Stable partial nitritation for low strength wastewater at low temperature in an aerobic granular reactor

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Abstract

Partial nitritation for a low strength wastewater at low temperature was stably achieved in an aerobic granular reactor. A bench-scale granular sludge bioreactor was operated in continuous mode treating an influent of 70 mg $N\text{-}NH_4^+$ L^{-1} to mimic pretreated municipal nitrogenous wastewater and the temperature was progressively decreased from 30 to 12.5 ºC. A suitable effluent nitrite to ammonium concentrations ratio to a subsequent anammox reactor was maintained stable during 300 days at 12.5ºC. The average applied nitrogen loading rate at 12.5ºC was 0.7±0.3 g N L^{-1} d^{-1}, with an effluent nitrate concentration of only 2.5±0.7 mg $N\text{-}NO_3^-$ L^{-1}. The biomass fraction of nitrite-oxidizing bacteria (NOB) in the granular sludge decreased from 19% to only 1% in 6 months of reactor operation at 12.5ºC. *Nitrobacter* spp. where found as the dominant

NOB population, whereas *Nitrospira* spp. were not detected. Simulations indicated that: (i) NOB would only be effectively repressed when their oxygen half-saturation coefficient was higher than that of ammonia-oxidizing bacteria; and (ii) a lower specific growth rate of NOB was maintained at any point in the biofilm (even at 12.5ºC) due to the bulk ammonium concentration imposed through the control strategy.

Keywords

Mainstream; NOB repression; partial nitritation; modeling

1. Introduction

For the achievement of sustainable (energy-neutral or even energy-positive) wastewater treatment plants the use of anammox for sewage treatment has been proposed (Kartal et al., 2010). The performance of one-stage nitrogen (N) removal of pretreated municipal nitrogenous wastewater has been tested with sequencing batch reactors (SBR) as a first approach (Winkler et al., 2011; Hu et al., 2013). In many of the studies, the known weak point of those trials is that nitrite-oxidizing bacteria (NOB) developed in the long term operation, triggering the production of nitrate, and decreasing importantly the N-removal performance with anammox (Winkler et al., 2011; De Clippeleir et al., 2013). Even in the treatment of the sidestream (reject water), with more advantageous conditions for anammox-based N-removal, the development of NOB in one-stage granular reactors was a problematic issue during the maintenance routines, like short-term aeration pulses, which are thought to lead to healthy NOB population (Joss et al., 2011).

A two-stage N-removal system operating in continuous mode could be thought also as an appealing solution for sewage treatment (Regmi et al., 2014). In fact, a two-stage N-removal system was proposed as a potential alternative (nitritation with activated sludge, (Ma et al., 2011); nitritation with granular reactor, (Torà et al., 2013)). In the past, poor results were reported, and little attention had been paid to partial nitritation with biofilm reactors either because such a process was thought difficult to be maintained in the long term (Garrido et al., 1997; Fux et al., 2004) or because trials yielded not the expected results (Bernet et al., 2005). However, stable nitritation in biofilm reactors operating in continuous mode have been reported for the specific treatment of the sidestream (Torà et al., 2013) and other types of rich ammonium wastewaters (Tokutomi, 2004; Bougard et al., 2006; Bartrolí et al., 2010) at temperatures over 20ºC. Also the process has been deeply studied through mathematical modeling (Pérez et al., 2009; Brockmann et al., 2010; Jemaat et al., 2013). The success of such a treatment relies in the use of a control strategy to maintain the adequate ratio between oxygen and ammonium concentrations in the reactor bulk liquid, as to repress NOB activity in the biofilm (automatic control for partial nitrification to nitrite in biofilm reactors, ANFIBIO) (Bartrolí et al., 2010; Jemaat et al., 2013). However, when applying such a strategy to the mainstream, two different challenges could be outlined: (i) the partial nitritation reactor would need to produce the adequate ratio between ammonium and nitrite concentrations as to feed a subsequent anammox reactor and (ii) to the best of our knowledge, a stable partial nitritation reactor (achieving an effluent with a nitrite/ammonium ratio of 1), with floccular, attached or granular biomass, at temperatures lower than 15 ºC and treating low-strength ammonium wastewaters has not been reported. Only some studies achieved stable full nitritation in SBRs at these conditions but treating low nitrogen loading rates, NLR: 0.05-0.10 g N L^{-1} d^{-1} (Yuan

and Oleszkiewicz, 2011; Gu et al., 2012). Other reactors operated at higher NLRs showed sudden deterioration of the nitritation at temperatures lower than 15 ºC (Yamamoto et al., 2006).

Here, we would like to demonstrate the feasibility of partial nitritation in a granular reactor operating in continuous mode at low temperatures, treating a wastewater with low N concentrations. Microbiological analyses and mathematical modeling tools will be used to better understand the experimental results.

2. Materials and Methods

2.1 Reactor set-up, inoculum and wastewater

An airlift reactor with working volume of 2.5 L was used; see a detailed diagram in Figure 1. Compressed air was supplied through an air diffuser placed at the bottom of the reactor and manually manipulated to maintain the dissolved oxygen (DO) concentration in the bulk liquid in the range 1–5 mg O_2 L^{-1}. There was not a closed loop DO control and the air flow rate was weekly selected to maintain the DO level inside the mentioned range. The DO concentration in the bulk liquid was measured on-line by means of a DO electrode (DO 60-50, Crison Instruments, Spain). The pH was measured online with a pH probe (pH 52-10, Crison Instruments, Spain) and automatically controlled at 8.0±0.1 by dosing a Na_2CO_3 0.5 M solution. Temperature was controlled at different values in the experiments, 30.0±0.1, 20.0±0.1, 15.0±0.1 and 12.5±0.1 ºC by means of a cooling system (E100, LAUDA, Germany) and an electric heater (HBSI 0.8m, HORST, Germany) connected to a temperature controller (BS-2400, Desin

Instruments, Spain). The total ammonia nitrogen (TAN = $N\text{-}NH_4^+ + N\text{-}NH_3$) in the bulk liquid was controlled varying the inflow rate. Before day 71, the TAN control was made manually based on the off-line bulk liquid TAN concentration measurement. From day 71 to day 250, the TAN control was automated by using an on-line TAN probe (NH4Dsc probe with a Cartrical cartridge, Hach Lange, Germany) using a proportional controller. From day 250 onwards, the TAN control was again made manually to check the feasibility of implementing this technology without a TAN on-line sensor.

The airlift reactor was inoculated with a nitrifying granular sludge. The origin of the inoculum was a granular sludge reactor treating real reject water, which was inoculated with activated sludge (Tora et al., 2013). *Nitrobacter* spp. was quantified through Fluorescence in situ hybridization (FISH) technique as the dominant NOB population in the inoculum, but small amounts of *Nitrospira* spp. could be expected, although were not quantified through FISH. The nitrifying granules were stored at 4ºC for 7 months after collection from the pilot reactor treating the reject water. The nitrifying granules had an average particle size of 0.5 mm (Torà et al., 2013).

The biofilm airlift reactor was fed with a synthetic influent mimicking the pretreated municipal wastewater from an anammox-based WWTP (Kartal et al., 2010). The pretreatment of the wastewater would consist of a classical WWTP primary treatment followed by the removal of the organic matter in a very high-loaded activated sludge reactor. The ammonium concentration of the wastewater also accounts for the ammonium coming from the sidestream produced after the digested sludge dewatering. Therefore, the resulting mineral medium contained (Hu et al., 2013), in average, 70 mg

126 $N\text{-}NH_4^+$ L^{-1} , added as NH_4Cl. The synthetic wastewater also contained 45 mg L^{-1}
127 KH_2PO_4, 90 mg L^{-1} $MgSO_4$, 40 mg L^{-1} $CaCl_2$ and 1 mL of trace elements solution per L
128 of influent (Guerrero et al., 2011).
129
130 2.2 Analytical methods
131
132 Liquid samples were periodically withdrawn from the reactor effluent to determine the
133 concentrations of TAN, total nitrite nitrogen (TNN = $N\text{-}NO_2^-$ + $N\text{-}HNO_2$) and nitrate.
134 TAN concentration was measured with an ammonium analyzer (AMTAX sc, Hach
135 Lange, Germany). Nitrate and TNN concentrations were analyzed with ionic
136 chromatography using an ICS-2000 Integrated Reagent-Free IC system (DIONEX
137 Corporation, USA) which performs ion analyses using suppressed conductivity
138 detection. Mixed liquor total suspended solids (TSS) and mixed liquor volatile
139 suspended solids (VSS) were analyzed according to Standard Methods (APHA, 1998).
140 Average particle size was measured by a laser particle size analysis system (Malvern
141 MasterSizer Series 2600, Malvern instruments Ltd., UK). The average settling velocity
142 was measured by recording the time taken for at least 40 individual granules to fall from
143 a certain height in a measuring cylinder filled with the synthetic medium described in
144 the previous section.
145
146 2.3. Fluorescence in situ hybridization (FISH)
147
148 FISH technique coupled with confocal laser scanning microscopy (CLSM) was used to
149 determine the relative abundances of ammonia-oxidizing bacteria (AOB), NOB and
150 anammox bacteria in the granules. Hybridizations were carried out using at the same

time a Cy3-labeled specific probe and Cy5-labeled general bacteria probe. The general probe was a 1:1:1 mixture of EUB338I, EUB338II, and EUB338III for all Bacteria. Specific and general probe sequences are described in Supporting Information (Table SI1 in Supporting Information). A Leica TCS SP2 AOBS confocal laser scanning microscope at a magnification of x63 (objective HCX PL APO ibd.B163 x1.4 oil) equipped with two HeNe lasers with light emission at 561 and 633nm was used for biomass quantification. For biomass fractions quantification, the granules were crushed using a mortar and a pestle and then, FISH procedure was followed. The quantification was performed following a modification of the procedure described in Jubany et al. (2009), where 40-50 microscopic fields were analysed, and a single z-position was selected based on the highest intensity for each sample.

2.4. Mathematical modeling

2.4.1. Biofilm model, kinetics and parameters

A one-dimensional biofilm model was developed to simulate the nitrifying biofilm airlift reactor performance based on Wanner and Reichert (1996) and implemented in the software package AQUASIM v.2.1d (Reichert, 1998).

The biomass species described as particulate compounds in the biofilm matrix were three: AOB, NOB and inert biomass. Biofilm area was described as a function of the granule radius, to correctly simulate the biofilm geometry (for further details see Jemaat et al., 2013). Total biofilm area was defined as a function of granule size and number of granules. A detachment rate was used to keep a constant biofilm thickness in steady

state at a predefined value. Detached biomass from the biofilm was considered as active following the same kinetics defined for the biomass in the biofilm. Attachment of biomass onto the biofilm surface has been neglected. For the sake of simplicity external mass transfer has been neglected. The porosity of the biofilm was fixed as 80% and kept constant during all the simulations. The microbial kinetics and the stoichiometry used are detailed in Supporting Information (Tables SI2-SI4). Other parameters related to the biofilm and used in the model are detailed in Supporting Information (Table SI5).

2.4.2. Process control modeling

The control strategy was also described by the model. Two different closed-loops were used: (i) one to maintain the TAN concentration in the bulk liquid of the nitritation reactor (i.e., the reactor effluent, considering a well-mixed liquid phase in the reactor) and (ii) a second one to control the DO concentration in the bulk liquid.

TAN concentration was maintained by regulating the inflow rate (as done experimentally in the reactor). The closed DO control loop was used to keep in the simulations the same DO applied in the experiments. For details about the mathematical expressions used, see Supporting Information.

2.4.3. Modeling strategy

For the description of the experiments, experimental data like pH, temperature, reactor volume, biomass concentration, granule size and characteristics of the influent were input to the model. Also the TAN set point imposed in the experiments and the

measured DO concentration were inputs for the model. The main model outputs are the values of TNN and nitrate concentrations in the effluent for each of the temperatures tested (20, 15 and 12.5 ºC) and flow-rate. The conditions used in the real start-up were imposed in the simulation, pursuing a fully dynamic description of the experiments. The initial biomass fractions of AOB and NOB in the biofilm were assumed to be 67 and 23% to adequately describe the measured initial nitrate production.

To investigate the mechanisms leading to stable partial nitritation at low temperatures, additional simulations were run. The impact of the oxygen affinity constant of NOB ($K_{O2,NOB}$) was investigated. NOB repression was investigated when (i) the oxygen affinity constants of NOB and AOB were assumed equal to the AOB affinity constant reported by Guisasola et al. (2005) ($K_{O2,NOB} = K_{O2,AOB} = 0.74$ mg N L^{-1}), and (ii) assuming NOB with a higher oxygen affinity, as the reported by Regmi et al. (2014), $K_{O2,NOB} < K_{O2,AOB}$ (0.16 and 0.74 mg N L^{-1}, respectively). Also, the model predictions at lower bulk ammonium concentration were explored to determine the importance of the ammonium concentration in NOB repression.

3. Results and Discussion

3.1. Reactor start-up

After the inoculation, the reactor was first left overnight in batch mode with an initial TAN concentration of 192 mg N L^{-1} and the temperature was set to 30 ºC to wake up the biomass. The day after, the continuous operation was started with an initial NLR of 0.27 g N L^{-1} d^{-1}. During the first 36 days, the temperature was progressively reduced

226 from 30 to 20 ºC, whereas the NLR was increased from 0.27 to 1.4 g N L^{-1} d^{-1} (Figure
227 2). Only during the first days of operation a significant amount of the ammonium was
228 oxidized to nitrate (up to 18 mg N-NO_3^- L^{-1} on day 2, see Figure 2C). However, the
229 nitrate production progressively decreased to only 0.4 mg N-NO_3^- L^{-1} on day 35. From
230 day 35 onwards, stable partial nitritation to nitrite was maintained in the reactor (Figure
231 2), see details in the next section.
232
233 From day 0 to 16, a fast decrease of the biomass concentration from 2.9 to 1.3 g VSS L^{-1}
234 occurred, while the particle size increase from 0.5 on day 0 to 1.2 mm on day 45
235 (Figure 2A).
236
237 3.2. Reactor performance at low temperatures
238
239 On day 36 the reactor temperature was decreased first to 20 ºC for a period of 40 days,
240 then to 15 ºC during 70 days and finally to 12.5 ºC during 300 days. Therefore, the
241 reactor was operated during more than 12 months at a temperature equal than or below
242 15 ºC.
243
244 As shown in Figure 2, stable partial nitritation was successfully maintained at any of the
245 temperatures tested by maintaining a very low DO/TAN concentrations ratio in the bulk
246 liquid of the reactor (i.e. very strong oxygen limiting conditions) to repress NOB
247 activity (Bartrolí et al., 2010; Jemaat et al., 2013). The on-line DO concentration is
248 plotted in Figure 2B to show that rather than the DO level, it is the high residual TAN
249 concentration (ca. 32 mg N L^{-1}, Fig. 2B) the reason why a very low DO/TAN
250 concentrations ratio is maintained throughout the reported period in the reactor bulk

liquid. This means that in the conditions tested, the influence of the DO concentration on the NOB repression is rather reduced, because the residual TAN concentration is high. Low effluent nitrate concentrations were measured; 0.3±0.1 and 0.4±0.1 mg N-NO_3^- L^{-1} at 20 and 15 ºC, respectively. When the temperature was decreased to 12.5 ºC, the effluent nitrate concentration slightly increased to an average value of 2.5±0.7 mg N-NO_3^- L^{-1}. Interestingly, the nitrate production did not increase further and remained rather stable. Note how an effluent nitrate concentration of 2.5±0.7 mg N-NO_3^- L^{-1} only means a 3.6 % of the influent ammonium oxidized to nitrate.

The average TNN/TAN concentrations ratio in the reactor effluent was 1.0±0.2 from days 40 to 150 and 1.2±0.3 from days 150 to 450. This ratio is suitable for a subsequent anammox step.

The ammonium oxidation rate (AOR) showed a decreasing trend with temperature during the first 200 days but AOR slightly increased after 100 days operating at 12.5 ºC (Figure 2B). This gain was probably due to the increase of the biomass concentration from day 200 onwards (Figure 2A). The specific ammonium oxidation rate (sAOR) achieved at 12.5 ºC (0.2±0.1 g N g^{-1} VSS d^{-1}) was considerably high compared with other reported nitrifying systems (Yuan et al., 2011; Gu et al., 2012). The sAOR at 12.5 ºC, 15 ºC (0.32±0.06 g N g^{-1} VSS d^{-1}) and 20 ºC (0.55±0.04 g N g^{-1} VSS d^{-1}) are comparable with the sAOR achieved in other nitrifying granular reactors also operated with a similar control strategy at 20 ºC (0.63±0.05 g N g^{-1} VSS d^{-1}, Jemaat et al., 2013) and 30 ºC (1.4±0.1 g N g^{-1} VSS d^{-1}, Bartrolí et al., 2010), and much higher than the 0.03 g N g^{-1} VSS d^{-1} reported by Hu et al. (2013) in a one-stage anammox reactor at 12ºC. The influence of the temperature in the nitritation process can be fitted to an Arrhenius-

276 type equation ($r_{nitT1} = r_{nitT2} \cdot \theta^{(T1-T2)}$), achieving a temperature coefficient of θ=1.13±0.03
277 which is in the range of the coefficients for nitrifying reactors in two-stage systems
278 (Carrera et al., 2003).
279
280 The size of the nitrifying granules quickly increased from the initial 0.5 mm to more
281 than 1.2 mm in only 45 days. After that, the particle size showed a decreasing trend to a
282 stable size around 0.7 mm (Figure 2A). The biomass concentration progressively
283 increased from 1.1 g VSS L^{-1} on day 77 to 2.1 g VSS L^{-1} on day 450 (Figure 2A). The
284 settling velocity of the granules had a rather constant value at ca. 25±2 m h^{-1} at any of
285 the temperatures tested.
286
287 On day 99 (at 15 ºC) a failure of the feeding pump left the reactor without feeding for
288 6h. Also on day 115, an electricity cut left the reactor with neither feeding nor pH
289 control during 3h, but with the aeration and temperature control correctly working.
290 Those days have been plotted with a NLR of 0 g N L^{-1} d^{-1} (Figure 2B). In both cases,
291 the DO concentration in the reactor increased to 8 mg O_2 L^{-1} for 1-2 h after the accident
292 due to the decrease of the AOB activity once ammonium was consumed. Both the high
293 oxygen concentration and the ammonium depletion enhanced the nitrate production
294 during the accidental periods, consequently, 4.5 and 2.6 mg N-NO_3^- L^{-1} were measured
295 on days 99 and 115, respectively. However, in all cases, the day after the restoring the
296 operational conditions, the nitrite and nitrate concentrations in the reactor were very
297 similar to those measured before the operational accident, indicating that the operational
298 strategy of applying a low DO/TAN concentrations ratio to repress NOB activity was
299 robust.
300

From day 250 onwards, the TAN control was switched from automatic to manual mode but the process remained stable. The slight oscillations in the TNN/TAN concentrations ratio around days 260 and 360 (Figure 2C) can be attributed to changes in the biomass concentration rather than control problems. The successful operation of the partial nitritation process with manual control at 12.5 ºC during more than 200 days demonstrated the high stability of this technology.

3.3. Microbial characterization of granules

Biomass samples from days 72 (20 ºC), 147 (15 ºC), 211 (12.5 ºC) and 391 (12.5 ºC) were analyzed using the FISH-CLSM technique to quantify the AOB, NOB and anammox biomass fractions at the different temperatures at which the reactor was operated. AOB was found to be the predominant microbial population at all temperatures with similar biomass fractions, around 70±8 %, in biomass samples from day 72, 147 and 211, while slightly increased to 81±12 % on day 391 (Table 1).

The NOB biomass fraction determined by FISH was relatively high and constant, around 18±8%, in biomass samples from day 72, 147 and 211 (Table 1) despite the NOB activity was effectively repressed. It could seem odd that there was such a high presence of NOB without nitrate production. One possible explanation could be that simultaneous denitrification occurred in the granules with organic matter coming from the biomass death. However, if this process was significant in the airlift reactor, there would be a non-fulfillment of the N-balance. To check this hypothesis, the N-balance fulfillment was calculated as:

% N-balance fulfillment = $[(TAN_{inf} - (TAN_{eff} + TNN_{eff} + Nitrate_{eff})/ TAN_{inf}]*100$

Equation 1

Where, TAN_{inf} was the TAN concentration in the influent and TAN_{eff}, TNN_{eff} and $Nitrate_{eff}$ were the TAN, TNN and nitrate concentrations in the effluent.

With the current definition of Eq. 1, N losses will be positive % N-balance fulfillment values. Although showing rather wide variation (see Figure SI1 in Supporting Information), the average % N-balance fulfillment value was 1.3%. This result indicates that, in average, 1.3% of the nitrogen of the influent is not in the effluent in form of ammonium, nitrite and nitrate. This nitrogen could be in form of nitrogen gas produced by denitrification but also in form of nitrous oxide produced by AOB through nitrifier denitrification. In any case, the non-fulfillment of the N-balance is too low to confirm the occurrence of a simultaneous denitrification process in the granules.
However, on day 391, NOB fraction decreased to 1±1%, indicating that NOB were almost washed out from the nitrifying granules.

Therefore, the long term application of a low DO/TAN concentrations ratio at low temperatures tended to wash out NOB, similarly to what Bartrolí et al. (2010) observed at 30ºC in a similar nitrifying granular reactor. The wash out process was much slower at 12.5 ºC (more than 6 months, Table 1) than that at 30ºC (ca. one month, Bartrolí et al., 2010). The granular biomass is essential to produce the oxygen gradients through the biofilm which are the main reason behind the efficiency of the strategy for NOB repression. The competition for space together with the oxygen limitation are needed. Consequently, the proposed strategy could not be used in activated sludge systems,

where there is no such competition for space and lower oxygen gradients are expected in the flocular sludge.

The main NOB genus at all the temperatures was *Nitrobacter*, whereas *Nitrospira* was not detected through FISH (Table 1). *Nitrobacter* are r-strategist microorganisms and they have higher maximum growth rate and nitrite half-saturation constants than those of *Nitrospira* which are K-strategist microorganisms (Blackburne et al., 2007; Downing and Nerenberg, 2008). Hence, *Nitrobacter* are favored in environments with accumulation of nitrite in the bulk liquid (Kim and Kim, 2006; Schramm et al., 2000), such as that in the granular reactor of this study (nitrite concentration ca. 30±5 mg N L^{-1}). In contrast, *Nitrospira* are favored in nitrite limiting environments, such as those in one-stage partial nitritation-anammox reactors (Winkler et al., 2011; De Clippeleir et al., 2013) or nitrifying reactors with low nitrite concentration in the bulk liquid (Regmi et al., 2014).

Finally, anammox could not be detected in any of the analyzed samples, neither in the sample on day 211, which was taken 7 months after the start-up of the nitrifying airlift reactor (Table 1). It is possible that anammox was not detected simply because it was not present in the inoculum. Anammox was included in the biofilm model to check what would be the impact on the reactor performance. The simulations indicated that less than 1.5% N conversion to N_2 by anammox was achieved at steady state. The biomass profiles in the biofilm obtained in the simulation (Figure SI2 in Supporting Information), show how anammox, despite colonizing the granule core, would poorly contribute to the overall ammonium conversion, and partial nitritation would remain still steady. The results are in agreement with those reported by Perez et al. (2014) and

376 Gilbert et al. (2014), in which the high performance of N removal for single-stage N
377 removal at low temperatures would require high residual ammonium and high granule
378 size.
379
380 3.4. Model-based assessment of NOB repression at low temperatures
381
382 As it was demonstrated in previous studies, the NOB repression in granular reactors is
383 achieved due to the strong oxygen limitation, assured by the control strategy, in which a
384 low DO/TAN concentrations ratio is maintained in the bulk liquid assuring an excess of
385 ammonium in the biofilm (Bartrolí et al., 2010; Jemaat et al., 2013). Reasons behind the
386 efficiency of this control strategy have been already discussed in depth in those
387 publications (Bartrolí et al., 2010; Jemaat et al., 2013), as well as the fundamental
388 aspects of how NOB repression is achieved in biofilm reactors (Pérez et al., 2009;
389 Brockmann and Morgenroth, 2010). However, all those studies were carried out at
390 temperatures above 20ºC and it is not clear if the same reasons are valid at the
391 experimental conditions of this study, because at temperatures below 20ºC, the specific
392 NOB growth rate is higher than that of AOB (Hunik et al., 1994). A mathematical
393 model was used to confirm the NOB repression mechanism in granular reactors at low
394 temperatures postulated in this study.
395
396 3.4.1. Model evaluation
397
398 The first 240 days of operation were used for model evaluation. This period was chosen
399 since both, the biomass concentration and the granular size achieved stable values
400 (Figure 2A). The simulated effluent concentrations of TAN, TNN and nitrate were

401 directly compared with those obtained in the experiments (Figure SI3 in Supporting
402 Information). The model predicted no nitrate accumulation for the three temperatures,
403 as obtained experimentally. The second major output of the model is the NLR applied to
404 the reactor, model predictions showed agreement with the experiments as shown in the
405 direct comparison of experimental and predicted flow-rates (Figure SI3 in Supporting
406 Information).
407
408 Maintaining the same operating conditions at 12.5ºC in long term simulation (i.e. steady
409 state) NOB were washed out from the reactor, in agreement with the experimental trend
410 (see NOB biomass fraction at day 391 in Table 1).
411
412 3.4.2. Importance of the difference in oxygen affinity between AOB and NOB for
413 effective NOB repression
414
415 When the oxygen affinity constants of NOB and AOB were assumed equal ($K_{O2,NOB}$ =
416 $K_{O2,AOB}$= 0.74 mg N L^{-1}) the model predictions indicate that nitrate accumulates in the
417 effluent because NOB repression is not efficient (Table 2). When a higher oxygen
418 affinity is considered for NOB $K_{O2,NOB} < K_{O2,AOB,}$ (0.16 and 0.74 mg N L^{-1},
419 respectively), even a higher nitrate concentration was found with the model (Table 2).
420 The simulation results indicated that if AOB oxygen affinity is equal or lower than that
421 of NOB, nitrite oxidizing activity is not effectively repressed despite applying low
422 DO/TAN concentration ratios in the bulk liquid, and nitrate is produced. In both cases
423 (for $K_{O2,AOB}$ = 0.74 and 0.16 mg N L^{-1}) for long term simulations (i.e. steady state) NOB
424 are not washed out from the reactor, contrary to what is observed experimentally (Table
425 1) and when using $K_{O2,NOB} > K_{O2,AOB}$.

Although it is generally accepted that NOB have lower oxygen affinity than AOB (Sin et al., 2008), Liu and Wang (2013) recently demonstrated that NOB can become better oxygen competitor than AOB under long term low DO conditions and as a result, nitrate is produced. Also, Regmi et al. (2014) found that the NOB population in their nitrifying reactor had a lower DO half-saturation constant than that of AOB and could compete with AOB for oxygen uptake at low DO concentrations. Interestingly, both studies found that the dominant NOB genus in their systems was *Nitrospira* instead of *Nitrobacter*.

Therefore, a scenario in which NOB have higher oxygen affinity than that of AOB is possible if the NOB population is enriched in *Nitrospira* instead of *Nitrobacter* (Liu and Wang, 2013; Regmi et al., 2014). Thus we hypothesize that a key aspect for achieving stable partial nitritation at temperatures below 20ºC could be to select a NOB population composed mainly by *Nitrobacter* instead of *Nitrospira*.

3.4.3. Importance of ammonium excess for effective NOB repression

As previously described, the control strategy applied in this study is based on maintaining a very low value of the DO/TAN concentrations ratio in the bulk liquid, thus assuring strong limiting conditions required to obtain the NOB repression in the granules. The importance of the oxygen affinity has been demonstrated in the previous section but also the importance of the ammonium excess in the bulk liquid was assessed with the model.

451 The usual $[TAN]_{SP}$=32 mg N L^{-1} used in previous section was the average experimental
452 TAN concentration in the airlift reactor throughout the study (Figure 2C). This TAN
453 concentration resulted from the oxidation to nitrite of 38 mg N L^{-1} of the influent TAN
454 concentration (70 mg N L^{-1}). Thus, the effluent of the airlift reactor had the proper
455 TNN/TAN ratio (38 mg N-NO_2^- L^{-1} / 32 mg N-NH_4^+ L^{-1} = 1.2) for a subsequent
456 anammox reactor. The inflow concentration (70 mg N L^{-1}) was chosen because is the
457 inflow concentration previously used in similar studies (De Clippeleir et al., 2013; Lotti
458 et al., 2014; Winkler et al., 2011). This inflow concentration would be the result of the
459 sum of the TAN coming with the urban wastewater plus the TAN coming with the
460 reject water. However, the inflow TAN concentration values may be lower if the reject
461 water was separately treated. In this case, a too low TAN concentration in the reactor
462 may compromise the stability of the partial nitritation, resulting in NOB proliferation.
463 An extremely low inflow TAN concentration could be around 20-25 mg N L^{-1}. In that
464 case, the TAN concentration in the reactor would be around 10 mg N L^{-1}.
465 A new dynamic simulation was run as to switch from the usual $[TAN]_{SP}$=30 mg N L^{-1} to
466 10 mg N L^{-1} and the ammonium and DO concentration profiles in the biofilm were
467 investigated at these two different ammonium setpoint values (Figure 3). When
468 decreasing the $[TAN]_{SP}$ to 10 mg N L^{-1}, ammonium was still in excess (see Figure 3B),
469 but the corresponding Monod term of the kinetics changed importantly (Figures 4A and
470 4B). This was the major change since oxygen was kept constant in the bulk at 2.2 mg O_2
471 L^{-1}. At $[TAN]_{SP}$= 10 mg N L^{-1} the AOB specific growth rate in the biofilm was lower
472 than that of NOB (see Figure 4D), reversing the previous trend found at $[TAN]_{SP}$=30
473 mg N L^{-1} (see Figure 4C). With the $[TAN]_{SP}$= 10 mg N L^{-1}, nitrate increased very fast in
474 the reactor (in a period of only hours, see Figure SI4 in Supporting Information), which
475 would lead to a stable NOB development in the biofilm.

Therefore, despite partial nitritation can be achieved in granular reactors in oxygen limiting conditions when the AOB population has a higher affinity for DO than that of the NOB, a certain excess of ammonium in the bulk liquid is required to keep the specific AOB growth rate higher than that of NOB. This finding is in full agreement with the results of a modelling study for single-stage N-removal in granular sludge, where the NOB repression also was related to the residual ammonium concentration (Perez et al., 2014).

3.5. Suitability of a two-stage autotrophic N-removal system for mainstream treatment

The strategy to split the N-removal in two different reactors has as main advantage that the anammox reactor can be operated anoxically, to avoid competition for nitrite by NOB, which is one of the main weak points of the use of an one-stage N-removal biofilm reactor to remove nitrogen in these conditions (Winkler et al., 2011; De Clippeleir et al., 2013), like genuinely proposed by Kartal et al. (2010).

Additionally, since the effluent of the partial nitritation reactor should be fed to a subsequent anammox reactor, nitrite will be always in excess. This type of operation favors the selection of *Nitrobacter*-like bacteria (r-strategist) instead of *Nitrospira*-like bacteria (K-strategist), enhancing the stability of the partial nitritation process in granular reactors due to the lower oxygen affinity of *Nitrobacter* compared to that of *Nitrospira* (as discussed in section 3.4.2). Furthermore, the presence of *Nitrobacter* can inhibit the growth of *Nitrospira*, increasing the stability of partial nitritation (Ahn et al., 2011; Wagner et al., 2002).

501

502 The operation of anammox reactors treating pretreated municipal wastewater at
503 temperatures below 20 ºC, as the one required in the two-stage N-removal system, has
504 been previously demonstrated (Lotti et al., 2014). In that study, a NLR of 0.6 g N L^{-1} d^{-1}
505 was achieved at 10 ºC using and upflow fluidized granular sludge reactor. Since the
506 average NLR applied in our partial nitritation reactor at 12.5 ºC was 0.7±0.3 g N L^{-1} d^{-1}
507 (Figure 2B), both processes could be easily integrated in series, in a two-stage
508 autotrophic N-removal system.

509

510 4. Conclusions

511

512 • Partial nitritation at low temperature was stably maintained at long-term in a
513 granular airlift reactor using a control strategy based on assuring an adequate ratio
514 between oxygen and ammonium concentrations in the reactor bulk liquid.
515 • NOB present in the granules during most of the operational period were identified as
516 *Nitrobacter* spp., in contrast to other autrotrophic N-removal systems operated at
517 low temperatures in which the predominant NOB were *Nitrospira* spp.
518 • In spite of the low temperature (12.5 ºC), NOB were completely washed-out from
519 the granules by long term effects of the control action.
520 • As demonstrated by modeling, despite partial nitritation can be achieved in granular
521 reactors in oxygen limiting conditions when the AOB population has a higher
522 affinity for DO than that of the NOB, a certain excess of ammonium in the bulk
523 liquid is required to keep the specific AOB growth rate higher than that of NOB.

524

525 5. Acknowledgements

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6. Supporting information available

Supporting information contains detailed description related to the modeling section, supplementary figures and tables.

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Figure captions and table legends

Figure 1. Schematic diagram of the reactor set-up showing the peripheral instrumentation and control loops. DO: dissolved oxygen; TAN: total ammonia nitrogen (TAN = $N\text{-}NH_4^+ + N\text{-}NH_3$).

Figure 2. Performance of the continuous granular airlift reactor treating a synthetic municipal mainstream wastewater. (A) Biomass concentration and particle size; (B) Nitrogen Loading Rate (NLR), Ammonium Oxidation Rate (AOR) and dissolved oxygen (DO); (C) Temperature in the airlift reactor; (D) Nitrogen compounds concentrations throughout the operational period.

Figure 3. Results obtained with the model. (A) Simulated biomass and oxygen biofilm profiles in the granular sludge at 12.5 ºC, day 248 when NOB are repressed. (B) Simulated dissolved oxygen (DO) and total ammonia nitrogen (TAN) biofilm profiles in the granular sludge at 12.5ºC for a $[TAN]_{SP}$ of 32 and 10 mg $N\text{-}NH_4^+$ L^{-1}, corresponding to days 248, and 254 respectively in the dynamic simulation detailed in SI2 in the Supporting information. Average concentrations of nitrite in the biofilm: 35 mg $N\text{-}NO_2^-$ L^{-1} at $[TAN]_{bulk}$=32 mg $N\text{-}NH_4^+$ L^{-1}; 34 mg $N\text{-}NO_2^-$ L^{-1} at $[TAN]_{bulk}$=10 mg $N\text{-}NH_4^+$ L^{-1}. Average concentrations of nitrate in the biofilm: 3.7 mg $N\text{-}NO_3^-$ L^{-1} at $[TAN]_{bulk}$=32 mg $N\text{-}NH_4^+$ L^{-1}; 34 mg $N\text{-}NO_3^-$ L^{-1} at $[TAN]_{bulk}$=10 mg $N\text{-}NH_4^+$ L^{-1}.

Figure 4. Results obtained with the model at 12.5ºC. Oxygen, ammonium and nitrite Monod terms in the granular sludge when maintaining the ammonium concentration in

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703

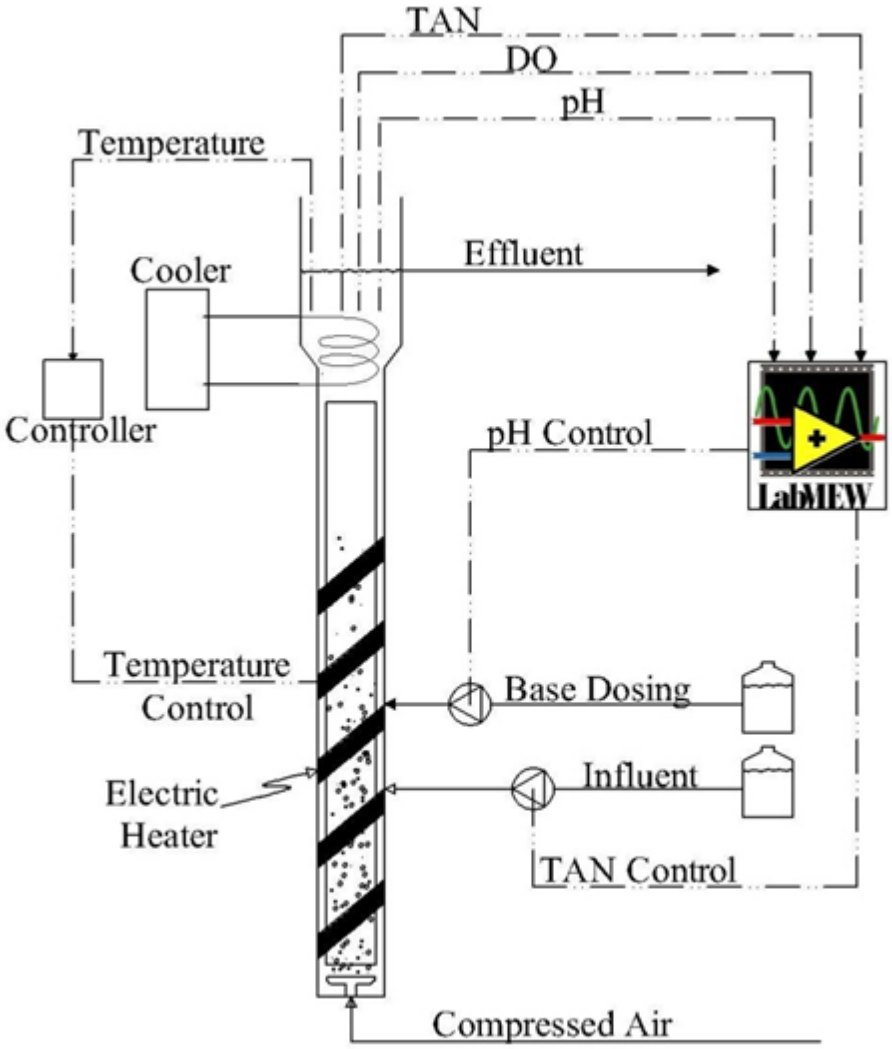
Table 1. Microbial fractions of AOB, NOB and anammox at the different temperatures tested, as determined by using the FISH technique. n/d=not detectable.

	20 ºC	15 ºC	12.5 ºC	
Day	72	147	211	391
AOB (%)	74±11	67±13	72±8	81±12
Nitrobacter spp. (%)	17±6	18±8	19±4	1±1
Nitrospira spp. (%)	n/d	n/d	n/d	n/d
Anammox (%)	n/d	n/d	n/d	n/d

Table 2. Effluent concentration of N-species on day 237 as predicted by the model for different values of the NOB oxygen half-saturation coefficient ($K_{O2,NOB}$) at 12.5ºC. NOB repression is efficient only for $K_{O2,NOB} > K_{O2,AOB}$.

	$K_{O2,NOB}$ (mg N L^{-1})	TAN (mg N L^{-1})	TNN (mg N L^{-1})	Nitrate (mg N L^{-1})
$K_{O2,NOB} > K_{O2,AOB}$ (model evaluation)	1.75	30	37	1.1
$K_{O2,NOB} = K_{O2,AOB}$	0.74	30	19	19
$K_{O2,NOB} < K_{O2,AOB}$	0.16	30	11	28

Figure



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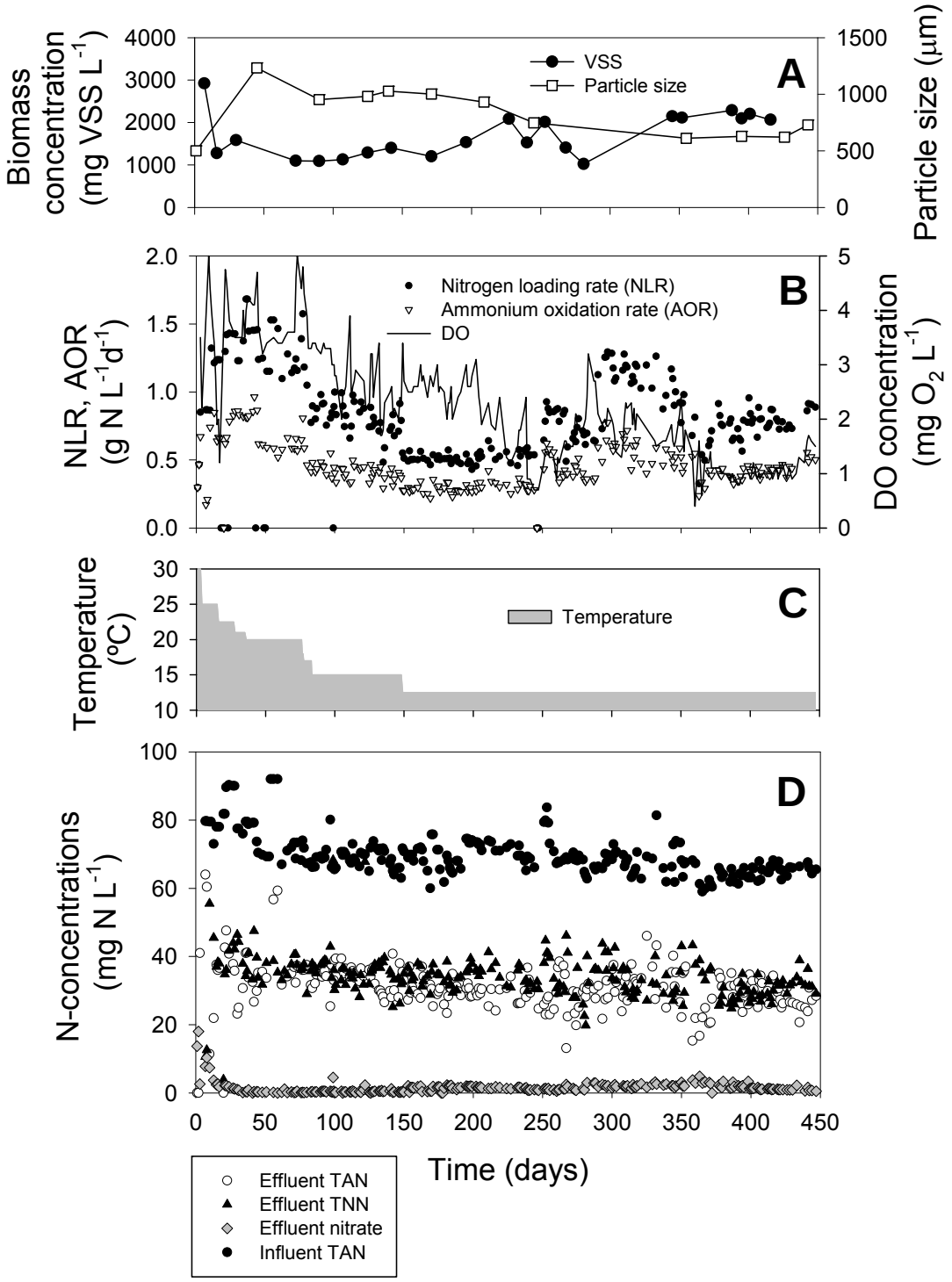

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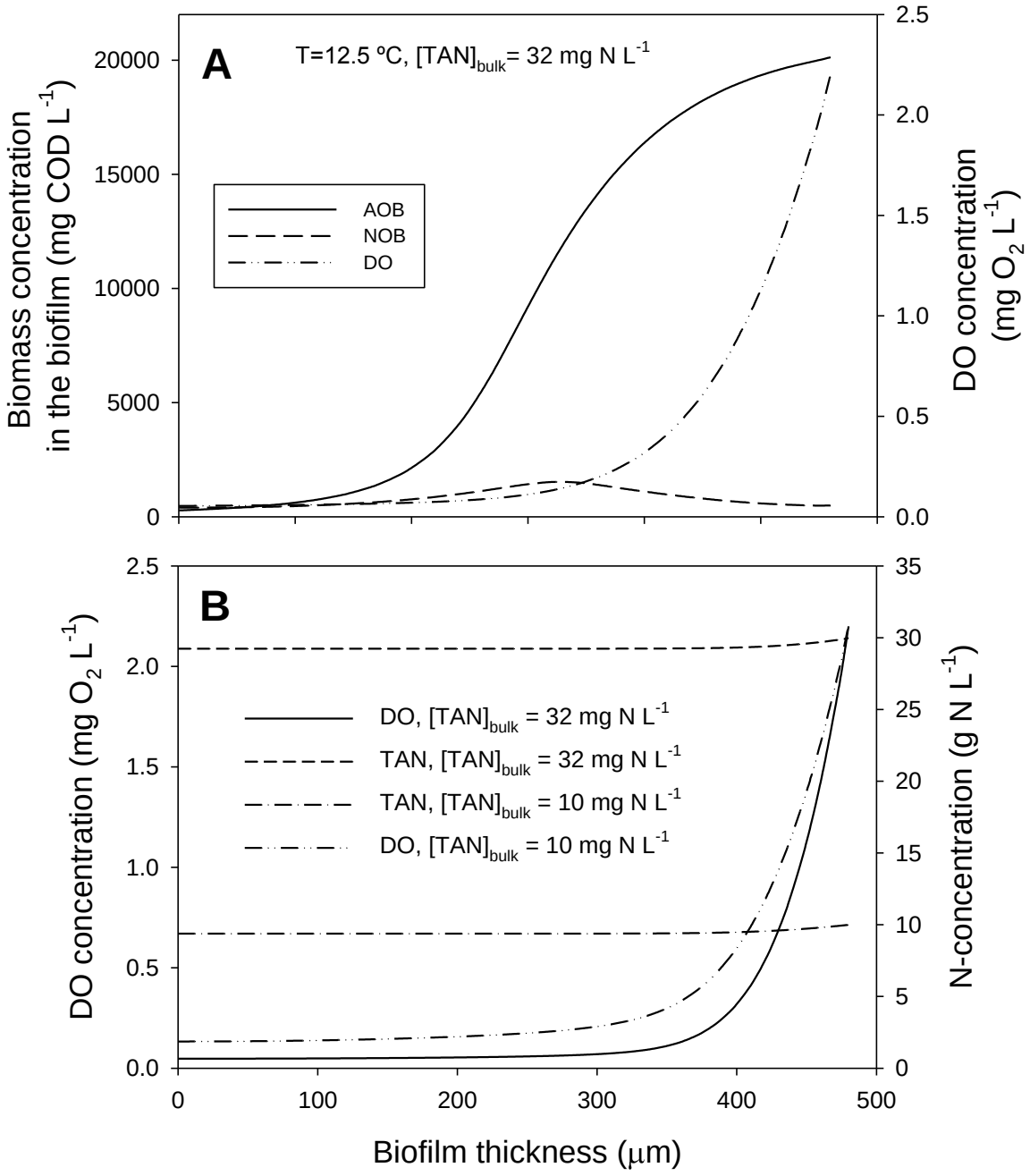

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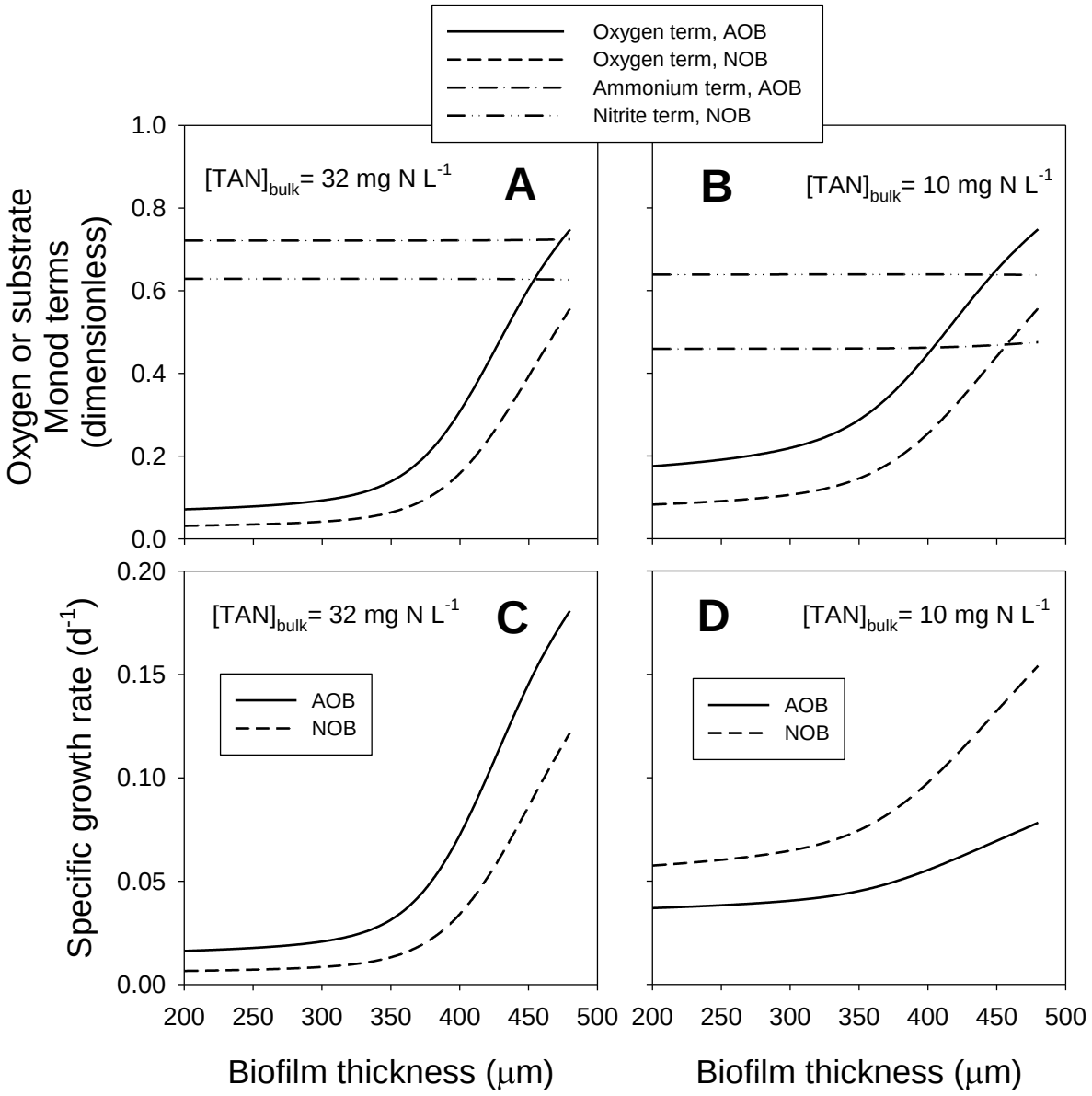

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