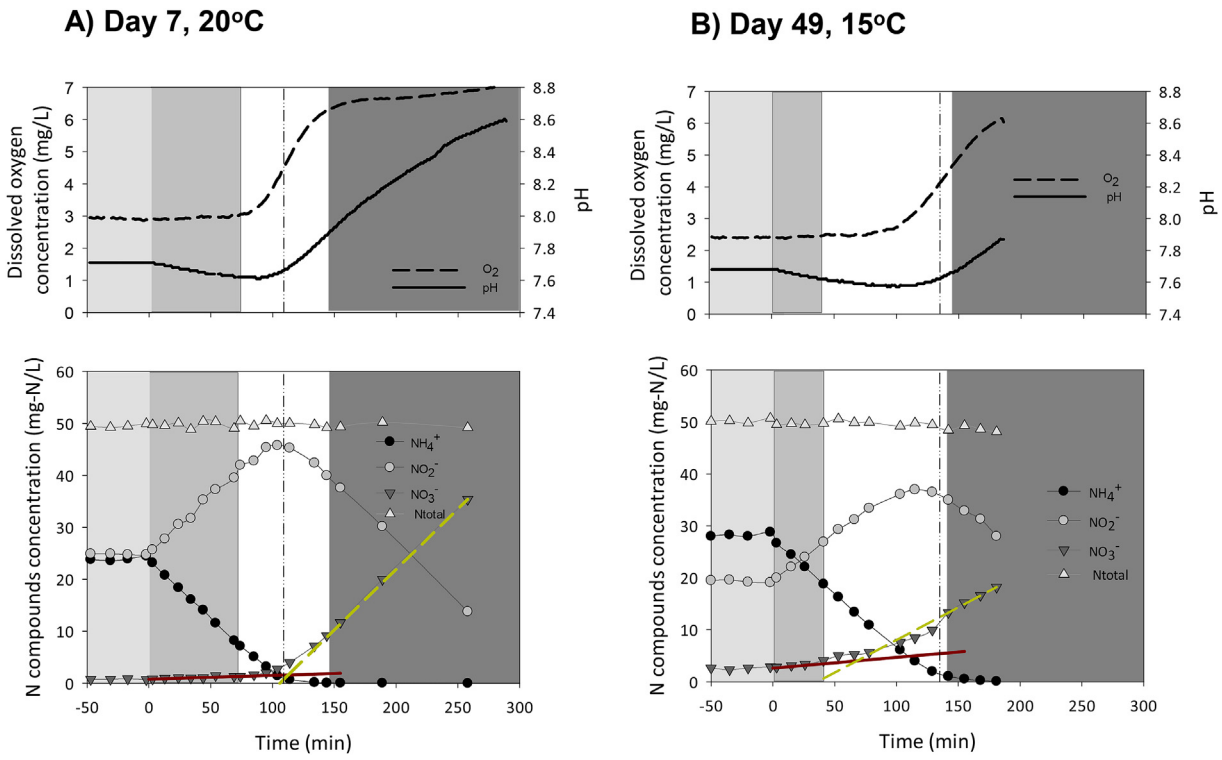

Fig. 4. Experimental batch tests. Effect of temperature on NOB repression. Continuous operation (left light grey area) was stopped at time zero and then the batch test was performed. pH and dissolved oxygen concentration were followed but not controlled. Nitrogen (N) compound concentrations were measured at different times from withdrawn samples. Red solid line corresponds to calculated nitrate concentration with continuous R_{NO3}, grey area represents measured nitrate that corresponds with calculated nitrate. Green dashed line corresponds to calculated nitrate concentration with maximum R_{NO3}, dark grey area represents measured nitrate that corresponds with calculated nitrate. Transition period with a non-linear R_{NO3} corresponds to the white area. Finally, dashed-dot-dot vertical black line indicates the threshold value of 3 g-O_2/g-NH_4^+ where oxygen switches from stoichiometric limitation to excess (for ammonium oxidation). A) Batch test performed on day 7 at 20 °C, B) Batch test performed on day 49 at 15 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
In-situ batch tests conditions and results. DO – Dissolved oxygen concentration. q_{NO3} - Specific nitrate production rate. q_{NH4} – Specific ammonium oxidation rate.

Day	Nitrate production continuous operation	Temperature (ºC)	DO continuous operation (mg-O_2/L)	DO batch (mg-O_2/L)	NH_4^+ concentration start transition phase (mg-N-NH_4^+/L)	q_{NO3} (mg-N-NO_3/gVSS/h)			q_{NH4} (mg-N-NH_4^+/gVSS/h)	
						Continuous	Initial	Maximum	Continuous	Initial
7	Low	20	2.9	2.9	7	0.3 ± 0.2	0.6 ± 0.3	9 ± 5	10 ± 5	10 ± 5
49	Low	15	2.4	2.4	19	0.6 ± 0.3	1.0 ± 0.5	4 ± 1	6 ± 2	7 ± 3
92	High	15	2.2	1.1	12	3 ± 2	3 ± 2	17 ± 10	5 ± 3	6 ± 3
111	High	15	1.8	3.7	28	3 ± 2	9 ± 5	17 ± 10	9 ± 5	15 ± 9
149	Low	15	1.0	0.9	19	0.9 ± 0.3	1.3 ± 0.5	9 ± 3	5 ± 2	6 ± 2
153	Low	15	1.0	3.6	24	0.8 ± 0.2	4 ± 1	9 ± 3	5 ± 1	11 ± 5

to consume a large fraction of the oxygen before it reaches NOB layers.

- **Transition period**, a non-linear nitrate production rate is observed, with lower nitrate production rates at the higher ammonium concentrations. The ammonium concentration observed at the start of this period indicates the minimum residual ammonium concentration required to minimize nitrate production rate, i.e., to efficiently repress NOB (see Fig. S2 for an example).
- **(Maximum) Nitrate production after ammonium depletion**, no oxygen is consumed by AOB; thus, oxygen reaches inner layers resulting in a maximum R_{NO3} at the DO concentration applied.

$$R_{\text{continuous}} = \frac{Q \cdot (C_{N,\text{out}} - C_{N,\text{in}})}{V_L} \quad (1)$$

$$C_{N,t_i} = R \cdot (t_i - t_0) + C_{N,t_{0\ or\ end}} \quad (2)$$

$$q_N = \frac{R}{C_x} \quad (3)$$

Where, $R_{\text{continuous}}$ is the volumetric rates of consumption or production of nitrogen compound during continuous operation in mg-N/L/h. Q stands for the inflow rate of the reactor in L/h. $C_{N,\text{in}}$ and $C_{N,\text{out}}$ are the influent and effluent nitrogen concentrations during continuous reactor operation, in mg-N/L. V_L is the reactor liquid volume in L. t is the time when the sample was taken in hours, using subscript i when referring to any time, subscript 0 for the time when the batch was started and the subscript *end* for the last measurement performed. Finally, C_x is the biomass concentration in the reactor during the batch test in gVSS/L. q is the specific nitrogen consumption or production rate in mg-N/gVSS/L, where N stands for the selected nitrogen species.

2.7. Fluorescence in-situ hybridization (FISH)

FISH was performed in both pottered biomass and cryosectioned granules. The aim of pottered biomass was to assess relative abundances of populations in the granule, whereas cryosectioning granules aimed to locate the distribution of different populations.

To obtain pottered biomass, granules were disrupted by pottering, washed in 1xPBS 3 times (PBS, pH 7.2), fixed 1 h in paraformaldehyde solution (4% paraformaldehyde in phosphate-buffer) and stored in the freezer until further use. The pottered cells were put onto pre-coated slides and then immersed in 50%, 80% and 98% ethanol for 3min each.

Granules cryosectioning technique was performed as previously described (Poot et al., 2016). Briefly, granular sludge was taken from the upper section of reactor and was suspended in 1x PBS immediately for 3 h. After that, the sludge was washed with 1x PBS and fixed with paraformaldehyde solution for 1 h and stored in the freezer until further use. A freeze-microtome (Leica CM1990) was used to cut the granules at 25 °C to obtain slices (10–15 μm thick). The obtained slices were put onto pre-coated slides and then immersed in 50% ethanol for 5min and in 98% ethanol for 5 s.

The pottered biomass was hybridized with EUB338, AOBmix, NOBmix and Ntoga1424 oligonucleotide probes, while the cryosectioned granules were hybridized with EUB338, AOBmix and NOBmix (see Table S4).

A Zeiss Axioplan 2 Imaging microscope, an AxioCam MRm camera (Zeiss), an ebq100 lamp for fluorescent light and the Axiovision software were used to analyse the images.

3. Results & discussion

3.1. Reactor continuous operation

The partial nitritation granular sludge reactor was previously operated at 20 °C for 214 days with an average loading rate of 0.8 g-N/L/d (Poot et al., 2016). The last phase of the already published data is included in the present study for convenience (Day −23 to −1), to show that partial nitritation was stable at 20 °C (Fig. 1). In the present study partial nitrification at 20 °C was maintained from day 0–8. The effluent concentrations of ammonium, nitrite and nitrate in reactor were 24 ± 2, 25 ± 1 and 0.9 ± 0.1 mg-N/L respectively, indicating that approximately half of the influent ammonium was converted to nitrite by AOB. Low nitrate effluent (<1 mg-N-NO_3^-/L) was produced, so an efficient NOB repression was achieved. From day 9–15 low effluent ammonium concentration was targeted (ca. 10 mg-N-NH_4^+/L) by decreasing influent flow rate, which led to an accumulation of small amounts of nitrate (ca. 2 mg-N-NO_3^-/L). This indicates that NOB were still present and active in the granular sludge (Fig. 1).

A gradual acclimation of the biomass from 20 to 15 °C was achieved by decreasing the operational temperature 2 °C per week from day 16–40. The influent flow rate was adjusted to maintain an effluent ammonium concentration at ca. 25-30 mg-N-NH_4^+/L. Averaged effluent nitrogen concentration during this period remained rather constant at 26 ± 4, 21 ± 2 and 1.6 ± 0.7 mg-N/L for ammonium, nitrite and nitrate respectively (Fig. 1).

The reactor was operated at 15 °C from day 41 onwards with an average loading rate of 0.6 ± 0.2 g-N/L/d. Approximately 25% of the granular sludge (from ca. 2 to 1.5 g-VSS/L) was removed from the reactor at day 72. During the continuous operation at low temperature, nitrate concentration in the effluent increased in time from ca. 2.5 mg-N-NO_3^-/L up to maximum values of ca. 9 mg-N-NO_3^-/L on day 111 (Fig. 1). However, from day 112 onwards, effluent nitrate concentration started to decrease gradually to ca. 4 mg-N-NO_3^-/L on day 153, when this study was ended (Fig. 1). Thus, although nitrate production increased during the first period at 15 °C (days 41–111), low residual nitrate concentrations were obtained again simply by maintaining the operational conditions. Reasons for the recovery of low effluent nitrate concentrations are discussed sections 3.5 & 3.6.

3.2. Theoretical assessment of in-situ batch tests through mathematical modelling

Theoretical analysis of the short-term effects of temperature and DO on process performance was carried out by mathematical modelling. Two different scenarios were imposed for the location of the nitrifier guilds in the granular sludge: i) stratification of nitrifier guilds in the biofilm (Fig. 2A), ii) mixed nitrifier guilds (Fig. 2B).

Firstly, the time course concentrations of ammonium, nitrite, nitrate and DO in a batch test were simulated at 20 °C for a granular sludge reactor for both scenarios (Fig. 2C and D). When there is stratification of nitrifier guilds in the granular sludge, with AOB occupying the external granule shell (as shown in Fig. 2A), the time course concentration of nitrate switches from a phase at low nitrate production rate to a phase at high nitrate production rate (Fig. 2C). During the period with a low nitrate production rate, ammonium is in excess and mainly nitritation is taking place. Then, the switch to a high nitrate production rate is observed just after ammonium is depleted, when oxygen can reach inner layers of the biofilm. However, the time course concentration of nitrate is different when both nitrifier guilds are mixed (Fig. 2B), with a relatively high nitrate production rate also before ammonium is depleted (Fig. 2D). Consequently, the fact that the nitrate production rate does not increase strongly until ammonium concentrations are rather low is indicative of a granular sludge with stratified nitrifier guilds, with AOB occupying the outer shell.

Secondly, the short-term effect of temperature on the time course concentrations of ammonium, nitrite, nitrate and DO was evaluated with the model for the first scenario, in which nitrifier guilds are stratified in the granular sludge (Fig. 2A). The decrease in temperature from 20 to 15 °C impacts the maximum growth rate of both populations (AOB and NOB), reducing their activity (i.e., a decrease of the q_{O2max}). However, in the model, while at 20 °C the maximum specific growth rate of AOB and NOB are considered equal (see Equations S1 & S2), at 15 °C there is a difference, favouring NOB (i.e. $\mu_{max,NOB} = 0.56\ d^{-1} > \mu_{max,AOB} = 0.48\ d^{-1}$ at 15 °C, at a pH of 7.7, see Table 1). The simulations indicated that the nitrate production rate before ammonium is depleted increased at decreasing temperatures in a stratified biofilm (compare Figs. 2C and 3A). Therefore, the suppression of nitrite oxidation activity at lower temperatures is more challenging, as expected.

However, it is a complex problem, as temperature impacts not only the maximum specific growth rate, but also the oxygen penetration depth. At 15 °C the overall AOB activity decreases if compared to that at 20 °C. Thus, at 15 °C less oxygen is consumed (compared to consumption at 20 °C) in the AOB shell, leading to a deeper oxygen penetration in the biofilm (see Fig. 3C). Thus, the increase in nitrate production rate found in the simulations is not due to a higher specific growth rate of NOB at 15 °C, but to a deeper oxygen penetration derived from the decrease in AOB activity.

To confirm this reasoning and to rule out the contribution of the NOB kinetics, a new set of simulations was performed comparing four scenarios (see Table 1): i) *Standard kinetics* (as already computed in Fig. 2A); ii) *Same fitness*, with fitness of NOB equal to that used for AOB in the standard kinetics (i.e., same half saturation coefficient, same maximum specific growth rate and same oxygen yield), (iii) *Same kinetics*, with kinetics of NOB equal to those used for AOB in the standard kinetics (i.e., same half saturation coefficient and maximum specific growth rate), iv) *Same* μ_{max}, with

maximum specific growth rate of NOB equal to that used for AOB in the standard kinetics. The results obtained in the simulations demonstrated a marginal impact of NOB fitness on the short-term activity (Fig. S1). The time course concentrations of nitrate and oxygen are almost unaffected by the NOB kinetic parameter selection (Fig. S1). Thus, what determines the increase of nitrate production rate at 15 °C in the simulations is the decrease in AOB activity in the external shell, which directly impacts the oxygen penetration depth, in turn resulting in more oxygen available for NOB located in the inner layers. Overall this is the dominant effect on short term, independently of the specific kinetics assumed for NOB. In other words, the effect of the position of the nitrifier guilds overrides, in this case, the effects of kinetics and stoichiometry.

Finally, to study the impact of DO, an additional simulation was performed, in which aeration was decreased to obtain an initial DO concentration of 2 mg-O_2/L (instead of 3 mg-O_2/L) as in Fig. 3B. The modelling results indicated a decrease of the oxygen penetration depth, together with a decrease of the nitrate production rate (see Fig. 3B and C). Note how the DO profile affects the whole AOB shell, indicating that the activity of AOB would overall be (negatively) impacted by the decrease in the bulk DO, decreasing the process performance. Interestingly, the ammonium concentration required to keep a low nitrate production rate at 15 °C and at 2 mg-O_2/L can be identified in Fig. 3B as ca. 8-9 mg-N-NH_4^+/L. Therefore, R_{NO3} was not affected by a decrease in the DO concentration.

Overall, the theoretical study carried out with the model provides insight regarding the impact of kinetics, DO and temperature on a batch test in case of stratification of the nitrifier guilds. The modelling data suggest that the extent of stratification of nitrifier guilds can be estimated from nitrification batch experiments with granular biomass, which was the objective of the experiments in section 3.3 & 3.4.

3.3. Experimental assessment of the impact of residual bulk ammonium on NOB repression at different temperatures

To experimentally investigate the effect of temperature on NOB repression, in-situ batch tests on day 7 and 49 of continuous operation were performed at 20 and 15 °C, respectively. On both days, nitrate concentrations during continuous operation were low indicating an efficient NOB repression (2 and 4 mg-N-NH_4^+/L at 20 and 15 °C, respectively, see Fig. 1). Initial DO and ammonium concentrations were also comparable (3 and 2.5 mg-O_2/L, at 20 and 15 °C, respectively, see Fig. 1 & Table 2). The batch tests were analysed according to the proposed *ad-hoc* methodology allowing for comparison between experiments; the main results are presented in Fig. 4 & Table 2.

Both batch tests showed similar trends with an initial nitrate production rate similar to the rate observed during continuous operation (red solid line in Fig. 4). At a low ammonium concentration, the nitrate production rate increased (green dashed line in Fig. 4). Thus, between the red solid and green dashed line in Fig. 4 a gradual increase in nitrate production rate was observed, which was named transition period.

Considering the stoichiometry of nitritation and diffusivities of oxygen and ammonium, the nitritation process in the granular sludge is (stoichiometrically) limited by oxygen if the ratio of the bulk concentrations is below 3 gO_2/g-N-NH_4^+ (Bartrolí et al., 2010; Pérez et al., 2015; Poot et al., 2016). Interestingly, the change in the nitrate production rate can be observed at both temperatures (20 and 15 °C, see Fig. 4) well before reaching this stoichiometric threshold value (highlighted in Fig. 4 with a vertical dash-dot-dot type-line). For instance, at 20 °C the required ammonium concentration to minimize the nitrate production rate can be estimated from Fig. 4A as 7 mg-N-NH_4^+/L for a DO concentration of 2.9 mg-O_2/L (Table 2, see Fig. S2 for more details), which is indeed in agreement with the value determined by Poot et al. (2016) during continuous operation (between 2 and 5 mg-N-NH_4^+/L for DO lower than 4 mg-O_2/L). Therefore, the bulk ammonium concentration is kinetically limiting AOB activity as previously demonstrated (Poot et al., 2016). At 15 °C this concentration strikingly increased to 19 mg-N-NH_4^+/L for a DO of 2.4 mg-O_2/L (see Table 2 and Fig. 4B).

This means that a higher ammonium concentration is required at lower temperatures for effective NOB repression, assuming a comparable distribution of nitrifier guilds in the granular sludge. This observation is in agreement with the theoretical simulations performed in section 3.2. In case of a granular sludge with stratified nitrifier guilds the simulations indicated that a decreased temperature affects the AOB activity in the external shell, leading to a higher oxygen penetration depth. Thus, to avoid a high nitrate production, higher ammonium concentrations are needed to promote higher consumption of oxygen by the external AOB shell.

It is considered proven that the required ammonium concentration for efficient NOB repression increased when the temperature decreased from 20 to 15 °C (Fig. 4 and Table 2). However, given the heterogeneity of the system, the exact values are probably system dependent and difficult to be precisely determined.

In the batch test of day 149 (Fig. 5B), nitrate production during continuous operation was equivalent to that measured in day 49 (ca. < 5 mg-N-NO_3^-/L). When performing the batch test in day 149 DO was 1 mg-O_2/L, 2-fold lower than in the batch of day 49. Nevertheless, the ammonium concentration at the start of the transition phase was ca. 19 mg-N-NH_4^+/L on day 149, comparable to the value measured on day 49 (ca. 19 mg-N-NH_4^+/L, see Table 2). This suggested that the bulk ammonium concentration required for efficient NOB repression is not much affected (in the short or long term) by a decrease in the DO concentration. The same trend was found by theoretical simulations (compare Fig. 3 A with Fig. 3B).

3.4. Use of the time course concentration of DO to determine the required bulk ammonium concentration for NOB repression

The increase in the time course for nitrate production rate assessed with the described *ad-hoc* methodology is correlated to an increase in the time course concentration of DO during the batch test (see Fig. 4B and S3A). Therefore, the bulk ammonium concentration required for efficient NOB repression can be determined assessing the changes in the time course concentration of DO during the in-situ batch test (Fig. S3B). When DO concentration increased 5% over the initial bulk DO concentration value, the bulk ammonium concentration was 7, 16 and 16 mg-N-NH_4^+/L for the batch tests performed in days 7, 49 and 149 respectively (see Figs. 4 and 5B). This is in agreement with the results presented in Table 2 which are based on the changes of the nitrate production rate.

There are two advantages when using the DO concentration -instead of the time course concentration of nitrate- to determine the required bulk ammonium concentration for NOB repression: (i) DO concentration is a rate measurement because it is the resultant from mass transfer and reaction (aeration is continuous during the batch test), whereas nitrate –or ammonium-concentration require slope estimation to identify kinetics. (ii) the measurement of DO concentration is often available online, which might result in a more reliable estimation, as more data points are available compared to the proposed method in section 2.6, which relies on ammonium and nitrate off-line measurements. However, the selection of the threshold value of the change of DO concentration has a direct impact on the obtained bulk ammonium concentration. In this study, a 5% change in the bulk DO concentration was chosen as threshold value; the rationale behind it can be found in the **Supplementary Information**.

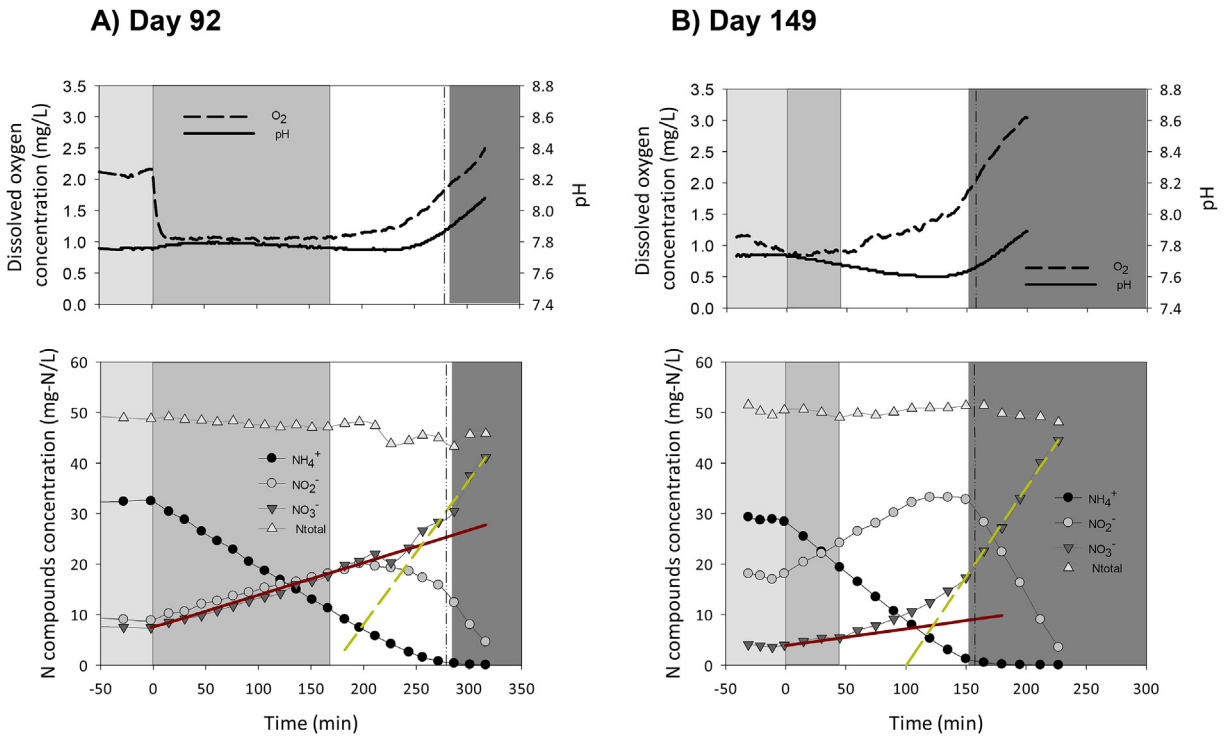

Fig. 5. Experimental batch tests. Continuous operation (left light grey area) was stopped at time zero and then the batch test was performed. pH and dissolved oxygen concentration were followed but not controlled in batch test of day 149. Air flow was manually manipulated in day 92 to target the desired DO set point. Nitrogen (N) compound concentrations were measured at different times from withdrawn samples. Red solid line corresponds to calculated nitrate concentration with continuous R_{NO3}, grey area represents measured nitrate that corresponds with calculated nitrate. Green dashed line corresponds to calculated nitrate concentration with maximum R_{NO3}, dark grey area represents measured nitrate that corresponds with calculated nitrate. Transition period with a non-linear R_{NO3} corresponds to the white area. Finally, dashed-dot-dot vertical black line indicates the threshold value of 3 g-O_2/g-NH_4^+ where oxygen switches from stoichiometric limitation to excess (for ammonium oxidation). A) Batch test performed on day 92 at 15 °C and ca. 1.1 mg-O_2/L, B) Batch test performed on day 149 at 15 °C and ca. 0.9 mg-O_2/L. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5. Proper stratification rather than DO plays a more important role when repressing NOB

A common strategy used for NOB repression in biofilm like systems or granular sludge is operation at a limiting DO concentration (Bartrolí et al., 2010; Garrido et al., 1997; Peng and Zhu, 2006; Pérez et al., 2004; Picioreanu et al., 1997). The bulk DO concentration affects the oxygen penetration depth. In case of a granular sludge with stratified nitrifier guilds a decrease in DO concentration results in a decrease of nitrate production (i.e. an increase of nitrite production) during continuous operation. However, if the stratification of the nitrifier guilds is deteriorated, and a fraction of NOB are growing close to the granule surface (i.e. close to the biofilm-liquid interface), decreasing DO would have a reduced impact on nitrate production. In other words, the location of AOB and NOB in the granule influences the response in terms of nitrate production at decreasing DO concentrations. Furthermore, a lower DO concentration would also imply overall lower conversion rates which would challenge the process performance. During the period with increasing nitrate production in continuous reactor the bulk DO concentration decreased from 2.2 to 1.2 mg-O_2/L (days 40–45) and even to 1 mg-O_2/L (days 58–70). Still, the effluent nitrate concentrations increased slightly. This indicates that a fraction of NOB was growing close to the biofilm-liquid surface, i.e. indicating deterioration of the stratification.

To further investigate this hypothesis, the batch test of day 92 was performed at a lower DO concentration (1.1 mg-O_2/L) than that used during the continuous operation (2.2 mg-O_2/L at day 92, see Fig. 5A and Table 2). On day 92 the nitrate accumulation during the continuous operation was rather high (ca. 9 mg-N-NO_3^-/L) at 2.2 mg-O_2/L. The results of the in-situ batch test in day 92 (Table 2, Fig. 5A) indicated how the nitrate production rate was not severely impacted by a decrease in DO concentration. Specific nitrate production rate (q_{NO3}) was measured as 3 mg-N-NO_3^-/gVSS/h in continuous operation at 2.2 mg-O_2/L, whereas in the batch test q_{NO3} was 3 mg-N-NO_3^-/gVSS/h at 1.1 mg-O_2/L (see Table 2). This comparable nitrate production rate despite the oxygen concentration was decreased by 50% could be explained by a not fully segregated growth of AOB and NOB, resulting in NOB colonies located close to the granule surface. For comparison, see the theoretical results obtained with the model in case of stratification of nitrifier guilds in the granular sludge (compare Fig. 3A with 3B).

The assessment of the nitrifier guild locations in granular sludge has been shown to be possible by performing batch tests at a decreased DO concentration compared to the continuous operation (Fig. 5A). Also, a strong switch of the time course of nitrate concentration was shown to be indicative of a stratified granule (Figs. 2 and 4).

As already suggested in previous sections, when limiting the DO concentration not only NOB conversion is decreased, but also AOB overall conversion is affected. That can challenge the overall process performance. Another application of in-situ batch tests could be to optimize the DO concentration applied during continuous operation, aiming for maximization of the AOB activity, while NOB activity is repressed. For instance, the short-term impact of a higher DO concentration was investigated using batch tests performed on days 111 and 153 (Fig. S5). Both batch tests were performed at higher DO concentration (ca. 3.6 mg-O_2/L) than that used during

continuous operation (1.8 and 1.0 mg-O_2/L for day 111 and 153, respectively). Thus, in both cases the increased DO during the batch tests resulted in an initial R_{NO3} higher than that measured during continuous operation, independently of the putative location of the nitrifier guilds (see red lines in Fig. S5). However, these tests also showed that the q^{+}_{NH4} can be much higher than what it is actually being measured during continuous operation. As an example, on day 153, specific ammonium consumptions rate (q_{NH4}) during continuous operation was 5 mg-N-NH_4^+/gVSS/h, whereas during the initial phase of the batch tests it reached values of 11 mg-N-NH_4^+/gVSS/h (Table 2). So, there was an AOB overcapacity that was not being used during continuous operation. By performing batch tests at different DO concentration, the optimal operation at which high q_{NH4} is promoted, while NOB are efficiently repressed could be identified.

In summary, location of the nitrifier guilds rather than DO played an important role when determining the ratio of AOB-NOB overall conversion rates. Strategies to minimize NOB activity should be focused on combining both the optimization of the DO concentration and, sufficient excess of ammonium to enhance and maintain adequate stratification of the nitrifier guilds. Even though this will not allow for NOB washout from the sludge in the short term, even if their activity can effectively be repressed (Jemaat et al., 2013).

3.6. Microbial population dynamics

In order to investigate the population dynamics and link it to the reactor continuous operation and in-situ batch tests results, FISH was performed using either pottered or cryosectioned granules.

First, pottered samples from days 16, 71 and 153 were fixed for FISH analysis. Initially, EUB338, AOBmix and NOBmix probes (Table S4) were used (Fig. S6 A, C& E). In all three samples AOB and NOB were detected, implying that NOB and AOB always coexisted in the granule throughout the period of continuous operation, despite hardly any nitrite oxidation occurred over long periods. Furthermore, EUB338 and Ntoga1424 (Table S4) were used to detect *Nitrotoga* in the same samples, as this genus was reported to develop in full-scale wastewater treatment plants operating at low temperature (7–16 °C) (Lücker et al., 2015; Reino et al., 2017). In samples fixed on day 16 and 153, no *Nitrotoga* was detected (Fig. S6 B&F). However, in samples fixed on day 71, *Nitrotoga* was detected, even though in a small fraction (Fig. S6D).

Once AOB and NOB showed to be the predominant microbial community members in pottered samples, EUB338, AOBmix and NOBmix probes were used to stain cryosectioned granules from day 71, 82 and 153 for visualisation of the distribution of AOB and NOB in the granular sludge (Fig. 6). Granules from day 71 and 82 had gaps on the surface layer, most likely due to the cutting process. For both samples, most of the bacteria detected were AOB and were located on the surface layer (Fig. 6A and C). NOB were found both on the surface layer of the granule and in the inner core, although few active clusters were detected (Fig. 6B and D). For a cryosectioned granule fixed on day 153, NOB were mainly located inside of the granules while AOB dominated the surface layer (Fig. 6E and F).

However, obtaining statistically solid evidence demonstrating that the stratification was deteriorated in days 71 and 82 only based on the analysis of FISH pictures, would require a large number of samples (granules) and fields per sample being analysed. Of course, this would be a time consuming and costly task. Here we used the pictures obtained through FISH coupled to confocal microscopy on cryosectioned granules just as indicative of the findings achieved with the in-depth analysis of the experimental batch tests and the theoretical considerations discussed in view of the results obtained through mathematical modelling.

Both *Nitrospira* and *Nitrobacter* genera were detected in the granular sludge before starting the operation here reported (Poot et al., 2016). Their relative abundances during the present study were not further investigated. However, after the period reported in this manuscript (ca. 150 days) we found a very comparable response of the system through in-situ batch tests (at days 49 and 149), thus even if there were putative microbial population dynamics, the operation or kinetic response of the system was comparable. Some NOB species i.e. *Nitrotoga* were not detected on day 153, whereas they were present on day 71 (Fig. S6 D, F). Both recovery of stratification and decay of *Nitrotoga* correlated to the recovery of efficient NOB repression, although the cell numbers were so low that a solid conclusion could not be drawn.

3.7. Short & long term effects of DO concentration when lowering temperature

The response of the reactor after decreasing the temperature presents a pattern known as inverse response. Inverse response is typical of processes in which two opposite additive effects simultaneously occur (Stephanopoulos, 1984). One of the processes acts faster, but the second (slower) one dominates the final response of the process. When decreasing the temperature in the reactor, there is a decrease of the ammonium oxidation rate which left more oxygen available to NOB (this is a rather fast response), resulting in an increase of the nitrate production rate. However, the second (slower) process is that the higher DO concentration in the AOB layer would result in a thicker (or denser) AOB layer in the granular sludge. That could eventually deplete the oxygen, preventing nitrite oxidation by NOB occupying inner positions in the granule. This (slower) process dominates the overall response of the system in the long term, and this is possibly the reason why a stable nitritation recovered. This overall response of the system is visible when analysing the reactor operation (Fig. 1).

The short-term effects could be easily assessed by means of the in-situ batch tests described here. As a consequence, if the operator could decide to decrease the DO concentration to decrease nitrate production, a smaller DO concentration imposed would slow down the transient period towards stable nitritation. The smaller DO concentration imposed would impact the growth rate of AOB. At a lower DO the time required to obtain a thicker or denser AOB layer is expected to be longer. So, in that situation, tolerating a transient nitrate production might be of interest for a fast recovery of the process performance in the long term.

3.8. Further implications of the findings

The need for a higher ammonium concentration at low temperatures (i.e. below 20 °C) to maintain stable nitritation (i.e. efficient NOB repression) fits with a process strategy of a two-stage N-removal. Compared to one-stage nitrogen removal, where bulk ammonium must be low to accomplish with discharging limits, two-stage nitrogen removal allows for an ammonium concentration usually in a range 20–30 mg-N-NH_4^+/L in the partial nitritation reactor. However, the stability of PN reactors requires of a biofilm structure in which nitrifier guilds are stratified, with AOB occupying the external shell (Picioreanu et al., 2016, Poot et al., 2016, this study). Given the importance of the thickness of the AOB layer for the process performance at low temperatures, an overcapacity in terms of AOB should be achieved/maintained during the operation at high temperatures. This overcapacity for ammonium oxidation is required to buffer the (negative) effects of temperature on AOB activity, as demonstrated with the experimental study in continuous operation (Fig. 1), in-situ batch tests (Fig. 4) and with the aid of the mathematical model (Figs. 2 and 3 & S1).

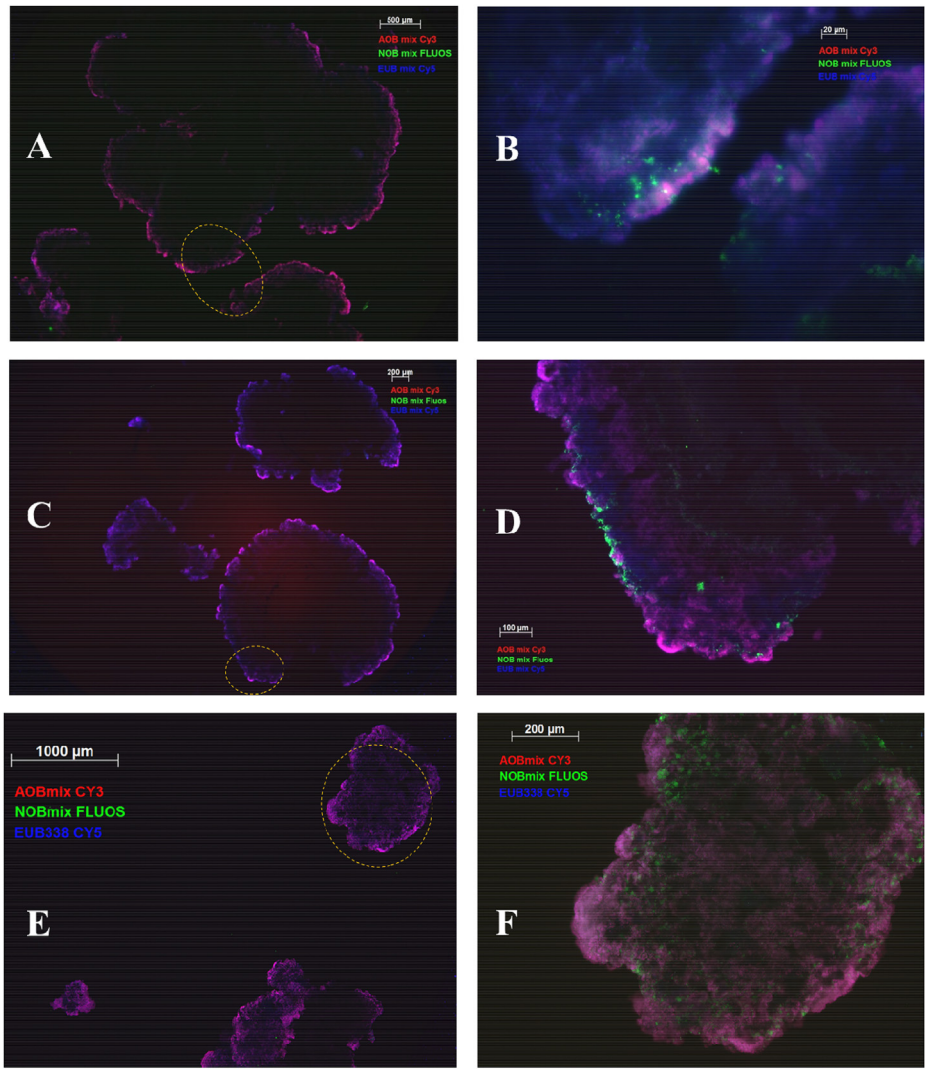

Fig. 6. FISH-cryosectioning of granules. Cy3(red), Fluos (green) and Cy5 (dark blue) were used to detect AOB, NOB and most bacteria, respectively. Slicing of granules at different days of continuous operation and magnification. Deteriorated stratification observed on: A) Day 71 (x2.5), B) Day 71 (x10), C) Day 82 (x2.5), D) Day 82 (x10). Recovered stratification on: E) Day 153 (x2.5) F) Day 153 (x10). (Reader is referred to the online version for colour images). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Regarding the inoculation of a PN reactor at mainstream conditions, a good recommendation would be to use granular sludge grown producing nitrite, by treating side-stream at high DO concentrations. Operating at high DO to produce the inoculum (as done in Bartrolí et al., 2010, with DO as high as 7 mg-O_2/L) treating side-stream wastewater (i.e. with high bulk ammonium concentrations 50-100 mg-N-NH_4^+/L) would result in a thick AOB layer. This thick AOB layer would enhance an initial overcapacity. When lower DO (ca. 1 mg-O_2/L) is used in mainstream conditions to minimize the energy consumption, the overcapacity of the inoculum would be of importance to buffer the effects of decreasing reactor temperatures towards the cold season. Therefore, this could be a good start-up strategy for two-stage PN/A in the main water line of WWTP.

This experimental study indicated how the trend for stratification of nitrifier guilds in granular sludge was happening at 15 °C, despite the maximum specific growth rate of AOB is (generally reported) lower than that of NOB. This finding would indicate that the stratification is happening due to a cross-feeding effect. In other words, the fact that the substrate of NOB is produced by AOB results in a stratified biofilm if there is enough substrate for AOB (i.e., ammonium is in excess), as previously hypothesized by Pérez et al. (2009). Possibly, this stratification trend is enhanced at high temperature (higher maximum growth rate for AOB) and due to the higher oxygen yield for AOB (i.e., higher maximum oxygen respiration rate, q_{O2max}).

3.9. Diagnose of stratification by in-situ batch tests

Generally, cryosectioning combined with FISH is used to assess the stratification of microbial populations in lab scale research (Almstrand et al., 2014; Kouba et al., 2014; Poot et al., 2016). However, this technique is time consuming and costly. A quick and cheap method to easily check if nitrifier guilds are mostly stratified could be the use of in-situ batch tests. The time course concentration of nitrate as obtained in the batch test of day 7 or 49, with an initial low nitrate production followed by a strong increase in the nitrate production rate when bulk ammonium concentration decreased is strongly indicative of a granular sludge with a high degree of stratification of the nitrifier guilds. Granular sludge (or biofilms) presenting a poor stratification of the nitrifier guilds

would present a nitrate time course in the in-situ batch test similar to that in Fig. 5A (day 92), in which initial nitrate production rate is very similar to the nitrate production rate when ammonium is depleted.

However, in-situ batch tests are a disturbance to reactor operation. The bulk ammonium concentration is allowed to become zero, leading to nitrate production (i.e. NOB growth). Therefore, it is advisable to use this diagnosis strategy not very frequently.

Finally, this methodology could be extrapolated to be used (or slightly adapted) to other similar cross-feeding cases, or to similar systems (like for instance PN/AMX granules).

4. Conclusions

- Continuous PN at 15 °C with granular sludge derived from a 20 °C culture, initially lead to proliferation of NOB in the granular sludge. Low nitrate effluent concentrations and stratification of nitrifier guilds were recovered by maintaining operational conditions with sufficient ammonium concentration to promote a faster growth of AOB than that of NOB.
- Batch tests can be easily used for diagnosis of both the required residual ammonium concentration for NOB repression and the stratification of nitrifier guilds.
- For a granular sludge with stratified nitrifier guilds (with AOB dominating in the external shell) a higher residual ammonium concentration is required to repress NOB activity at 15 °C compared to that at 20 °C (ca. 19 versus 7 mg-N/-NH_4^+/L at 15 and 20 °C, respectively). Therefore, a high residual ammonium concentration should be assured at low temperatures.

Acknowledgements

We would like to thank China Scholarship Council for offering funding for Ru Wang's stay in TU Delft. We would like to thank Udo van Dongen (formerly at TU Delft, currently at Bureau Waardenburg BV, The Nertherlands) for the help with the cryosectioning. This research was funded by the SIAM Gravitation Grant 024.002.002, the Netherlands Organization for Scientific Research. Support was also received from Ministerio de Economía, Industria y Competitividad, Gobierno de España (CTQ2017-82404-R).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2018.10.064.

References

Almstrand, R., Persson, F., Daims, H., Ekenberg, M., Christensson, M., Wilén, B.-M., Sörensson, F., Hermansson, M., 2014. Three-dimensional stratification of bacterial biofilm populations in a moving bed biofilm reactor for nitritation-anammox. Int. J. Mol. Sci. 15 (2), 2191–2206.

APHA, 2012. Standard Methods for the Examination of Water and Wastewater. A.P.H. Association. American Water Works Associationa (AWWA) & Water Environmental Federation (WEF), Washington, DC, USA.

Bartrolí, A., Pérez, J., Carrera, J., 2010. Applying ratio control in a continuous granular reactor to achieve full nitritation under stable operating conditions. Environ. Sci. Technol. 44 (23), 8930–8935.

Blackburne, R., Yuan, Z., Keller, J., 2008. Partial nitrification to nitrite using low dissolved oxygen concentration as the main selection factor. Biodegradation 19 (2), 303–312.

Cao, Y., van Loosdrecht, M.C., Daigger, G.T., 2017. Mainstream partial nitritation–anammox in municipal wastewater treatment: status, bottlenecks, and further studies. Appl. Microbiol. Biotechnol. 101 (4), 1365–1383.

De Clippeleir, H., Vlaeminck, S.E., De Wilde, F., Daeninck, K., Mosquera, M., Boeckx, P., Verstraete, W., Boon, N., 2013. One-stage partial nitritation/anammox at 15 °C on pretreated sewage: feasibility demonstration at lab-scale. Appl. Microbiol. Biotechnol. 97 (23), 10199–10210.

Garrido, J., Van Benthum, W., Van Loosdrecht, M., Heijnen, J., 1997. Influence of dissolved oxygen concentration on nitrite accumulation in a biofilm airlift suspension reactor. Biotechnol. Bioeng. 53 (2), 168–178.

Guisasola, A., Jubany, I., Baeza, J.A., Carrera, J., Lafuente, J., 2005. Respirometric estimation of the oxygen affinity constants for biological ammonium and nitrite oxidation. J. Chem. Technol. Biotechnol. 80 (4), 388–396.

Hellinga, C., Schellen, A., Mulder, J.W., Van Loosdrecht, M., Heijnen, J., 1998. The SHARON process: an innovative method for nitrogen removal from ammonium-rich waste water. Water Sci. Technol. 37 (9), 135–142.

Hendrickx, T.L., Wang, Y., Kampman, C., Zeeman, G., Temmink, H., Buisman, C.J., 2012. Autotrophic nitrogen removal from low strength waste water at low temperature. Water Res. 46 (7), 2187–2193.

Hunik, J.H., Bos, C.G., van den Hoogen, M.P., De Gooijer, C.D., Tramper, J., 1994. Co-immobilized *Nitrosomonas europaea* and *Nitrobacter agilis* cells: validation of a dynamic model for simultaneous substrate conversion and growth in κ-carrageenan gel beads. Biotechnol. Bioeng. 43 (11), 1153–1163.

Isanta, E., Reino, C., Carrera, J., Pérez, J., 2015. Stable partial nitritation for low-strength wastewater at low temperature in an aerobic granular reactor. Water Res. 80, 149–158.

Jemaat, Z., Bartrolí, A., Isanta, E., Carrera, J., Suárez-Ojeda, M.E., Pérez, J., 2013. Closed-loop control of ammonium concentration in nitritation: convenient for reactor operation but also for modeling. Bioresour. Technol. 128, 655–663.

Jubany, I., Carrera, J., Lafuente, J., Baeza, J.A., 2008. Start-up of a nitrification system with automatic control to treat highly concentrated ammonium wastewater: experimental results and modeling. Chem. Eng. J. 144 (3), 407–419.

Kouba, V., Catrysse, M., Stryjova, H., Jonatova, I., Volcke, E.I., Svehla, P., Bartacek, J., 2014. The impact of influent total ammonium nitrogen concentration on nitrite-oxidizing bacteria inhibition in moving bed biofilm reactor. Water Sci. Technol. 69 (6), 1227–1233.

Lackner, S., Gilbert, E.M., Vlaeminck, S.E., Joss, A., Horn, H., van Loosdrecht, M.C., 2014. Full-scale partial nitritation/anammox experiences – an application survey. Water Res. 55, 292–303.

Liu, G., Wang, J., 2013. Long-term low DO enriches and shifts nitrifier community in activated sludge. Environ. Sci. Technol. 47 (10), 5109–5117.

Lotti, T., Kleerebezem, R., van Erp Taalman Kip, C., Hendrickx, T.L., Kruit, J., Hoekstra, M., van Loosdrecht, M.C., 2014. Anammox growth on pretreated municipal wastewater. Environ. Sci. Technol. 48 (14), 7874–7880.

Lücker, S., Schwarz, J., Gruber-Dorninger, C., Spieck, E., Wagner, M., Daims, H., 2015. *Nitrotoga*-like bacteria are previously unrecognized key nitrite oxidizers in full-scale wastewater treatment plants. ISME J. 9 (3), 708–720.

Ma, B., Zhang, S., Zhang, L., Yi, P., Wang, J., Wang, S., Peng, Y., 2011. The feasibility of using a two-stage autotrophic nitrogen removal process to treat sewage. Bioresour. Technol. 102 (17), 8331–8334.

Manser, R., Gujer, W., Siegrist, H., 2005. Consequences of mass transfer effects on the kinetics of nitrifiers. Water Res. 39 (19), 4633–4642.

Nowka, B., Daims, H., Spieck, E., 2015. Comparison of oxidation kinetics of nitrite-oxidizing bacteria: nitrite availability as a key factor in niche differentiation. Appl. Environ. Microbiol. 81 (2), 745–753.

Peng, Y., Zhu, G., 2006. Biological nitrogen removal with nitrification and denitrification via nitrite pathway. Appl. Microbiol. Biotechnol. 73 (1), 15–26.

Pérez, J., Costa, E., Kreft, J.U., 2009. Conditions for partial nitrification in biofilm reactors and a kinetic explanation. Biotechnol. Bioeng. 103 (2), 282–295.

Pérez, J., Isanta, E., Carrera, J., 2015. Would a two-stage N-removal be a suitable technology to implement at full scale the use of anammox for sewage treatment? Water Sci. Technol. 72 (6), 858–864.

Pérez, J., Lotti, T., Kleerebezem, R., Picioreanu, C., van Loosdrecht, M.C., 2014. Outcompeting nitrite-oxidizing bacteria in single-stage nitrogen removal in sewage treatment plants: a model-based study. Water Res. 66, 208–218.

Pérez, J., Montesinos, J., Albiol, J., Godia, F., 2004. Nitrification by immobilized cells in a micro-ecological life support system using packed-bed bioreactors: an engineering study. J. Chem. Technol. Biotechnol. 79 (7), 742–754.

Picioreanu, C., Pérez, J., van Loosdrecht, M.C., 2016. Impact of cell cluster size on apparent half-saturation coefficients for oxygen in nitrifying sludge and biofilms. Water Res. 106, 371–382.

Picioreanu, C., Van Loosdrecht, M., Heijnen, J., 1997. Modelling the effect of oxygen concentration on nitrite accumulation in a biofilm airlift suspension reactor. Water Sci. Technol. 36 (1), 147–156.

Poot, V., Hoekstra, M., Geleijnse, M.A., van Loosdrecht, M.C., Pérez, J., 2016. Effects of the residual ammonium concentration on NOB repression during partial nitritation with granular sludge. Water Res. 106, 518–530.

Regmi, P., Miller, M.W., Holgate, B., Bunce, R., Park, H., Chandran, K., Wett, B., Murthy, S., Bott, C.B., 2014. Control of aeration, aerobic SRT and COD input for mainstream nitritation/denitritation. Water Res. 57, 162–171.

Reino, C., Suárez-Ojeda, M.E., Pérez, J., Carrera, J., 2016. Kinetic and microbiological characterization of aerobic granules performing partial nitritation of a low-strength wastewater at 10°C. Water Res. 101, 147–156.

Reino, C., Suárez-Ojeda, M.E., Pérez, J., Carrera, J., 2018. Stable long-term operation of an upflow anammox sludge bed reactor at mainstream conditions. Water Res. 128, 331–340.

Reino, C., van Loosdrecht, M.C., Carrera, J., Pérez, J., 2017. Effect of temperature on N_2O emissions from a highly enriched nitrifying granular sludge performing partial nitritation of a low-strength wastewater. Chemosphere 185, 336–343.

Sliekers, A.O., Derwort, N., Gomez, J.C., Strous, M., Kuenen, J., Jetten, M., 2002. Completely autotrophic nitrogen removal over nitrite in one single reactor. Water Res. 36 (10), 2475–2482.

Stephanopoulos, G., 1984. Chemical Process Control: an Introduction to Theory and Practice. Prentice Hall, New Jersey.

Third, K., Sliekers, A.O., Kuenen, J., Jetten, M., 2001. The CANON system (completely autotrophic nitrogen-removal over nitrite) under ammonium limitation: interaction and competition between three groups of bacteria. Syst. Appl. Microbiol. 24 (4), 588–596.

Volcke, E., Picioreanu, C., De Baets, B., Van Loosdrecht, M., 2010. Effect of granule size on autotrophic nitrogen removal in a granular sludge reactor. Environ. Technol. 31 (11), 1271–1280.

Wiesmann, U., 1994. Biological nitrogen removal from wastewater. In: Biotechnics/wastewater. Advances in Biochemical Engineering/Biotechnology, vol. 51. Springer, Berlin, Heidelberg.

Winkler, M.K., Kleerebezem, R., Kuenen, J.G., Yang, J., van Loosdrecht, M.C., 2011. Segregation of biomass in cyclic anaerobic/aerobic granular sludge allows the enrichment of anaerobic ammonium oxidizing bacteria at low temperatures. Environ. Sci. Technol. 45 (17), 7330–7337.