OPERATIONAL STRATEGIES TOWARDS NITRITATION-ANAMMOX IMPLEMENTATION FOR MAINSTREAM MUNICIPAL WASTEWATER TREATMENT

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DOCTORAL THESIS

Operational strategies towards nitritation-anammox implementation for mainstream municipal wastewater treatment

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Certificate of thesis direction

The thesis entitled "*Operational strategies towards nitritation-anammox implementation for mainstream municipal wastewater treatment*" has been supervised by Dr. Jesús Colprim Galceran, Dr. Maël Ruscalleda Beylier, and Dr. Maria Dolors Balaguer Condom, from University of Girona and it fulfil the requirements for the degree of Doctor. This certificate, original and signed by the supervisors, proves that the doctoral candidate has carried out the research work under the supervisor's guidance.

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Notation and abbreviations

µM	Micromolar
μ_{max}	Maximum growth rate
Aer	Aerobic
ALR	Ammonium loading rate
AMO	Ammonia monooxygenase
Anammox	Anoxic ammonium oxidation
AnAOB	Anammox bacteria
AnMBR	Anaerobic membrane bioreactor
Ano	Anoxic
ANR	Autotrophic nitrogen removal
AOA	Ammonium-oxidizing archaea
AOB	Ammonium-oxidizing bacteria
AOR	Maximum ammonium (AOR)
BNR	Biological nitrogen removal
BOD	Biochemical oxygen demand
CLSM	Confocal laser scanning microscopy
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
DO	Dissolved oxygen
Eff	Effluent
FA	Free ammonia
FISH	Fluorescence in situ hybridization
FNA	Free nitrous acid
HAO	Hydroxylamine oxidoreductase
HB	Heterotrophic bacteria
HDH	Hydrazine dehydrogenase

HRT	Hydraulic retention time
HZS	Hydrazine synthase
IC	Inorganic carbon
IFAS	Integrated fixed film activated sludge
In	Influent
kLa	Oxygen transfer rate coefficient
K_O	Half-saturation constant for oxygen
MBBR	Moving-bed biofilm reactor
MBR	Membrane bioreactor
MLE	Modified Ludzack and Ettinger
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
NCBI	National Center for Biotechnology Information
NirS	Nitrite oxidase
NLR	Nitrogen loading rate
NOB	Nitrite-oxidizing bacteria
NOR	Maximum nitrite rate
NRR	Nitrogen removal rate
OTR	Oxygen transfer rate
OTUs	Operational taxonomic units
OUR	Oxygen uptake rate
OUR_{AOB}	Oxygen uptake rate by ammonium-oxidizing bacteria
OUR_{NOB}	Oxygen uptake rate by nitrite-oxidizing bacteria
PN	Partial nitritation
PNA	Partial nitritation-anammox
qPCR	Quantitative polymerase chain reaction
rAnAOB	Conversion rates by AnAOB
rAOB	Conversion rates by AOB
$rNH_4^+{}_{AOB}$	Ammonium conversion rate by ammonium-oxidizing bacteria
$rNH_4^+{}_{max}$	Maximum ammonium oxidation rate

$rNO_2^-{}_{max}$	Maximum nitrite oxidation rate
$rNO_2^-{}_{NOB}$	Nitrite conversion rate by nitrite-oxidizing bacteria
rNOB	Conversion rates by NOB
rRNA	Ribosomal RNA
SAA	Specific anammox activity
SBR	Sequencing batch reactor
SHARON	Single reactor high activity ammonia removal over nitrite
Sobs	Observed operational taxonomic units
SOUR	Specific oxygen uptake rate
SRT	Solids retention time
TAN	Residual total ammonium
TCA	Reductive tricarboxylic acid cycle
UASB	Upflow anaerobic sludge bed (UASB)
VER	Volume exchange ratio
VSI	Volume settling index
VSS	Volatile suspended solids
WWTP	Wastewater treatment plant

Summary

The need for moving the current paradigm to sustainable and energy-neutral wastewater treatment plants (WWTP) is pushing for the development of novel approaches to obtaining efficient processes with less energy and chemical inputs. In this context, nitrogen removal from wastewater through anoxic ammonium oxidation (anammox)-based processes has pointed up due to their cost effectiveness compared to the conventional nitrification-denitrification processes. Anammox process has been commonly applied for the treatment of reject water, in the sidestream line of the urban WWTP. However, the mainstream line accounts for the majority of nitrogen in the WWTP and anammox implementation has been focused on dealing with sewage. The downsides of anammox at mainstream conditions are (i) the low growth rate, mainly during winter season when temperature can drop far below the optimum temperature for anammox growth, and (ii) robust operational strategy to control nitrite-oxidizing bacteria (NOB) growth, once they can compete for oxygen and nitrite, reducing nitrogen removal efficiencies. In this Thesis, operational strategies to achieve stable nitrogen removal by anammox process at mainstream conditions were studied.

An operational strategy that consisted in controlling the oxygen transfer according to the ammonium loading rate was evaluated in a one-stage partial nitritation-anammox (PNA) carried out in a sequencing batch reactor (SBR). This strategy resulted in extremely low bulk-liquid dissolved oxygen (DO) concentration. Both sidestream (high N strength- around 600 mg $N \cdot L^{-1}$, and temperature of 25 ^{o}C) and mainstream conditions (low N strength- around 75 mg $N \cdot L^{-1}$, temperatures of 25 and 15 ^{o}C) were assessed. At moderate temperature, average nitrogen removal rates (NRR) of 0.34 ± 0.05 and 0.37 ± 0.07 kg $N \cdot m^{-3} \cdot d^{-1}$ at sidestream and mainstream, respectively, were observed. NOB activity was completely suppressed at sidestream conditions due to a low DO availability and assisted by free ammonia (FA) inhibition. However, the results obtained demonstrate that uniquely low DO is not sufficient for complete NOB repression at mainstream

conditions in one-stage PNA, although the possibility of maintaining NOB activity limited at long term is highlighted. At 15 °C, anammox activity deteriorated and led to increased nitratation rates. Hence, removal rates were limited by lower anammox activity and higher oxidation of nitrite by NOB. Results showed that NOB suppression strongly relies on anammox process performance and activity imbalance with nitritation.

How the operational strategies applied to the one-stage PNA-SBR affected the microbial community was also explored with high-throughput techniques (16S rRNA sequencing and quantitative real-time polymerase chain reaction, qPCR). Sequencing analysis revealed the dominant bacterial groups in the microbial community that clustered within the phyla *Planctomycetes*, *Proteobacteria*, *Chloroflexi*, and *Bacteroidetes*. Aerobic ammonium-oxidizing (AOB) and anammox bacteria (AnAOB) were affiliated to the genera *Nitrosomonas* and *Candidatus* Kuenenia, respectively, and furthermore, they remained unaltered despite lowering N and temperature values. *Nitrospira* was the main NOB obtained when the SBR was switched from sidestream to mainstream conditions.

Studies were also conducted aiming to obtain one-stage PNA in a plug-flow reactor with an aerobic and anaerobic compartment. The temperature in this reactor was maintained at room temperature (between 16 and 27 °C), and DO lower than 0.20 mg $O_2 \cdot L^{-1}$ most of the experimental time. Nitrogen removal rate lower than 20 mg $N \cdot L^{-1} \cdot d^{-1}$ was achieved, because AnAOB growth was limited by NOB activity.

Finally, the effect of different inorganic carbon (IC) availability on nitrifying activities in a SBR operated at mainstream conditions (temperature of 15 °C) was also investigated. An influent NH_4^+:IC of 0.73 ± 0.03 allowed nitrite build-up in the SBR, and therefore, alongside DO control at lower level, a ratio of 80% for nitrite accumulation was reached and maintained for several weeks. Activity tests have also shown that the maximum ammonium conversion rates increased throughout the reactor operation, while the maximum nitrite oxidation dropped after IC limitation imposition.

In summary, this PhD Thesis brings insights into the strategies for anammox application at mainstream conditions, limitations which can be found at long-term operation and alternative parameters which have the potential to enhance process robustness.

Resum

La necessitat de canviar el paradigma actual de les plantes depuradores d'aigües residuals (EDARs) perquè siguin més sostenibles i amb un consum d’energia gairebé nul, està impulsant el desenvolupament de nous enfocaments per obtenir processos eficients en el requeriment d’energia i consums químics. En aquest context, la utilització de processos basats en l’oxidació anòxica d’amoni (anammox) per l’eliminació del nitrogen han destacat degut a la seva major efectivitat en relació amb els processos convencionals de nitrificació-desnitrificació. El procés anammox és habitualment aplicat per tractar l'aigua de retorns de digestió de fangs (amb alta concentració de nitrogen i temperatura de moderada a alta). Tanmateix, la línia principal d’aigua conté la majoria de nitrogen de l'EDAR (presenta baixa concentració de nitrogen i temperatura de moderada a baixa), i la implementació d’anammox s'ha centrat els darrers anys per a l’eliminació d’aquest nitrogen. Les dificultats per aplicar anammox en aquestes condicions són: (i) la baixa velocitat de creixement, principalment durant la temporada d'hivern quan la temperatura pot baixar molt per sota de la temperatura òptima pel creixement de les bactèries anammox; i (ii) absència d’una estratègia d’operació robusta que pugui controlar el creixement de les bacteris oxidants de nitrit (NOB), un cop aquestes poden competir per l'oxigen i el nitrit, reduint l'eficiència d'eliminació de nitrogen. En aquesta Tesi s’han estudiat estratègies operatives per aconseguir una eliminació estable del nitrogen a través d’un procés anammox aplicat a la línia principal d’aigües residuals.

Una estratègia d’operació va consistir en controlar la transferència d’oxigen segons la càrrega d’amoni que s’aportava al reactor, la qual va ser avaluada operant un reactor seqüencial en discontinu (SBR) amb els processes de nitritació parcial i anammox (PNA) en una única etapa. Aquesta estratègia va donar com a resultat una concentració d'oxigen dissolt (DO) molt baixa dins del reactor. L’estratègia va ser avaluada utilitzant l'aigua de retorns de digestió de fangs amb una elevada concentració de nitrogen (al

voltant de 600 mg $N \cdot L^{-1}$ a 25 ºC) i aigües de la línia principal de l'EDAR (al voltant de 75 mg $N \cdot L^{-1}$ a temperatures de 25 - 15 ºC). Per a la temperatura de 25 ºC, es van assolir valors mitjans d'eliminació volumètrica de nitrogen (NRR) de 0,34 ± 0,05 i 0,37 ± 0,07 kg $N \cdot m^{-3} \cdot d^{-1}$ per aigües amb alta i baixa concentració, respectivament. L'activitat de NOB va ser suprimida completament en condicions amb una elevada concentració de nitrogen, degut a la baixa disponibilitat de DO i per la inhibició d'amoníac lliure (FA). Tanmateix, els resultats obtinguts demostren que la DO excessivament baixa no és suficient per a la repressió completa de NOB en les condicions operades amb baixa concentració de nitrogen, tot i que es destaca la possibilitat de mantenir l'activitat NOB limitada a llarg termini. A la temperatura de 15 ºC, l'activitat d'anammox es va deteriorar i va provocar un augment de la producció de nitrat, degut a l'augment de la nitratació. Per tant, les velocitats d'eliminació van ser limitades per una menor activitat anammox i una major oxidació del nitrit per les NOB. Els resultats van mostrar que la supressió de NOB es basa en el rendiment del procés anammox i el desequilibri d'activitat amb la nitritació.

Els efectes de les estratègies d'operació aplicades als SBR en la comunitat microbiana també es van estudiar mitjançant l'ajuda de tècniques d'alt rendiment (seqüenciació 16S rRNA i reacció en cadena quantitativa de polimerasa en temps real, qPCR). L'anàlisi de seqüenciació va revelar els grups bacterians dominants en la comunitat microbiana que es van agrupar dins del fílums *Planctomycetes*, *Proteobacteria*, *Chloroflexi* i *Bacteroidetes*. Els bacteris aerobis d'oxidació d'amoni (AOB) i anammox (AnAOB) es van associar als gèneres *Nitrosomonas* i *Candidatus* Kuenenia, respectivament, i a més, es van mantenir inalterables tot i que van disminuir els valors de nitrogen i la temperatura. *Nitrospira* van ser els principals bacteris dins del grup de les NOB, quan el SBR va ser operat en condicions similars a les de la línia principal d'aigües d'una EDAR.

També es van realitzar estudis amb l'objectiu d'obtenir un PNA en un escenari amb un reactor en règim de flux de pistó amb dos compartiments, un aeròbic i altre anòxic. La temperatura en aquest reactor es va mantenir a temperatura ambient (entre 16 i 27 ºC) i DO inferior a 0,20 mg $O_2 \cdot L^{-1}$ la major part del temps experimental. Es va aconseguir una taxa d'eliminació de nitrogen inferior a 20 mg $N \cdot L^{-1} \cdot d^{-1}$, perquè el creixement d'AnAOB estava limitat per l'activitat de NOB.

Finalment, també es va investigar l'efecte de la disponibilitat de carboni inorgànic (IC) en les activitats nitrificants en el SBR amb una baixa concentració de nitrogen i temperatura de 15 °C. Un influent amb una ratio NH_4^+: IC de 0,73 ± 0,03 va permetre l'acumulació de nitrit en el SBR i, per tant, juntament amb el control de DO a baixa concentració, es va aconseguir mantenir una ratio del 80% per a l'acumulació de nitrit durant diverses setmanes. Els assajos d'activitat també han demostrat que les velocitats màximes de conversió de l'amoni es van incrementar durant tota l'operació del reactor, mentre que la màxima oxidació de nitrit es va reduir després de la limitació del IC.

En resum, aquesta Tesi doctoral aporta informació sobre les estratègies per a l'aplicació d’anammox a la línia principal d’aigües de l’EDAR, i les limitacions que es poden trobar en l'operació a llarg termini, juntament amb paràmetres alternatius que tenen el potencial de millorar la robustesa del procés.

Resumen

La necesidad de cambiar el paradigma actual de las plantas depuradoras de aguas residuales (EDARs), para que sean más sostenibles y con un consumo de energía casi nulo, se está impulsando el desarrollo de nuevos enfoques para obtener procesos eficientes en el requerimiento de energía y consumos químicos. En este contexto, la utilización de procesos basados en la oxidación anóxica de amonio (anammox) para la eliminación de nitrógeno han destacado debido su mayor efectividad en relación con los procesos convencionales de nitrificación-desnitrificación. El proceso anammox es comúnmente aplicado para tratar agua de retornos de digestión de fangos (con alta concentración de nitrógeno y temperatura de mediana a alta). Sin embargo, es en la línea principal del agua donde hay la mayoría del aporte de nitrógeno en la EDAR (baja concentración de nitrógeno y temperatura de moderada a baja), y la implementación de anammox se ha centrado en los últimos años en la eliminación de nitrógeno en esta línea principal de aguas de la EDAR. Las dificultades para aplicar anammox en éstas condiciones son: (i) la baja velocidad de crecimiento, principalmente durante la temporada de invierno cuando la temperatura puede bajar muy por debajo de la temperatura óptima para el crecimiento de las bacterias anammox; y (ii) ausencia de una estrategia de operación robusta que pueda controlar el crecimiento de bacterias oxidantes de nitrito (NOB), una vez que éstas pueden competir por el oxígeno y el nitrito, reduciendo la eficiencia de la eliminación de nitrógeno. En esta Tesis, se han estudiado estrategias operativas para conseguir una eliminación estable del nitrógeno mediante un proceso anammox aplicado a línea principal de aguas residuales.

Una estrategia de operación consistió en controlar la transferencia de oxígeno según la carga de amonio que se aportaba al reactor, y fue evaluada operando un reactor secuencial en discontinuo (SBR) con los procesos de nitritación parcial y anammox (PNA) en una única etapa. Ésta estrategia dio como resultado una concentración de oxígeno disuelto (DO) muy baja dentro del reactor. Dicha estrategia fue evaluada utilizando

aguas de retornos de digestión de fangos con una elevada concentración de nitrógeno (alrededor de 600 mg $N \cdot L^{-1}$ y temperatura de 25 ºC) y con aguas de la línea principal de la EDAR (alrededor de 75 mg $N \cdot L^{-1}$ y temperaturas de 25 y 15 ºC). Para la temperatura de 25 ºC se alcanzaron valores medios de eliminación volumétrica de nitrógeno (NRR) de 0,34 ± 0,05 y 0,37 ± 0,07 kg $N \cdot m^{-3} \cdot d^{-1}$ para aguas con alta y baja concentración, respectivamente. La actividad de NOB fue suprimida completamente en condiciones con una elevada concentración de nitrógeno, debida a la baja disponibilidad de DO y ayudada por la inhibición del amoníaco libre (FA). Sin embargo, los resultados obtenidos demuestran que la DO excesivamente baja no es suficiente para la represión completa de NOB en las condiciones operadas con baja concentración de nitrógeno, aunque se destaca la posibilidad de mantener la actividad NOB limitada a largo plazo. A la temperatura de 15 ºC, la actividad de anammox se deterioró y provocó un aumento en la producción de nitrato, debido al aumento de la nitratación. Por lo tanto, las velocidades de eliminación fueron limitadas por una menor actividad anammox y una mayor oxidación del nitrito por las NOB. Los resultados mostraron que la supresión de NOB se basa en el rendimiento del proceso anammox y el desequilibrio de la actividad con la nitritación.

Los efectos de las estrategias de operación, aplicadas en el SBR, sobre la comunidad microbiana también se estudiaron mediante la ayuda de técnicas de alto rendimiento (secuenciación 16S rRNA y reacción en cadena cuantitativa de polimerasa en tiempo real, qPCR). El análisis de secuenciación reveló los grupos bacterianos dominantes en la comunidad microbiana que se agruparon dentro de los filos *Planctomycetes*, *Proteobacteria*, *Chloroflexi* y *Bacteroidetes*. Las bacterias aeróbicas de oxidación de amonio (AOB) y anammox (AnAOB) se asociaron a los géneros *Nitrosomonas* y *Candidatus* Kuenenia, respectivamente, y además, se mantuvieron inalterables todo que disminuyó los valores nitrógeno y la temperatura. *Nitrospira* fueron las principales bacterias dentro del grupo de las NOB, cuando el SBR fue operado en condiciones similares a las de la línea principal de aguas residuales.

También se realizaron estudios con el objetivo de obtener un PNA de una etapa en un reactor en regimiento de flujo de pistón con dos compartimentos, uno aeróbico y otro anóxico. La temperatura en este reactor se mantuvo a temperatura ambiente (entre 16 y 27 ºC) y DO inferior a 0,20 mg $O_2 \cdot L^{-1}$ la mayor parte del tiempo experimental. Se logró una tasa de eliminación de nitrógeno inferior a 20 mg $N \cdot L^{-1} \cdot d^{-1}$, porque el crecimiento de AnAOB estaba limitado por la actividad de NOB.

Finalmente, también se investigó el efecto de la disponibilidad de carbono inorgánico (IC) en las actividades nitrificantes en el SBR con baja carga y temperatura de 15 ° C. Un afluente con una ratio NH_4^+: IC de 0,73 ± 0,03 permitió la acumulación de nitrito en el SBR y, por tanto, junto al control de DO a baja concentración, se consiguió mantener una ratio del 80% para a la acumulación de nitrito durante varias semanas. Los ensayos de actividad también han demostrado que las velocidades máximas de conversión del amonio se incrementaron durante toda la operación del reactor, mientras que la máxima oxidación de nitrito se redujo tras la limitación del IC.

En resumen, esta Tesis doctoral aporta información sobre las estrategias para la aplicación de anammox en la línea principal de la EDAR, y las limitaciones que se pueden encontrar en la operación a largo plazo, juntamente con parámetros alternativos que tienen el potencial de mejorar la robustez del proceso.

Chapter 1.

General introduction

1.1. Nitrogen on earth: major biological nitrogen conversion processes

The current biogeochemical nitrogen cycle took billions of years to be formed; however, due to the use of nitrogen fertilizing facing the global growing demand for food, nitrogen cycle has been severely modified over the last century, leading to artificial eutrophication of fresh water and coastal zones, and also rising the releasing of greenhouse gas nitrous oxide (N_2O) (Canfield et al., 2010). In lakes, the main symptom of eutrophication is cyanobacterial blooms, which can cause hypoxia, disrupt food webs, and can be also toxic (Conley et al., 2009). The increase of ammonia (NH_3) in fresh water has also harmful effects on aquatic animals. Other environmental problem regarding the alteration of nitrogen cycle is the presence of nitrate (NO_3^-) in groundwater due to anthropogenic sources (Almasri, 2007). Nitrate in drinking water has been epidemiologically associated to methaemoglobinaemia and thyroid effects, and it is recommended an exposition to a maximum of 11 mg NO_3^--N·L^{-1} (WHO, 2017).

Nitrogen species are among the most important nutrient sources in wastewater and its discharges can impact the water quality. Aiming at preventing water quality deterioration as a consequence of nitrogen concentration increase, several countries have been imposing strict legislation regarding nitrogen streams emissions from urban wastewater treatment plants (WWTP). In countries member of European Union, the directive 91/271/EEC of 21st May 1991 established a nitrogen threshold value of 10 mg N·L^{-1} (population equivalent > 10,000) for sensitive areas (aquatic bodies which are or may become eutrophic state, drinking water subjected to nitrate concentration higher than 50 mg N·L^{-1} and aquatic systems for the conservation of natural habitats).

Nitrogen circulates among lithosphere, atmosphere, and hydrosphere, traditionally comprising three major biological mediated processes: nitrogen fixation, nitrification, and denitrification. Thanks to a wide modern techniques, such as metagenomic-based and stable isotope, recent scientific advances have discovered and revealed other biological transformation processes and several microorganisms involved (Daims et al., 2016; Ferrera and Sánchez, 2016). These different nitrogen pathways are shown in Figure 1.1.

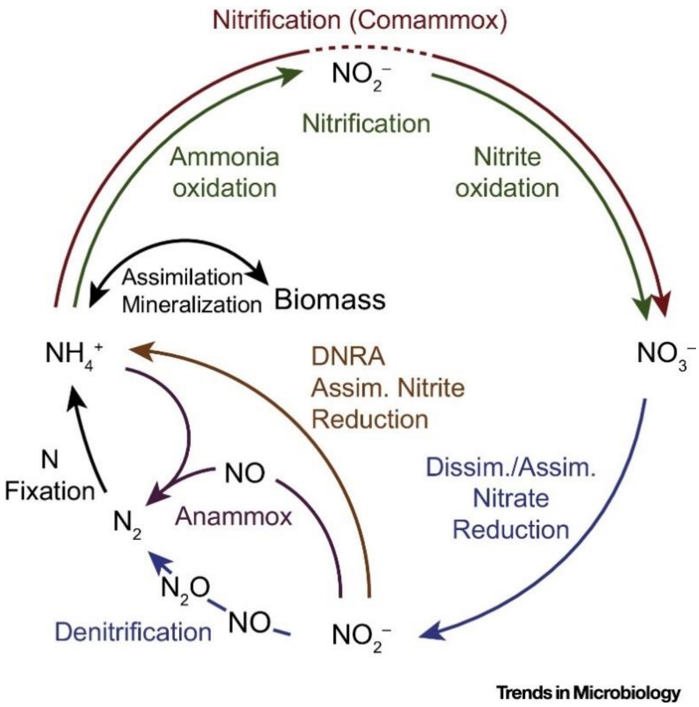

Figure 1.1. Schematic illustration of the key processes of the nitrogen cycle (Daims et al., 2016)

The knowledge achieved on nitrogen cycle have contributed to the implementation of biological wastewater treatment processes to remove nitrogen from wastewater. The nitrogen present in the water (mainly as ammonium – NH_4^+) is cycled by microorganisms through various biological processes, from the most conventional applied worldwide – nitrification and denitrification- to recent discovered processes, such as anammox. Whilst a broad range of processes has been showed in Figure 1.1, in this chapter only conventional nitrification-denitrification and anammox process will be addressed.

1.1.1. Autotrophic nitrification

Nitrification is an aerobic process carried out by two sequential steps: nitritation and nitratation. The first one consists in the oxidation of ammonium to nitrite by aerobic ammonium-oxidizing bacteria (AOB) and archaea (AOA). However, only AOB role will be discussed in this chapter due to their major importance on nitrification in WWTP, despite of the participation of AOA and some heterotrophs. The second step is the oxidation of nitrite to nitrate by nitrite-oxidizing bacteria (NOB). Among the bacteria responsible to trigger nitritation are *Nitrosomonas* and *Nitrosospira*, belonging to the class of β-Proteobacteria, and *Nitrosococcus* in the γ-Proteobacteria class (Schmidt et al., 2003). The bacteria within NOB genera identified so far and most widespread in WWTP are *Nitrobacter*, *Nitrospira*, and *Nitrotoga* (Koch et al., 2015). Nitrifying bacteria are chemolithoautotrophic and use CO_2 as carbon source for biomass synthesis. In nitritation, the first step of nitrification, ammonium is the electron donor, while nitrite is the electron donor for nitratation. Both AOB and NOB respiration use oxygen as electron acceptor. The overall stoichiometry of nitritation and nitratation are presented in equations 1.1 and 1.2, respectively (Grady et al., 1999):

$$\begin{aligned} &\mathrm{NH_4^+} + 1.38\ \mathrm{O_2} + 1.98\ \mathrm{HCO_3^-} \\ &\rightarrow 0.018\ \mathrm{C_5H_7NO_2} + 0.98\ \mathrm{NO_2^-} + 1.04\ \mathrm{H_2O} + 1.89\ \mathrm{H_2CO_3} \end{aligned} \qquad \text{(Eq. 1.1)}$$

$$\begin{aligned} &\mathrm{NO_2^-} + 0.0025\ \mathrm{NH_4^+} + 0.4875\ \mathrm{O_2} + 0.01\ \mathrm{H_2CO_3} + 0.0025\ \mathrm{HCO_3^-} \\ &\rightarrow 0.0025\ \mathrm{C_5H_7NO_2} + \mathrm{NO_3^-} + 0.0075\ \mathrm{H_2O} \end{aligned} \qquad \text{(Eq. 1.2)}$$

Two enzymes have been recognized as catalyzers of the nitritation step (Figure 1.2). At first, ammonia monooxygenase (AMO) acts on the oxidation of ammonia to hydroxylamine (NH_2OH). Further the hydroxylamine is oxidized to nitrite, by the enzyme hydroxylamine oxidoreductase (HAO). Four electrons are released during the hydroxylamine oxidation, being two of them returned to AMO, while the other two electrons become available for cell metabolism, specifically for inorganic carbon assimilation (Arp and Stein, 2003).

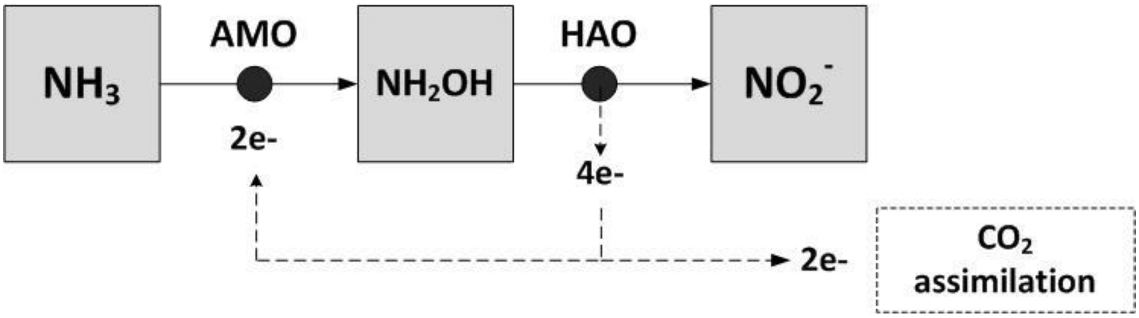

Figure 1.2. Nitritation pathway. Arrow line and arrow dashed line indicate reaction and electron flow. Modified from Arp and Stein (2003).

1.1.2. Heterotrophic denitrification

Denitrification consists in a heterotrophic process which nitrate is reduced to dinitrogen (N_2) under anoxic environments, and together with nitrification, both processes are usually applied in the biological nitrogen removal in WWTP. The process can be performed not only by bacteria, but also fungi and archaea (Thorndycroft et al., 2007). Several denitrifying bacteria have been identified in wastewater treatment, such as *Comamonadaceae*, *Azoarcus*, *Thauera*, *Dechloromonas*, *Candidatus* Accumulibacter, *Hydrogenophilaceae*, *Denitratisoma*, *Haliangium*, *Pseudomonas*, and others (Y. Chen et al., 2016; Zielińska et al., 2016). Most denitrifiers are facultative anaerobes, which are capable to use nitrite and nitrate instead of oxygen as electron acceptor (Wrage et al., 2001).

The reduction of nitrate into dinitrogen comprises several stages and there is a specific reductase enzyme that catalyzes the transfer of electrons to the nitrogen. The stages and respective enzymes involved in denitrification are shown in Figure 1.3. Nitric oxide (NO) and nitrous oxide (N_2O) are intermediates compounds of denitrification, and they can be emitted in case incomplete denitrification occurs (Wiesmann et al., 2006).

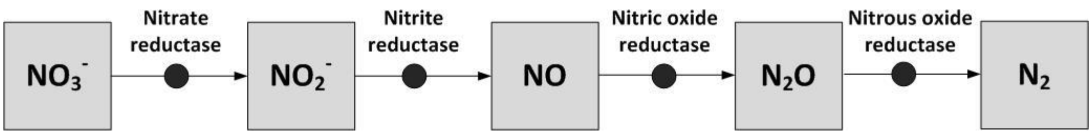

Figure 1.3. Overall denitrification pathway and enzymes involved (Wrage et al., 2001).

Organic matter is the carbon source and the electron donor for denitrifying bacteria. Wastewater harbor organic content for denitrification in WWTP, however, when wastewater does not contain enough organic matter several external carbon sources, such as methanol, ethanol, glucose, acetate, among others, can be also used (Akunna et al., 1993; Purtschert et al., 1996). The efficiency of denitrification relies on different factors, such as pH, temperature, DO concentration, etc. However, the C/N ratio is one of the most important factors, because it effects on the operating costs (Kim et al., 2008). Stoichiometric denitrification reaction with methanol, ethanol and "typical" organic matter from wastewater are presented in equations 1.3 - 1.5, respectively (Matějů et al., 1992).

$$6\ \mathrm{NO_3^-} + 5\ \mathrm{CH_3OH} \rightarrow 3\ \mathrm{N_2} + 5\ \mathrm{CO_2} + 7\ \mathrm{H_2O} + \mathrm{OH^-} \qquad \text{(Eq. 1.3)}$$

$$12\ \mathrm{NO_3^-} + 5\ \mathrm{C_2H_5OH} \rightarrow 6\ \mathrm{N_2} + 10\ \mathrm{HCO_3^-} + 9\ \mathrm{H_2O} + 2\ \mathrm{OH^-} \qquad \text{(Eq. 1.4)}$$

$$3.36\ \mathrm{NO_3^-} + \mathrm{C_5H_9NO} + 3.92\ \mathrm{H^+} \rightarrow 1.68\ \mathrm{N_2} + 0.36\ \mathrm{C_5H_7NO_2} + 3.2\ \mathrm{CO_2} + 3.92\ \mathrm{H_2O} + 0.64\ \mathrm{NH_4^+} \qquad \text{(Eq. 1.5)}$$

1.1.3. The anammox process

The discovery of the anoxic ammonium oxidation (anammox) process in the 90s brought the possibility of removing nitrogen from high-strength wastewater without needing to use organic matter. Broda (1977) first predicted the anammox process thermodynamically, but the nitrogen loss verified in an autotrophic denitrifying reactor treating high ammonium concentration wastewater and sulfide-limiting loading denitrification made it possible to demonstrate the process (Mulder et al., 1995; Siegrist et al., 1998). Since then, the anammox process has been applied in scaled up reactors in different geographical localizations (Ali and Okabe, 2015; Lackner et al., 2014).

In the anammox process, ammonium is oxidized into nitrogen gas by using nitrite as electron acceptor in anoxic conditions. Overall anammox process stoichiometry was first presented by Strous et al. (1998), however it was later revised by Lotti et al. (2014) (Equation 1.6).

$$\mathrm{NH_4^+} + 1.146\ \mathrm{NO_2^-} + 0.071\ \mathrm{HCO_3^-} + 0.057\ \mathrm{H^+} \rightarrow 0.986\ \mathrm{N_2} + 0.161\ \mathrm{NO_3^-} + 2.002\ \mathrm{H_2O} + 0.071\mathrm{CH_{1.74}O_{0.31}N_{0.20}} \quad \text{(Eq. 1.6)}$$

Anammox bacteria (AnAOB), differently from others prokaryotes, have an intracytoplasmic compartment called anammoxosome where anammox catabolism takes place (Van Niftrik et al., 2004). In the attempt of understanding AnAOB metabolism, it was originally stated that hydroxylamine and hydrazine (N_2H_4) were the main intermediates of the anammox process (van de Graaf et al., 1997). However, at that time, it was not clear that those intermediates started from the ammonium neither nitrite. Thus, the following anammox catabolism pathway (Figure 1.4) was proposed later by Kartal and collaborators (2011). The enzyme nitrite oxidase (NirS) reduces nitrite to nitric oxide (NO). Thus, NO is combined with ammonium to produce hydrazine (N_2H_4), process catalyzed by the enzyme hydrazine synthase (HZS). In the last step, N_2H_4 is oxidized to dinitrogen gas (N_2) by the enzyme hydrazine dehydrogenase (HDH) (Kartal et al., 2011). More recently, it was showed that anammox bacteria affiliated to the candidate genus Candidatus *Kuenenia stuttgartiensis* could conserve energy and grow by oxidizing ammonium in the absence of nitrite, but reducing NO instead (Hu et al., 2019). Additionally, it was also evidenced, by the cited study, that N_2 is the sole end product of this NO dependent anammox.

AnAOB are chemolithoautotrophic bacteria, which belongs to the phylum *Planctomycetes*. So far, it has been identified five candidate genera (Oshiki et al., 2016). The most common genera found in engineered environments are *Candidatus* Brocadia (Mulder, 1995), *Candidatus* Kuenenia (Schmid et al., 2000) and *Candidatus* Jettenia (Quan et al., 2008). However, *Candidatus* Anammoxglobus was also enriched in an anammox reactor (Kartal et al., 2007). The occurrence of *Candidatus* Scalindua had been mostly reported in marine environments (Schmid et al., 2007), but also found in a wastewater treatment plant (WWTP) treating landfill leachate (Schmid et al., 2003) and, recently, in rice paddy soils (Wang and Gu, 2013).

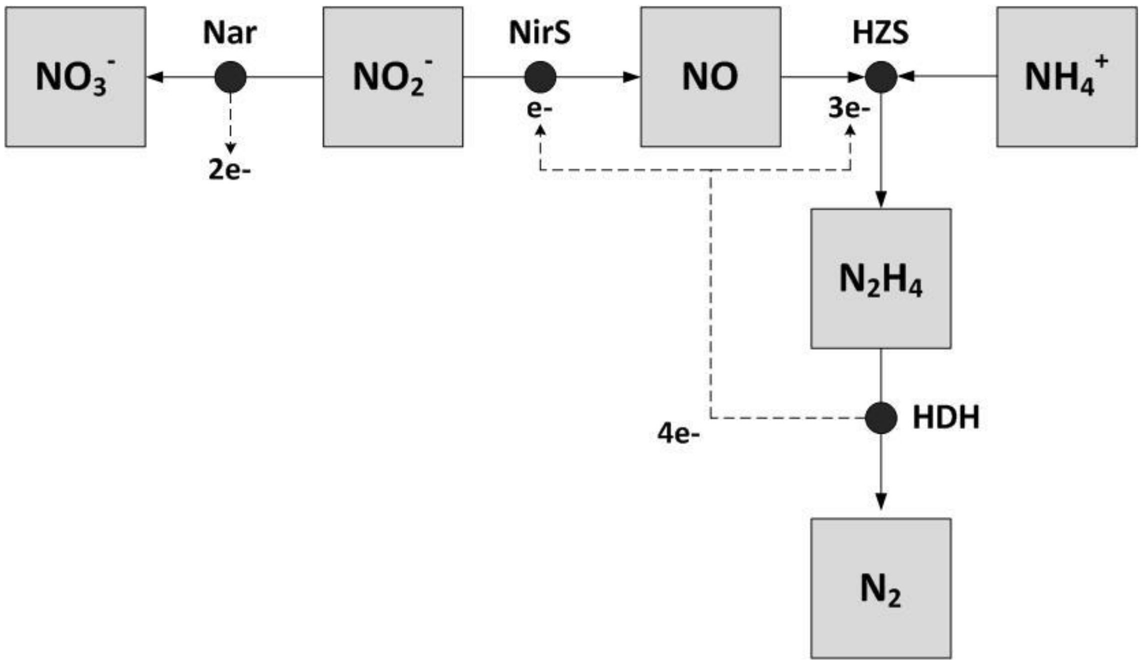

Figure 1.4. Anammox metabolism scheme. Arrow line and arrow dashed line indicate reaction and electron flow. Modified from Kartal et al. (2011) and Carvajal-Arroyo et al. (2016).

One of the obstacles for anammox process application is the relatively low growth rate presented by AnAOB compared to denitrifying and nitrifying bacteria. Thus, it demands long start-up time for an engineering point of view. Initially, it was reported a doubling time for AnAOB of 11 days (Strous et al., 1998), however, recent studies have pointed out that doubling time is lower than previously showed. Table 1.1 shows physiological characteristics of some AnAOB.

Table 1.1. Physiological characteristics of some AnAOB

Anammox species	μ_{max} (d^{-1})	Doubling time (d)	Biomass type	Temperature (°C)	Reference
Ca. Brocadia sinica	0.1	7	Granular	37	Oshiki et al. (2011)
Ca. Brocadia sinica	0.17[a] and 0.34[b]	4.1* and 2.1⁋	*Planktonic cells and ⁋Cells immobilized in gel beads	37	Zhang et al. (2017)
Ca. Jettenia caeni	0.11[a] and 0.18[b]	6.3* and 3.9⁋	*Planktonic cells and ⁋Cells immobilized in gel beads	37	Zhang et al. (2017)
Ca. Scalindua sp.	0.17	4.1	Planktonic cells	22	Zhang et al. (2017)
Ca. Brocadia sp.40	0.33	-	Planktonic cells	30	Lotti et al. (2015a)
Ca. Kuenenia stuttgartiensis	0.084	8.3	Planktonic cells	38	van Der Star et al. (2008)

1.2. Biological nitrogen removal in WWTP

1.2.1. Conventional nitrogen removal process

Nitrogen removal in WWTP has been commonly achieved by nitrification-denitrification processes. In those processes, influent ammonium is converted to nitrite/nitrate in the autotrophic nitrification processes carried out in the aerobic zone. The oxidized nitrogen species are reduced to gaseous nitrogen in the denitrification step, which occurs in the anoxic zone. Several reactors configuration have been applied to perform the nitrogen removal (Figure 1.5).

In the post-denitrification system, organic carbon removal and nitrate production occurs simultaneously in the aerobic reactor, which is placed before the anoxic reactor. The mixed liquor with the nitrate produced is sent to the anoxic reactor, where nitrate is

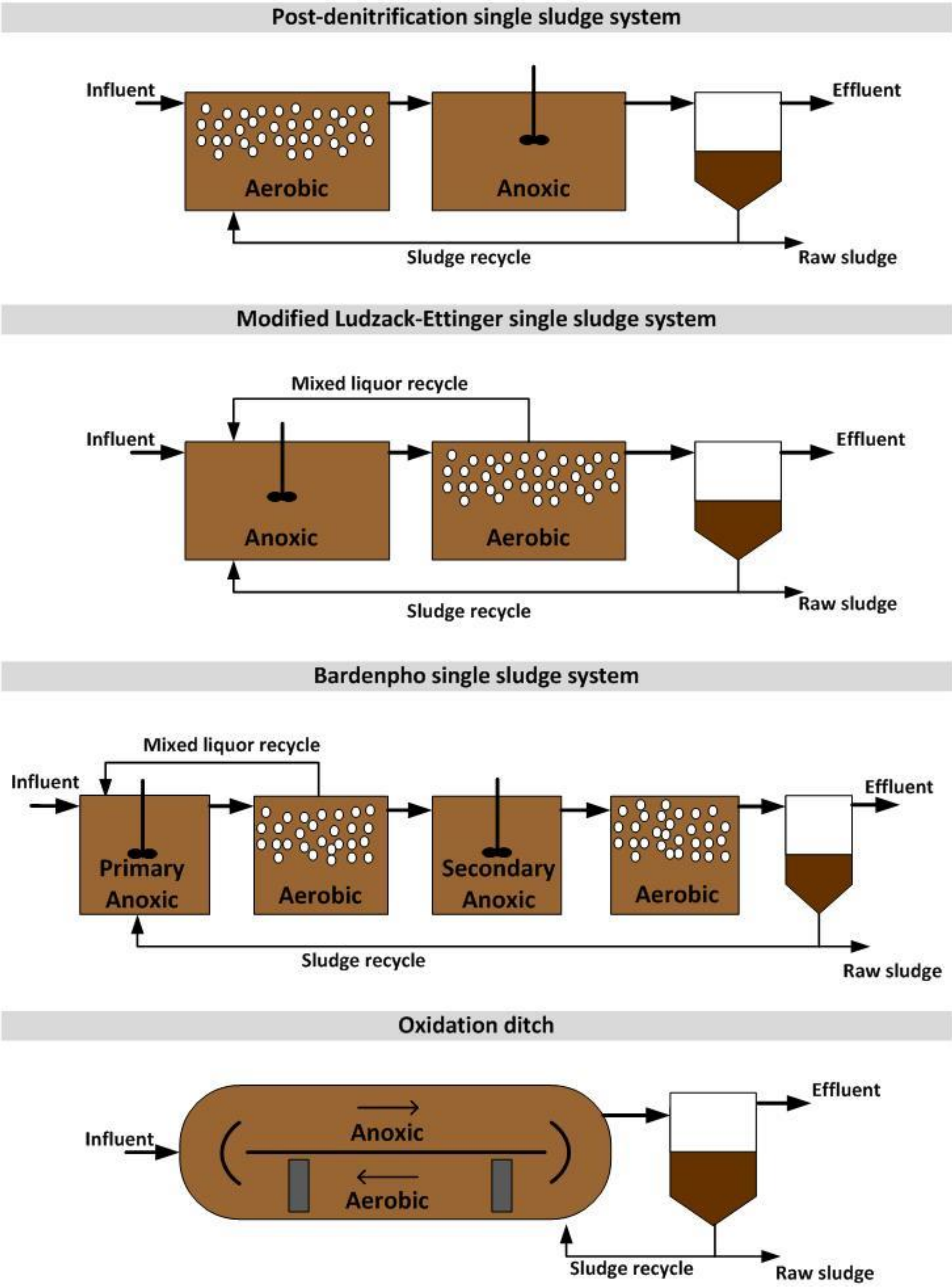

Figure 1.5. Schematic figures of suspended growth systems of nitrogen-removal treatment processes. Adapted from Wiesmann et al. (2006) and Ekama and Wentzel (2008).

then reduced. Since most of organic carbon is removed in the first reactor, denitrification occurs under endogenous conditions, by releasing organic matter from decaying biomass. Aiming at increasing denitrification rates, the addition of external carbon source, such as glycerol or methanol, and the direction of part of the raw sewage to the anoxic reactor can be done. The post-denitrification configuration have some drawbacks because of the need of adding external chemical carbon source to achieve better removal rates, as well as the release of ammonium due to biomass lysis in the anoxic reactor.

The modified Ludzack and Ettinger (MLE) system consists of an anoxic reactor followed by an aerobic reactor. In this system, nitrification takes place in the second reactor (aerobic) and the mixed liquor with produced electrons acceptor (nitrite/nitrate) is recycled to the anoxic reactor, where influent organic matter is used as electron donor for denitrification. The system also contains an external recirculation from the clarifier to the first reactor to maintain a proper sludge concentration and reach the biomass retention time.

The Bardenpho process is an arrangement of both configurations discussed above. In this system, the nitrite/nitrate discharged from the aerobic reactor has a second chance to be denitrified in the secondary anoxic reactor. Furthermore, an aerobic tank is set after the anoxic reactor in order to strip the gaseous nitrogen and nitrify the ammonia released in the previous step due to biomass lysis.

Finally, in the oxidation ditch, the mixed liquor circulates several times through the aerobic and anoxic zones. Aeration rotors and brushes provide aeration and circulation. In all the processes presented, part of the sludge from the settling tank is recycled to the first unit, in order to maintain the desirable sludge retention time.

1.2.2. Autotrophic nitrogen removal based on anammox process

Since the experimental proof of anammox pathway in the mid-nineties (Mulder, 1995; Siegrist et al., 1998) a great interest has arisen aiming at applying anammox-based technology to remove nitrogen from wastewater. It took about one decade for the first anammox full-scale facility to be implemented (van der Star et al., 2007). The technology become more mature and several facilities have been reported around the world, with the last reports indicating more than one hundred in total (Ali and Okabe, 2015; Lackner et al., 2014). Anammox process application results in several advantages compared to the conventional biological nitrogen removal (BNR) process, and for this reason anammox systems have been broadly applied. Table 1.2 presents the benefits that can be achieved by implementing partial nitritation-anammox (PNA) processes for nitrogen removal in urban WWTP (Fatone et al., 2017). It is highlighted in the table the cost-effectiveness of the process, because it represents up to 60% of aeration savings, no organic carbon supplies and lower sludge yields. Hence, anammox application allows bringing WWTP to neutral energy consumption (Kartal et al., 2010; Siegrist et al., 2008).

Table 1.2. Comparison of the conventional BNR with innovative processes

Process	**Oxygen requirements (kg O_2/kg $N_{rem.}$)**	**COD consumption (kg COD/kg $N_{rem.}$)**	**Sludge produced (kg VSS/kg $N_{rem.}$)**	**Total treatment cost (€/ kg $N_{rem.}$)**
Nitrification/Denitrification	4.33	2.86	1 – 1.2	3 – 5
Nitritation/Denitritation	3.26	1.72	0.8 - 0.9	1.5 – 2.5
Partial nitritation/Anammox	1.71	0	< 0.1	1 -2

Source: Fatone et al. (2017).

Autotrophic nitrogen removal from wastewater implies in two sequential processes: partial nitritation, where the required molar ratio of NH_4^+:NO_2^- for anammox is achieved by aerobic AOB activity, and the anammox process itself in anoxic conditions. Two-stage configuration – one aerobic reactor for partial nitritation followed by an anoxic reactor for anammox – was initially proposed, based on the different microorganisms' metabolism, because AnAOB could be inhibited by oxygen. However, since Strous and collaborators (1997) proved the reversible inhibition effects of oxygen to the anammox bacteria, research was focused to the possibility of combining partial nitritation and anammox processes in the same reactor, and it is named one-stage or single-stage configuration. The achieved knowledge allowed obtaining insights in the mechanisms for stable nitrogen removal in one-stage PNA, being the technology broadly implemented later (Joss et al., 2009; Wett, 2007; Windey et al., 2005). The lesser capital and operational costs coupled with lower footprint made one-stage configuration feasible, and mostly of the full-scale facilities are operated in one-stage (Lackner et al., 2014).

Both AOB and AnAOB have low growth rate, which demands the retention of the biomass in the reactor. It can be achieved in biofilm grown in an inert material or self-aggregated granular technologies. In these systems, mass transport of substrates and biological conversion within the biofilm results in a gradient where different feasible niches for AOB and AnAOB are created (Figure 1.6). In one-stage PNA biofilm, due to oxygen limitation in the bulk liquid, an aerobic layer is created in the external biofilm zone. The availability of both ammonium and oxygen induces nitritation by AOB and oxygen consumption in this external layer. The diffusion of substrates and no availability of oxygen create an anoxic layer feasible for AnAOB growth, and anammox process takes place.

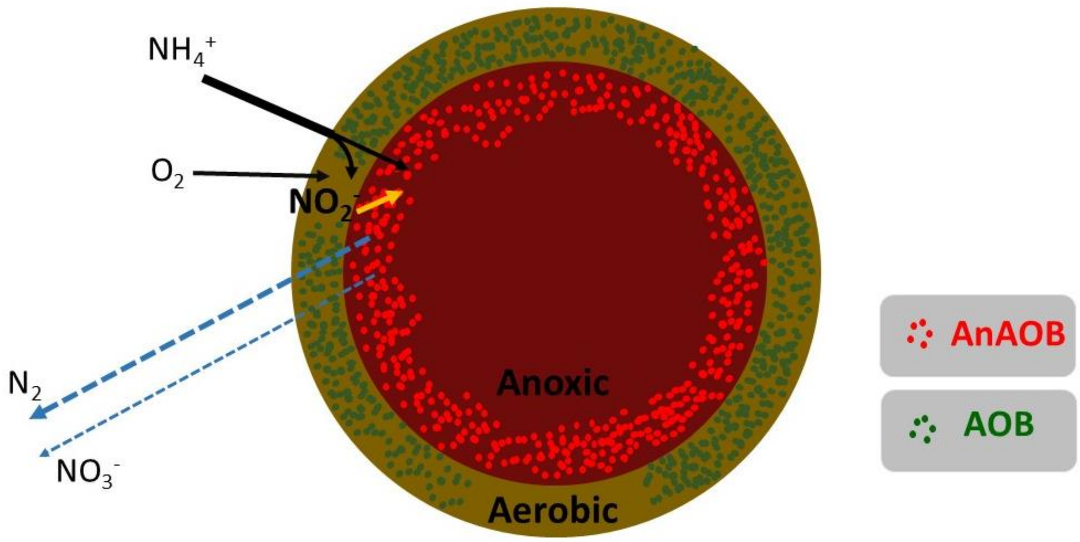

Figure 1.6. Scheme of one-stage PNA granule structure: Nitrogen species and oxygen diffusion creating niches for AOB and AnAOB.

The total exemption for organic matter makes anammox process feasible for wastewater treatment characterized with low C/N ratios, such as some industrial wastewater or post-treatment of effluent organic matter conversion processes. Over the years, PNA has been applied for the treatment of seafood, pharmaceutical, agro-digestate and landfill leachate (Lamsam et al., 2008; Ramos et al., 2007; Ruscalleda et al., 2008; Scaglione et al., 2015; F. Zhang et al., 2017). However, PNA processes have been widespread implemented at sidestream (reject) wastewater (Joss et al., 2011; Lackner et al., 2014; Wett, 2007). Due to its large application, it will addressed in the next section.

1.3. Partial nitritation-anammox in urban WWTP facilities

1.3.1. PNA for sidestream wastewater treatment

In urban wastewater system, reject water (also known as sidestream wastewater) is generated by sludge dewatering processes. This water is commonly recycled to the inlet WWTP and may constitute up to 30% of the incoming nitrogen load (Christensson et al., 2013). Reject water is characterized by high-strength nitrogen content, typically ranging from 400 to 1500 mg NH_4^+-N·L^{-1} (Abma et al., 2007; Morales et al., 2015), and low C:N, which is not suitable for the conventional nitrification-denitrification processes (Christensson et al., 2013). Additionally, the molar ratio $HCO_3^-:NH_4^+$ is around 1 (Dosta et al., 2015), under the amount required stoichiometrically for total ammonium oxidation by nitrification process (Grady et al., 1999), i.e., 1.98 $HCO_3^-:NH_4^+$. These referred conditions make anammox-based processes suitable for treating reject wastewater and mostly of current worldwide full-scale facilities are in the sidestream line (Lackner et al., 2014) as presented in Figure 1.7.

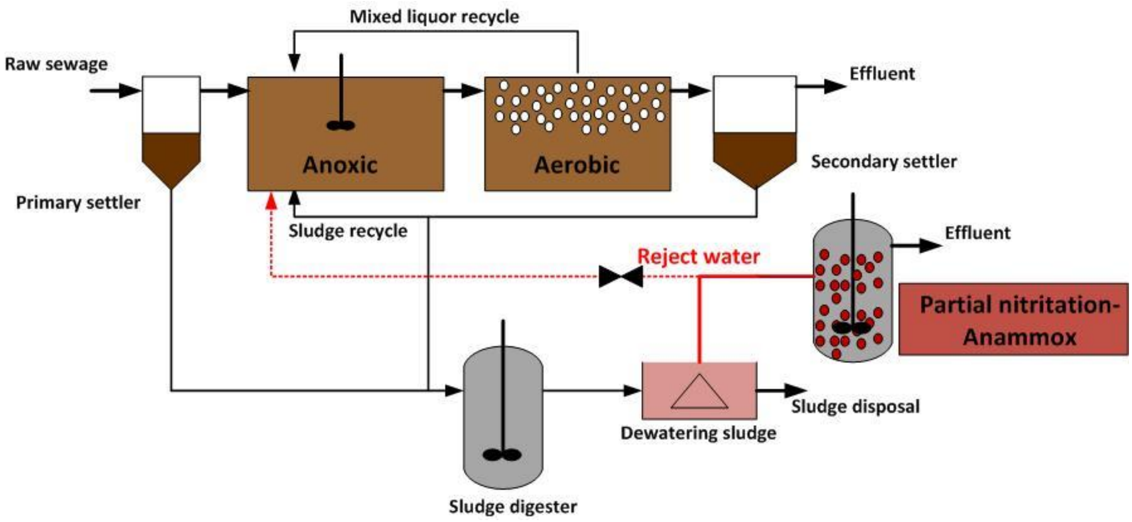

Figure 1.7. Flow-scheme indicating the actual layout of reject water flowing to the liquid train of the WWTP (dashed line), and the proposition of autotrophic N removal by PNA processes

Initial PNA implementation used the two-stage configuration to remove nitrogen from reject wastewater. The SHARON process - acronym for Single reactor High activity Ammonia Removal Over Nitrite - (Hellinga et al., 1998; Mulder et al., 2001) was proposed as the first step to carry out partial nitritation, which allowed obtaining the required NH_4^+:NO_2^- ratio for anammox process. However, other technologies, such as sequencing batch reactor (SBR) and airlift reactor were also proposed, only lab and pilot-scale studies have been reported in the reject water treatment (Bartrolí et al., 2010; Dosta et al., 2015). In the SHARON process, the operation of a continuous stirred tank reactor (CSTR) occurs at high temperature (ranging between 30 and 40 °C) and pH between 7 and 8. Moreover, in order to achieve the nitratation repression, hydraulic retention time (HRT) is equal solids retention time (SRT), i.e. HRT=SRT (Hellinga et al., 1998). The SHARON process has been tested at full-scale installation in a 1,800 m^3 reactor (Mulder et al., 2001), and the effluent of this reactor provided the feed for the first full-scale anammox reactor (van der Star et al., 2007).

SBR technology shows several operating advantages and flexibility, which allow dealing with variations in the influent characteristics. Several reports have showed the potential of using two-stage partial nitritation-anammox in SBR for reject wastewater, but they were limited at lab and pilot-scale (Dosta et al., 2015; Galí et al., 2007). However, SBR technology at full-scale in sidestream water has been applied in nitritation-denitritation processes (Fux et al., 2003; Gustavsson et al., 2008) or in one-stage PNA configuration, as obtained in a survey published elsewhere (Lackner et al., 2014). Compared to the SHARON process, SBR can be operated with higher SRT, because HRT and SRT are uncoupled, which can decrease both reactor footprint and volume to deal with higher nitrogen loads. Since SRT can be longer, this parameter is not applied to outcompete NOB activity in SBR. Hence, other operational parameters such as low DO and higher free ammonia (FA) and free nitrous acid (FNA) have been applied to prevent nitratation (Gustavsson et al., 2008).

AnAOB cultivation feasibility has been demonstrated in several lab and pilot-scale reactors configurations, such as SBR (Dosta et al., 2015; López et al., 2008), fixed-bed reactor (Fux et al., 2004), moving bed biofilm reactor (MBBR) (Zekker et al., 2012), and membrane bioreactors (MBR) (Van Der Star et al., 2008). However, at full-scale sidestream application, the only two-stage facility reported in literature is the SHARON/ANAMMOX® implemented by Paques in Rotterdam (The Netherlands) (van der Star et al., 2007). In this WWTP, reject water partially nitrified in the SHARON reactor is forwarded to the anammox reactor, which comprises a granule sludge bed reactor with two compartments (total volume of 70 m^3). The bottom compartment (volume of 40 m^3) is mixed by the influent (feed), downcomer flow and the gas recycled from the upper reactor. An additional recirculation flow from the effluent of the reactor to the bottom compartment was also designed, in order to keep an upflow velocity and shear stress to favor granule formation. The start-up time took about 3.5 years, and nitrogen removal rate (NRR) achieved in the reactor at full operation was higher than 9 kg $N \cdot m^{-3} \cdot d^{-1}$(van der Star et al., 2007).

When organic compounds are present in the feed, two-stage PNA is the most suitable configuration because the occurrence of denitrification in the first unit could prevent anammox instability in the second unit, since higher C:N process could lead to higher denitrification rather than anammox (Jenni et al., 2014). Since reject water presents low

C:N, one-stage configuration has been the most suitable application. Despite initial proposition of two-stage, one-stage rather than two-stage PNA configuration has been largely applied for reject water treatment at full-scale (Joss et al., 2009; Lackner et al., 2014; Wett, 2007). The main advantages of one-stage with respect to two-stage configurations are: (i) lower costs for installation; (ii) simplification of reactor operation (e.g., no pH control); (iii) lower footprint, because one-stage reactor is more compact; (iv) at regular operation, it is easier to avoid nitrite accumulation and its possible toxic effects on AnAOB, because feed can achieve improper NH_4^+:NO_2^- in two-stage systems; and (v) more flexibility face to very high, unstable and uncertain loads (Jaroszynski and Oleszkiewicz, 2011; Joss et al., 2009; Vázquez-Padín et al., 2009). Furthermore, it was also reported AnAOB detection in full-scale reactor operating at nitritation-denitritation (Park et al., 2017), and it also corroborates to one-stage by only adapting the operation to facilitate AnAOB growth.

One-stage PNA corresponds to 88% of anammox full-scale facilities in operation worldwide (Lackner et al., 2014). From this amount, SBR and MBBR are the most built reactors. SBR has been the system implemented by the DEMON® technology in 2004 at WWTP Strass (Austria) (Wett, 2007). Before the implementation of autotrophic N removal, from 1997 until 2004, the SBR was operated for nitrification/denitrification. For the start-up of DEMON® technology, 2.5 m^3 of anammox seed sludge were introduced in the 500 m^3 SBR. The control strategy to operate DEMON® is discussed below. SBR was also chosen by Eawag to be implemented first in the WWTP Zürich (Switzerland) in 2007 and further extended to other plants (Joss et al., 2009). Attached biofilm processes have been developed in MBBR by Veolia and AnoxKaldnes, and the first full-scale facility was in Malmö (Sweden) in 2011 (Christensson et al., 2013). The commercial MBBR technology is the ANITA™Mox. Bacteria grow as a biofilm attached on AnoxKaldnes carrier media, allowing the retention of AnAOB. Table 1.3 shows a summary of several reported studies regarded to pilot and full-scale one-stage partial nitritation-anammox process removing nitrogen from reject water.

Table 1.3. Overview of the nitrogen removal performance of several full-scale PNA reactors (one and two-stage) treating reject wastewater

Reactor type	**Configuration**	**Working volume (m^3)**	**NLR (kg N·m^{-3}·d^{-1})**	**NRR (kg N·m^{-3}·d^{-1})**	**Temperature (°C)**	**DO (mg O_2·L^{-1})**	**pH**	**Reference**
CSTR/ Gas-lift	Two-stage	1,800; 70	0.23; 7.1	9 (2nd stage)	30 - 40	-	7 - 8	Mulder et al. (2001); van der Star et al. (2007)
SBR	One-stage	500	0.68	0.5	27.8 ± 1.7	0.3	-	Wett (2007)
SBR	One-stage	2 x 1,400	0.4	-	30 ± 3	< 1	7.1 ± 0.2 (effluent)	Joss et al. (2011, 2009)
SBR	One-stage	550	0.20	0.18	31 ± 3	0.24 - 0.46	-	Lackner et al. (2015)
MBBR	One-stage	200	1.40	1.2	22 - 33	0.5 - 1.5	6.7 - 8.0	Christensson et al. (2013)
MBBR	One-stage	350	0.83	0.63	22 - 33	0.5 - 1.5	6.7 - 7.5	Christensson et al. (2013)

1.3.2. Operation and control of PNA at sidestream

Efficient nitrogen removal by PNA processes relies on the interaction between the main microorganisms involved, AOB and AnAOB. Since AnAOB depends on the ammonium and nitrite availability, AOB activity is the main step limiting the overall process (Sliekers et al., 2003; van der Star et al., 2007). Oxygen is the substrate utilized by AOB and NOB to perform nitritation and nitratation. Thus, oxygen has a key role in PNA because it has to allow ammonium oxidation by AOB to provide substrate by AnAOB, but at the same avoid nitrite oxidation by NOB. Moreover, according to Vázquez-Padín et al. (2010), in one-stage systems an adequate control of DO concentration in the liquid media is necessary to avoid AnAOB bacteria inhibition caused by higher DO concentrations levels.

Besides off-line measurements, real-time control, based on online monitoring, is largely applied in full-scale PNA installations (Lackner et al., 2014). Online sensors in the reactors allow obtaining the value of substrates and other operational parameters. According to the survey carried out by Lackner and et al. (2014), the most common measurements are pH and the DO concentration, but oxidation-reduction potential (ORP), ammonium and nitrate online probes are others alternative used in some installations as well.

A control strategy for full-scale SBR was developed by Joss and collaborators (2011) and it was applied in a nitritation-anammox process at WWTP of Zurich-Werdhölzli. This control strategy is based on ammonium online probe. By interpreting the online ammonium signal, accumulation of nitrite is detected indirectly and used to signal an imbalance of oxygen supply and AOB activity. The airflow rate is controlled to increase or decrease nitritation, avoiding nitrite accumulation, and consequently, optimizing and suppressing anammox and nitratation process, respectively.

In the ANITA Mox process, a DO control strategy is applied in order to avoid nitratation process (Christensson et al., 2013). DO concentrations are adjusted according to a control loop, which continuously calculates the ratio $NO_3^-{}_{produced}$:$NH_4^+{}_{removed}$ based on signals from the online sensors installed in the entrance of reactor (ammonium probe) and inside the reactor (ammonium and nitrate probes). It means, DO set-point is increased if the nitrate produced is lower than 11% of the inlet ammonium, or controversially, DO set-point is decreased in case nitrate over ammonium is higher than 11% because it indicates NOB activity is occurring.

The pH-based set-point governs the process on DEMON® (Wett, 2007). The control system determines the length of aeration intervals depending on the current production of H^+ ions or nitrite. During the ammonium oxidation by AOB, H^+ production drives the pH-value to the lower set-point and aeration is stopped. Then nitrite produced is consumed by anammox process, and some alkalinity produced plus alkalinity present in the reject water increase the pH-value to the upper set-point. The aeration system is activated only within a very tight pH-bandwidth of 0.01.

1.3.3. PNA for mainstream conditions

After the successful developing and implementation of PNA processes at sidestream conditions, nowadays a great interest on PNA at mainstream is going on. Studies have shown PNA competitiveness in the main WWTP water line compared to the conventional and currently mostly applied processes, because of the lower energy requirements and lesser biosolids production (Morales et al., 2014; Winkler and Straka, 2019). PNA application at mainstream has been mostly studied in lab and pilot-scale reactors, but efforts have been also done to investigate PNA feasibility at full-scale operation (Cao et al., 2018; Wett et al., 2013). Several publications from those studies have been published in recent years, as well review papers pointing out the main factors that affect PNA process, operational strategies and remaining drawbacks (Agrawal et al., 2018; Cao et al., 2017; Delgado Vela et al., 2015; Li et al., 2018; Ma et al., 2016; Tan and Shuai, 2015).

Since the presence of organic matter in wastewater can negatively affect anammox process (because it creates conditions for denitrification by heterotrophic bacteria-HB) and compete with AnAOB for the nitrite as electron acceptor), COD must be removed from sewage prior PNA treatment. In this case, the A-B process (Figure 1.8) is the recommended WWTP configuration for PNA implementation at mainstream conditions (Wan et al., 2016). Energy recovery potential can be improved at A-stage, with simultaneous reduction of energy consumption at B-stage. Several biotechnologies have the potential to recover COD into valuable products, such as biogas and biohydrogen, and in the last years some emergent technologies have been explored to sewage treatment (Puyol et al., 2016).

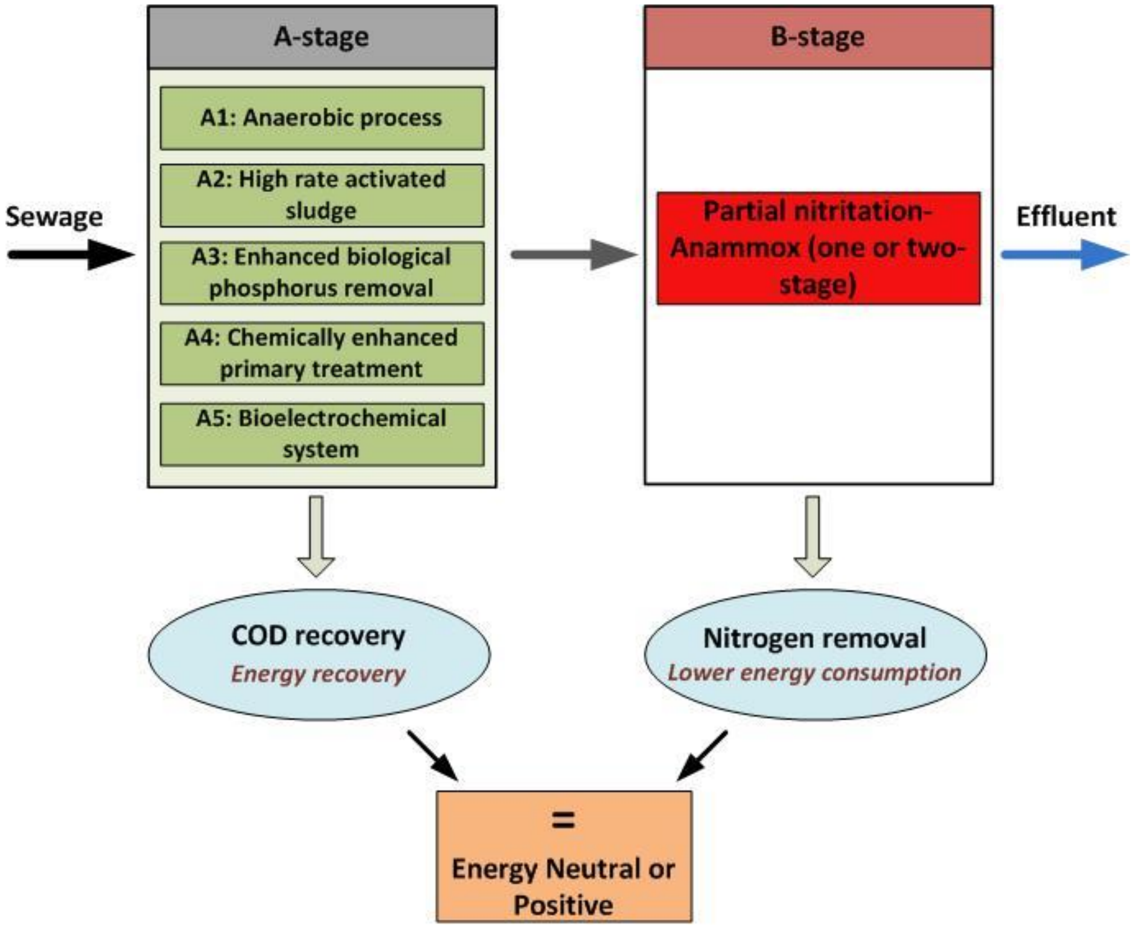

Figure 1.8. Potential combinations of autotrophic nitrogen removal based on A-B process. Modified from Li et al. (2018) and Wan et al. (2016).

Similar to sidestream wastewater, PNA can also be realized in one or two-stage reactors at mainstream conditions, but sewage characteristics make mainstream PNA more challenging. Among those characteristics are the (i) low N content of sewage, with concentration below 100 mg NH_4^+-N·L^{-1}; (ii) low temperature of the wastewater, which affects microbial activities and rise the required SRT; and (iii) higher C:N that can favor HB growth over AnAOB (De Clippeleir et al., 2013). A summary of the main nitrogen removal performance from several studies carried out by applying PNA as B-stage is shown in Table 1.4.

1.3.4. Approaches for anammox growth in sewage

Anammox application has been proved feasible to remove nitrogen from pre-treated sewage (Hendrickx et al., 2012; Laureni et al., 2015; Lotti et al., 2014b). Effluent sewage from A-stage, where organic matter is removed in an initial step, was used to cultivate AnAOB in those studies. Biomass retention was achieved by cultivating granules, allowing AnAOB growth even at temperatures of 12.5 ^{o}C (Laureni et al., 2015). Moreover, AnAOB doubling time is increased as a consequence of temperature reduction. By operating the reactor at 29 ^{o}C, a minimum doubling time of 18 d was obtained, but at 12.5 ^{o}C, it rose to 79 days (Laureni et al., 2015). In other study, the doubling time rose from 35 d to 77 d, for temperatures of 20 ^{o}C and 15 ^{o}C, respectively (Lotti et al., 2014b).

In one-stage, the main applied reactor configurations are SBR (Ma et al., 2015), MBBR (Gilbert et al., 2014b), upflow granular system (Li et al., 2017), plug-flow granular reactor (Lotti et al., 2015b) and step-feed reactor with aerobic and anoxic chambers (packed with AnAOB-attached carriers) (Gu et al., 2019). For two-stage, nitritation has been achieved in airlift (Isanta et al., 2015), while anammox has been developed in UASB (Reino et al., 2018). Successful anammox at sewage wastewater and temperature of 11 ^{o}C was reported in an upflow anammox sludge bed reactor (UAnSB), with average NRR of 1.2 ± 0.5 g $N \cdot L^{-1} \cdot d^{-1}$ (Reino et al., 2018).

Table 1.4. Review of studies performed PNA as B-stage to treat sewage

COD removal reactor		Nitrogen removal reactor (PNA)						Reference
Reactor type	**Working volume (L)**	**Reactor type**	**Working volume (L)**	**Influent C:N**	**NLR (g N·m^{-3}·d^{-1})**	**NRR (g N·m^{-3}·d^{-1})**	**Temperature (°C)**	
Hybrid anaerobic reactor: Upflow anaerobic sludge bed (UASB) with carrier medium)	1.28	Upflow membrane aerated biofilm reactor (one-stage)	2.5	1 to 3.5	32.5 ± 4.2 (Period I) and 104.8 ± 13.1 (Period IV)	24.4 ± 4.0 (Period I) and 84.2 ± 16.4 (Period IV)	24 ± 1 (Periods I and II) and 20 ± 1 (Periods III and IV)	Li et al. (2017)
Anaerobic membrane bioreactor (AnMBR)	4	MBR (one-stage)	4	0.85 ± 0.39	200 and 250	150 and 200	23 ± 3	Dai et al. (2015)
High rate activated sludge	Not reported (full-scale)	Plug-flow granular (one-stage)	4,000	2.3 ± 1.1	Not reported	182 ± 47	19 ± 1	Lotti et al. (2015b)
UASB	6,200	MBBR (one-stage)	200	1.2 to 2.3	40 to 72	8 to 26	25	Malovanyy et al. (2015b)

1.3.5. Operational strategies to suppress NOB at mainstream

- **DO concentration**

Earlier studies have showed that nitrite accumulation under oxygen limitation was achieved because AOB present lower half-saturation constant for oxygen than NOB (Bernet et al., 2001; Blackburne et al., 2008). Mainstream PNA operation under limited DO in the bulk liquid (concentration lower than 0.5 mg $O_2 \cdot L^{-1}$) obtained efficient nitratation suppression (Laureni et al., 2016; Li et al., 2016). However, it was also showed low DO operation was not enough to repress NOB activity (Miao et al., 2016; Wett et al., 2013), and a reliable DO control has to be defined.

In the study carried out by Blackburne and collaborators (2008), nitratation was controlled in a floc-based CSTR under low DO conditions because AOB presented lower oxygen half-saturation constant (0.033 ± 0.003 mg $O_2 \cdot L^{-1}$) than NOB (0.43 ± 0.08 mg $O_2 \cdot L^{-1}$). However, a mass transfer resistance is found in biofilm and granular sludge, and the substrates diffusion towards the inner core of biofilm or granule impacts the microbial spatial distribution. This mass transfer resistance affects the determination of the kinetics affinity constants. Oxygen half-saturation constant of 0.0736 mg $O_2 \cdot L^{-1}$ and 0.0416 mg $O_2 \cdot L^{-1}$ was obtained for AOB and NOB, respectively, in a granular system (Sliekers et al., 2005). The role of DO on limiting nitratation also depends of the NOB strain. *Nitrospira* sp. are K-strategists and present better growth under low amounts of substrate, whereas *Nitrobacter* sp. are r-strategists and can grow faster than *Nitrospira* in higher substrate availability (Blackburne et al., 2007; Kim and Kim, 2006). Hence, *Nitrospira* population instead of *Nitrobacter* have commonly been enriched under low DO concentration (Arnaldos et al., 2013; Liu and Wang, 2013).

- **Transient anoxia**

The use of intermittent aeration pattern to induce nitrite accumulation by transient anoxia has been applied in many studies (Mota et al., 2005; Yoo et al., 1999). By imposing anoxic time, both AOB and NOB are inactivated due to the absence of oxygen, however, by turning on the aeration, NOB lag time is higher than AOB, and this unbalance between nitritation and nitratation rates led to an accumulation of nitrite (Gilbert et al., 2014a). An anoxic time length of 3 and 4 h resulted in lower NOB biomass fraction and nitrite accumulation during the aerobic phase (Mota et al., 2005). It was previously demonstrated that an anoxic time of 15-20 min and an aeration phase lower than the lag phase of NOB might suppress NOB (Gilbert et al., 2014a). Additionally, in one-stage PNA reactors, this strategy restored systems with NOB proliferating conditions (Miao et al., 2016; Pellicer-Nàcher et al., 2010). By using intermittent aeration, nitrate production can be stopped due to the lack of oxygen, and the presence of ammonium and nitrite in anoxic conditions benefits anammox process. Transient anoxia was also achieved by spatial shifting between aerobic and anoxic chambers in a step-feed reactor (Gu et al., 2019). The main outcomes obtained by several studies that applied intermittent aeration to outcompete NOB are summarized in Table 1.5.

- **Residual ammonium concentration**

Modelling (Corbalá-Robles et al., 2015; Pérez et al., 2014) and experimental studies (Bian et al., 2017; Isanta et al., 2015; Regmi et al., 2014) have showed that the maintenance of residual total ammonium (TAN) concentration in the bulk liquid infers in the bulk DO concentration to obtain an oxygen-limiting condition in biofilm reactors. Pérez and collaborators (2014) concluded that for a determined DO in the bulk liquid, a minimum ammonium concentration (S_{NH4MIN}) should be maintained to hinder NOB of competing for oxygen with AOB. In nitritation reactors at mainstream conditions, residual ammonium/oxygen control has been assessed at 25 °C (Regmi et al., 2014) and temperatures lower than 15 °C (Bian et al., 2017; Isanta et al., 2015; Reino et al., 2016). DO concentration in the bulk liquid in the range of 0.5 and 2.5 mg $O_2 \cdot L^{-1}$ and effluent ammonium concentration between 20 and 40 mg $N \cdot L^{-1}$, allowed a ratio DO/TAN of 0.04 ± 0.02 mg $O_2 \cdot mg^{-1} NH_4^+$-N to be supported in a granular airlift reactor for 250 days at 10 °C (Reino et al., 2016). This operational strategy allowed enriching an AOB community

Table 1.5. Studies summary of mainstream operation by intermittent aeration

PNA configuration	Reactor type	Wastewater characteristics in the influent		Aeration frequency	DO (mg $O_2 \cdot L^{-1}$)	Efficiency remarks	Reference
		NH_4^+ (mg $N \cdot L^{-1}$)	COD (mg $O_2 \cdot L^{-1}$)				
One-stage	SBR	51.2 to 67.5	41.4 to 70.2 (soluble COD)	7 min air on/ 21 min air off	0.5±0.1	Total nitrogen removal efficiency of 56.8 % and NO_3^- in the effluent of 2.1 mg $N \cdot L^{-1}$	Miao et al. (2016)
One-stage	MBBR	45.4 (Phase B) 47.1 (Phase C)	43.6 (Phase B) 71 (Phase C)	15 min air on/ 60 min air off	0.4 to 1.5 (Phase B) 1.5 mg (Phase C)	The molar ratio $NO_3^-{}_{produced}$:$NH_4^+{}_{removed}$ was 0.53 mol:mol By intermittent aeration and transition to IFAS mode, the $NO_3^-{}_{produced}$:$NH_4^+{}_{removed}$ molar ratio was 0.47 mol:mol Removal efficiency of 51% was reached	Trojanowicz et al. (2016)
One-stage	SBR	62.6±7.6	76.6±30.7 (soluble COD)	15 and 30 min air on/15 to 90 min air off	0.2	NO_3^- concentration was lower than 10 mg $N \cdot L^{-1}$ throughout all the reactor operation TN in the effluent of 6.6±7.7 mg $N \cdot L^{-1}$, at reactor temperature above 20 ºC NO_2^- was accumulated when the temperature in the reactor dropped, due to anammox activity decreasing	Ma et al. (2015)

Table 1.5 (Continued)

PNA configuration	Reactor type	Wastewater characteristics in the influent		Aeration frequency	DO (mg $O_2 \cdot L^{-1}$)	Efficiency remarks	Reference
		NH_4^+ (mg $N \cdot L^{-1}$)	COD (mg $O_2 \cdot L^{-1}$)				
One-stage	IFAS	35 to 50	44 to 88	15 min air on/ 15 to 45 min air off	0.7 to 1.5	Nitrogen removal efficiency ranged from 30 to 75%	Malovanyy et al. (2015)
One-stage	Step-feed	45	19	Anoxic HRT 14 to 17 min	1.2 to 1.5	Nitrogen removal efficiency higher than 80% Specific oxygen uptake rate (SOUR) of AOB 1.07 ± 0.02 g $O_2 \cdot g^{-1}$ $VSS \cdot d^{-1}$ and NOB 0.17 ± 0.07 g $O_2 \cdot g^{-1}$ $VSS \cdot d^{-1}$	Gu et al. (2019)
One-stage (Nitritation + Denitritation)	CSTR	29.7 ± 3.9	306 ± 87	4 to 10 min air on/ 2 to 8 min air off (real-time control)	> 1.5	SRT control (6.5 ± 4.3 days) and maintenance of residual NH_4^+ (7.3 ± 4.4 mg $N \cdot L^{-1}$) were applied as well. Nitrogen removal rate of 151 ± 74 mg $N \cdot L^{-1} \cdot d^{-1}$. Nitrite accumulation of 0.36 ± 0.27 (NO_2^--N/NO_X-N)	Regmi et al. (2014)

with higher growth rate than NOB, and thus nitrite accumulation was stable throughout the operation. It was also reported elsewhere that keeping ammonium concentration between 10 and 20 mg $N \cdot L^{-1}$ enabled the enrichment of r-strategist AOB population, with an increase of the μ_{max} from 0.39 to 1.45 d^{-1} (Wu et al., 2016).

- **Exposing the sludge to inhibitory FA and FNA concentrations**

Concentrations of FA and FNA can exert inhibition effects on both AOB and NOB (Anthonisen et al., 1976; Blackburne et al., 2007), however NOB inhibition occurs at lower substrate levels than AOB. To take advantage of this different inhibition conditions, it has been reported operational strategies by exposing the sludge to higher FA and FNA concentrations, which can be performed in-situ (Piculell et al., 2015; Wang and Gao, 2016) or ex-situ (Nan et al., 2019; Wang et al., 2017, 2014).

In order to achieve FA concentration that could cause NOB inhibition, a mainstream two-stage MBBR, operated at 15 ^oC, was subjected to periodical change in the feed medium from low (sewage) to high-strength (reject water) nitrogen content in the nitritation reactor for short periods (Piculell et al., 2015). FA in the range of 7.1 to 495 mg NH_3-$N \cdot L^{-1}$ was achieved in the first reactor, assisted by temperature increase to 30 ^oC as well, which allowed obtained a nitrite accumulation rate of 75-85% throughout the experimental study. DO was controlled from 4 to 7 mg $O_2 \cdot L^{-1}$, whereas the biofilm thickness was controlled bellow 200 µm. Another in-situ NOB inhibition was assessed by Wang and Gao (2016) in a one-stage lab-scale expanded granular sludge bed reactor (EGSB). In this study, influent concentrations of ammonium and nitrite were elevated aiming at controlling the overgrowth of NOB in the reactor. Both FA between 5 and 40 mg NH_3-$N \cdot L^{-1}$ and DO lower than 0.13 mg $O_2 \cdot L^{-1}$ were effective to achieve nitratation after 53 days, despite NOB washout being not reached.

Ex-situ sludge treatment has been also showed effective to sustain NOB inhibition at mainstream wastewater treatment (Wang et al., 2017, 2014). In the presented concept, the abundance of NOB reduced by recirculating 22% of the sludge from the mainstream reactor to a sidestream sludge treatment reactor with higher concentration of FA or FNA, with a residence time of 24 h. It has been reported that FA concentration of 210 mg $NH_3 \cdot L^{-1}$ and FNA of 1.35 mg $HNO_2\text{-}N \cdot L^{-1}$ allowed a nitrite accumulation rate of 90 and 80%, respectively (Wang et al., 2017, 2014). FNA ex-situ treatment can be softened by the increase in the mixed liquor volatile suspended solids (MLVSS) concentration, and it demands a control of FNA concentration to keep the FNA/MLVSS from 0.16 to 0.3 mg HNO_2-N/g MLVSS (Wang et al., 2019). It is also recommended to collect the sludge straight from the aerobic zone (without starvation) to use it in the ex situ FNA treatment (Nan et al., 2019). In this study, the authors reported that the resistance to FNA in the sludge collected from the secondary settling tank (anoxic pre-starvation) was enhanced, whereas nitritation accumulation reduced in the sludge subjected to a post-starvation period after passing through FNA treatment.

- **SRT control**

SRT control has been effective for suppressing NOB activity at sidestream conditions, being the base parameter of the SHARON reactor (Fux et al., 2003; Hellinga et al., 1998; Mulder et al., 2001). At shorter SRT values and high bulk ammonium concentration, greater nitritation is achieved by competitive growth rate AOB, while NOB is washed-out. Studies have also effectively repressed NOB by applying short SRT of 4.2 d (Wu et al., 2016) and 6.5 ± 4.3 days (Regmi et al., 2014) in mainstream floc-based nitritation reactors operated at 25 ^{o}C. Moreover, others strategies such as keeping higher both ammonium and oxygen in the bulk liquid, and intermittent aeration, also contributed to NOB repression. SRT as a key selector for NOB washout to achieve stable nitritation at both low temperature and nitrogen streams, has not been reported for floc-based systems.

In one-stage PNA based on biofilm/granular reactors, since AnAOB require higher biomass retention, two operational strategies have been studied. The first one is the separation of flocs from granules biomass by using screen and cyclones (Han et al., 2016b; Wett et al., 2013). With this strategy, SRT from granules and flocs are separated, and AnAOB can grow mostly in granules, whereas AOB and NOB grow in flocs, and NOB can be washed-out by wasting flocs (Han et al., 2016b). A second strategy has been to keep anammox in biofilm-based carrier while nitrifiers are kept in suspended floc-based sludge, and this system is called integrated fixed-film activated sludge (IFAS) (Laureni et al., 2019; Malovanyy et al., 2015a; Trojanowicz et al., 2016).

Chapter 2.

Thesis objectives

The general objective of this PhD thesis was to study suitable operational strategies for applying autotrophic nitrogen removal, based on anammox process, at the mainstream line of urban WWTP. The effects of the evaluated strategies on (partial) nitritation-anammox processes performance and on the involved microbial community, were investigated pointing at gaining insights towards the key parameters for PNA stability and efficiency. Special attention is paid on assessing the operation at low nitrogen content and seasonal temperature variations.

To achieve this general objective, several specific objectives were determined:

- To develop and assess a strategy based on limiting dissolved oxygen conditions (operation at micromolar DO concentrations) in order to achieve stable nitritation and NOB suppression at sidestream and mainstream conditions (Chapters 3 and 5);
- To study reactor performance response to abrupt temperature reduction from moderately to low temperature operation (25 ^oC to 15 ^oC), without biomass adaptation (Chapter 5);
- To characterize the microbial community of the PNA and link its dynamics to operating conditions changes: effects of transition from sidestream to mainstream conditions (Chapter 4) and abrupt temperature reduction (Chapter 5);
- To test the suitability of one-stage PNA in a plug-flow reactor with an aerobic and an anoxic compartments, operated at room temperature varying between 16 ^oC and 27 ^oC (Chapter 6);
- To stablish the effects of limiting inorganic carbon (IC) availability on microbial nitrifying activities at mainstream conditions in a SBR (Chapter 7).

Chapter 3.

Effects of extremely low bulk liquid DO on autotrophic nitrogen removal performance and NOB suppression in side- and mainstream one-stage PNA

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Effects of extremely low bulk liquid DO on autotrophic nitrogen removal performance and NOB suppression in side- and mainstream one-stage PNA

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Abstract

BACKGROUND: Results in the literature are divergent regarding dissolved oxygen (DO) level to ensure nitrite-oxidizing bacteria (NOB) suppression in mainstream conditions. In this study, a one-stage granular partial-nitritation (PNA) was operated controlling the oxygen transfer over ammonium loading rate (OTR/ALR). The effects of extremely low DO availability on autotrophic nitrogen removal (ANR) performance and NOB suppression were assessed.

RESULTS: The strategy applied in the sequencing batch reactor (SBR) in sidestream and mainstream conditions (temperature at 25 °C) allowed micromolar bulk liquid DO concentrations to be obtained. Nitrite production rate (NPR) by aerobic ammonium-oxidizing bacteria (AOB) was sufficient to provide nitrite to anammox bacteria (AnAOB) and sustain nitrogen removal rates (NRR) of 0.34 ± 0.05 and 0.37 ± 0.07 kg N m^{-3} d^{-1} during sidestream and mainstream operation, respectively. Extremely low DO assisted by free ammonia (FA; $\leq$6.2 mg N L^{-1}) in sidestream conditions resulted in complete nitratation suppression. In mainstream conditions, *Nitrospira* spp. growth led to limited nitratation rates. Decreasing the OTR/ALR resulted in lower oxygen consumption by AOB than by NOB, as well as reduced nitrite consumption by AnAOB, which is likely to be due to granule structure.

CONCLUSIONS: Low DO availability did not compromise ANR. Micromolar DO allowed complete NOB suppression only with FA assistance (sidestream) but was sufficient to keep nitratation limited in mainstream conditions.

Supporting information may be found in the online version of this article.

Keywords: nitritation–anammox; extremely low DO; NOB suppression; *Nitrospira*; mainstream

INTRODUCTION

The cost-effectiveness of autotrophic nitrogen removal (ANR) based on nitritation–anammox processes has already been proven and there are several full-scale applications worldwide for nitrogen removal from high-strength wastewater, mainly in sidestream treatment (digested sludge liquor) in urban wastewater treatment plants (WWTP).[1,2] Recently, efforts have been made in order to apply partial nitritation–anammox (PNA) processes in the mainstream line of WWTP[3,4] in order to develop energy self-sufficient facilities.[5] Although sidestream PNA reactors are commonly operated at warmer temperatures (higher than 30 °C),[4,6] mainstream anammox faces seasonal temperature variations, including low temperatures in winter, which can affect microbial activities and process stability.[4] Furthermore, in one-stage PNA reactors, where nitritation and anammox take place in the same unit, the nitrite-oxidizing bacteria (NOB) competes for oxygen with ammonium-oxidizing bacteria (AOB) and for nitrite with anammox bacteria (AnAOB). Thus, nitrogen removal efficiency can be compromised. Nitrate production by NOB in one-stage PNA reactors results in a $NO_3^-{}_{produced}$:$NH_4^+{}_{removed}$ molar ratio above 0.11.[7]

Nitratation has been experienced in many studies carried out in both sidestream[6,8] and mainstream conditions.[3,9,10] In mainstream conditions, due to the low nitrogen concentration of the wastewater, free ammonia (FA) and/or free nitrous acid (FNA) concentration cannot achieve inhibitory levels for NOB suppression.[9] The main strategy pointed out in several works to achieve NOB suppression without FA/FNA inhibition assistance relies on adjusting the reactor bulk liquid dissolved oxygen (DO) concentration to a

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Table 1. Comparison of one-stage PNA reactor performance under sidestream and mainstream conditions

Applied condition	Reactor type	Wastewater	NRR (kg N m^{-3} d^{-1})	DO (mg O_2 L^{-1})	DO (μmol L^{-1} O_2)	$NO_3^-{}_{produced}$: $NH_4^+{}_{removed}$ (%)	Temperature (°C)	Reference
Sidestream	MBBR	Reject water	0.3 ± 0.1	2.3 ± 0.8	71.9 ± 25	NP	24 ± 2.5	18
		Reject water	0.63	0.5–1.5	15.7–46.9	8–22	22–33	19
	SBR	Synthetic	1.1	0.3–0.7	9.4–21.9	NP	33 ± 1	20
		Synthetic	0.08	< 0.1	< 3.1	NP	30	21
		Reject water	0.36	0.5	15.7	NP	21	22
Mainstream	UMABR[a]	Synthetic	0.0776 ± 0.005	0.6	18.75	≤ 8	25	12
	Plug- flow[b]	Pre-treated domestic ww	0.189 ± 0.59	≥1	≥31.2	35 ± 16	19 ± 1	3
	OLAND[c]	Synthetic wastewater	0.529 ± 0.75	3.1 ± 0.2	96.9 ± 6.2	21 ± 4	14 ± 0.4	4
	MBBR	Pre-treated domestic ww	0.02–0.04	0.15–0.18	4.7–5.6	≤30	15	11
	SBR[d]	Pre-treated domestic ww	–	0.17 ± 0.08	5.3 ± 2.5	88	32 ± 1	23

NP, not presented.
[a] Upflow membrane-aerated biofilm reactor.
[b] Data obtained from the period with best performance without technical failures.
[c] Data obtained from operational Period VII.
[d] Data obtained from operational Period II.

proper level which should promote AOB over NOB growth, based on the affinity for oxygen.[11–13] Model-based studies have demonstrated that the desirable DO concentration to repress NOB activity depends directly on the residual ammonium concentration in the bulk liquid, which means that higher DO operation requires higher residual ammonium.[14,15] Similarly, some works highlight the need of adjusting DO set-point according to the ammonium loading rate (ALR) to properly control AOB and NOB activities.[16,17]

The required DO level that needs to be maintained to suppress NOB activity is not conclusive so far and a lot of research in the field is concentrated on this topic. Table 1 summarizes the results obtained by relevant reported PNA studies. In mainstream conditions, only a few works have reported effective NOB activity repression. Better reactor performance has been obtained in reactors where DO concentration was maintained lower than 0.6 mg O_2 L^{-1}. Controversially, in other studies low DO set-points did not ensure NOB suppression (Table 1). Moreover, similar nitratation activities were reported in studies operating either at low or high DO set-point (values higher than 1 mg O_2 L^{-1}). This overview presented in the current study did not account the effects of heterotrophic denitrification on nitrate reduction, which could cause underestimation of the nitrate production. Furthermore, other studies also have shown that biomass aggregate size affects the DO threshold where NOB is suppressed.[24,25] Accordingly, floccular biomass and/or small granule diameter biomass would require low DO concentrations. Hence, bulk liquid DO concentration plays a major role in the achievement of efficient mainstream PNA, but the possibility of suppressing NOB by low DO in low-strength wastewater remains unclear.

In order to produce stable one-stage nitritation–anammox and test the effect of extremely low DO on suppressing NOB activity, a strategy based on controlling the oxygen transfer rate (OTR) according to the ALR (OTR/ALR) was assessed in a PNA-SBR. The strategy was tested by operating the reactor under sidestream and mainstream conditions at 25 °C. The applied OTR/ALR ratio was based on stoichiometric requirements for PNA, which resulted in micromolar DO concentration in the bulk liquid. Specific bacterial activities and substrate competition were also evaluated, as well as process responses to transition from side to mainstream conditions.

MATERIAL AND METHODS

PNA-SBR setup and inoculation

The study was carried out in 10 L laboratory-scale sequencing batch reactor (SBR) with granular sludge. The reactor consisted of a water-jacketed glass vessel fermentor (Biostat B Plus, Sartorius, Germany). The system was equipped with pH (405-DPAS-SC-K8S/325, Mettler Toledo), ORP (4805 DPAS-SC-K8S/325), and DO (InPro 6850i polarographic oxygen sensor, Mettler Toledo) probes. Air was supplied with a mass flow meter and a stainless steel fine-bubble diffuser. Biomass suspension was ensured by mechanical stirring.

The reactor was inoculated in two steps. First, the reactor was seeded with floccular sludge from a pilot-scale PN-SBR treating landfill leachate, and then it was fed with a synthetic medium. A stable NH_4^+:NO_2^- effluent ratio around 1 was achieved by aerating the reactor, controlling the mass flow meter and keeping the bulk liquid DO concentration under the detection limit of the probe. According to manufacturer information, the detection limit of the probe is 6 ppb O_2 which results in a DO concentration of 0.2 μmol O_2 L^{-1}. Secondly, on day 14, the reactor was seeded with anammox-enriched sludge from a laboratory-scale anammox SBR fed with mineral medium; the specific nitrogen removal rate (sNRR) of the anammox sludge was about 430 mg N g VSS^{-1} d^{-1}.

PNA-SBR operation

Table 2 shows the main characteristics of the four operational periods considered in this study. In Period II, from days 133 to

Table 2. Characteristics of each operational condition

Operational period	Period I: Sidestream condition	Period II: Transition from side to mainstream condition	Period III: Mainstream condition	Period IV: Mainstream condition
Wastewater	Synthetic	Synthetic	Pre-treated municipal wastewater	Synthetic
Days of operation	0 - 133	133 - 173	173 - 297	297 - 390
Hydraulic retention time (HRT)	24 h	3 h and 12 h	3 h	3 h
Reactor working volume (L)	8	8 and 10	10	10
Volume exchange ratio (VER) (%)	25	25 and 50	50	50
NLR (kg $N \cdot m^{-3} \cdot d^{-1}$)	0.512 ± 0.05	0.608 ± 0.01	0.602 ± 0.02	0.585 ± 0.02
NH_4^+ inf. (mg $N \cdot L^{-1}$)	510.25 ± 55.12	169.55 ± 111.84	70.82 ± 3.93	69.51 ± 3.80
HCO_3^-:NH_4^+ inf. (mol:mol)	1.31 ± 0.12	1.55 ± 0.39	2.42 ± 0.58	3.90 ± 0.87
pH inf.	8.09 ± 0.11	8.00 ± 0.08	7.77 ± 0.23	8.48 ± 0.16
pH eff.	7.29 ± 0.21	7.09 ± 0.19	7.14 ± 0.18	7.35 ± 0.07
Conductivity inf. ($mS \cdot cm^{-1}$)	18.11 ± 0.66	10.71 ± 6.89	2.23 ± 0.11	2.52 ± 0.05
FNA ($\mu g\ HNO_2 \cdot L^{-1}$)	2.72 ± 5.12	0.07 ± 0.09	0.17 ± 0.21	0.03 ± 0.02

146, the transition from sidestream to mainstream conditions was performed by maintaining the nitrogen loading rate (NLR) and reducing the hydraulic retention time (HRT) from 24 h to 3 h and ammonium influent concentrations reduced from around 600–75 mg N L^{-1}. The cycle time decreased from 6 to 1.5 h. In both configurations, reactor filling and aeration took place simultaneously and comprised 77% of the total cycle length in the first cycle configuration and 72% in the second one (Fig. SI1 in File S1). From days 145 to 152, salinity in the influent synthetic medium was gradually decreased, and the conductivity dropped from 17 to 2 mS cm^{-1} to obtain similar values as mainstream conditions. The temperature was maintained at 25 °C throughout the entire experimental period to avoid its effects on microbial activities, allowing for the assessment of the process performance in each period.

Except in Period III, the reactor was fed with synthetic wastewater containing ammonium as the only nitrogen source. $(NH_4)_2SO_4$ and $NaHCO_3$ provided the ammonium and bicarbonate, respectively. The macro- and micronutrient concentration of the mineral medium is presented elsewhere.[26] In Period III, the reactor was fed with real wastewater (effluent from a municipal WWTP performing COD and N removal). However, to simulate sewage after COD removal, $(NH_4)_2SO_4$ and $NaHCO_3$ were added to the wastewater as ammonium and alkalinity source.

OTR/ALR ratio control

According to the oxygen mass balance, the variation of the bulk liquid DO concentration (*d*DO) over time is given by Eqn (1):

$$\frac{dDO}{dt} = OTR - OUR \qquad (1)$$

where OTR and OUR are the oxygen transfer and oxygen uptake rates, respectively, both in mg O_2 L^{-1} d^{-1}. Therefore, if OUR is equal to or higher than OTR, oxygen will not accumulate in the bulk liquid. The OTR from gas to liquid phase can be obtained by Eqn (2).

$$OTR = k_L a\ (DO^* - DO) \qquad (2)$$

where $k_L a$ is the oxygen mass transfer coefficient (d^{-1}) and DO^* is the saturation concentration of DO in the bulk liquid (mg L^{-1}). Prior to the reactor inoculation, assays were performed in the reactor to obtain the $k_L a$ at different airflow rates. These preliminary assays were carried out using tap water and the synthetic medium used to feed the reactor. In order to correct the OTR under side and mainstream conditions, new $k_L a$ tests were carried out in periods I and III. These tests were performed at the maximum working volume (8 and 10 L) and with mixed liquor (biomass plus feed medium).

In order to operate the reactor under strong DO limitation in the bulk liquid, the mass flow controller was manually manipulated in order to re-adjust the airflow rate to provide the OTR calculated according to the ALR (mg N L^{-1} d^{-1}). Stepwise adjustments avoid oxygen accumulation in the bulk liquid and DO levels remained undetected by the probe. Therefore, it was assumed that DO stayed below the minimum detection level of the DO probe. Stoichiometric oxygen demand to achieve partial nitritation and anammox process in the same reactor was obtained based on the one-stage PNA stoichiometry presented by Vlaeminck *et al.*,[7] as given in Eqn (3):

$$NH_4^+ + 0.79\ O_2 + 0.08\ HCO_3^- \rightarrow 0.11\ NO_3^- + 0.44\ N_2 + 1.03\ H^+ +$$
$$0.05\ CH_{1.4}O_{0.4}N_{0.2} + 0.03\ CH_2O_{0.5}N_{0.15} + 1.46\ H_2O \qquad (3)$$

OTR in bioreactors is affected by hydrodynamic conditions, medium composition and microbial activity,[27] so the applied airflow rate was slightly and carefully adjusted throughout the reactor operation to increase NRR during periods I to III, but avoiding higher oxygen supply than stoichiometrically required, which could become available for NOB growth. The $NO_3^-{}_{produced}$:$NH_4^+{}_{removed}$ molar ratio was used as reference to detect nitratation.

Chemical analysis

Ammonium and alkalinity were measured by titrimetric methods.[28] The concentrations of nitrite and nitrate were determined by ion chromatography (DIONEX ICS5000). DO, pH and ORP were measured by the aforementioned online probes.

Maximum specific anammox activity

The maximum specific anammox activity (SAA), which indicates the potential anammox conversion rates without substrate limitation, was measured *in situ*. The aeration was switched off in the reactor, and the substrate required was added in a spike. In sidestream conditions, only $NaNO_2$ was added in order to obtain equal NH_4^+:NO_2^- concentration, since ammonium was already present in the bulk liquid, while in mainstream conditions, $(NH_4)_2SO_4$ and $NaNO_2$ were added to reach equivalent concentrations of around 100 mg N L^{-1}. Samples were then collected to obtain nitrogen concentrations during the time course of the assay. SAA was calculated by linear regression of the ammonium and nitrite consumption, divided by the MLVSS concentrations in the reactor.

Microbial population analysis

Mixed liquor samples were taken from the reactor, and granules were disintegrated to obtain an homogeneous sample, washed with PBS and fixed in 4% paraformaldehyde. The samples were placed on gelatin-coated glass slides, and fluorescent *in situ* hybridization (FISH) was performed as described elsewhere.[29] The probes used to target the microorganisms are shown in Table SI1 in File S1. The images were obtained with a confocal microscope CLSM (confocal laser scanning microscopy) (Nikon A1R) and analysed with the ImageJ program to quantify the abundance of AOB, NOB and AnAOB. The abundances of each microbial population were quantified by calculating the area containing cells targeted by the Cy3-labelled (NOB and *Candidatus* Brocadia+Kuenenia) and Fluos-labelled (*Nitrosomonas* spp.) and comparing this as a percentage of the area of Cy5-labelled (Eubacteria) within each image. A total of 12 images were analysed per sample. Granule morphology was analysed by Nikon SMZ1000.

Calculations

Nitrogen mass balance

Volumetric nitrogen consumption rates (mg N L^{-1} d^{-1}) by AOB, NOB and AnAOB were determined by mass balance considering the nitrogen concentration species during the operation. The stoichiometry of anammox process was based on Strous *et al.*[30] Nitrogen assimilation by biomass and heterotrophic denitrification was not considered in the nitrogen mass balance. The equations and calculation procedures are presented in SI1.1 in File S1.

Oxygen uptake rate

OUR was calculated from the consumption rates of ammonium by AOB (OUR_{AOB}) and nitrite by NOB (OUR_{NOB}). Nitrification stoichiometry was based on Tchobanoglous *et al.*.[31]

Oxygen penetration depth

Oxygen penetration depth in the granule was calculated according to the procedures and assumptions presented in SI1.5 in File S1.

Free ammonia and free nitrous acid

FA and FNA concentrations were calculated according to Anthonisen *et al.*[32]

RESULTS

Long-term PNA-SBR nitrogen removal performance during sidestream and mainstream operation at extremely low DO (including the transition period) is presented in Figs 1 and 2. The start-up was carried out treating high-strength nitrogen concentrations wastewater (sidestream WW), by first obtaining a stable PN process. In spite of restrictive DO conditions in bulk liquid, the PN-SBR biomass remained active (specific nitrite production rate – sNPR – up to 1.25 g N gVSS^{-1} d^{-1}) and stable PN was achieved rapidly, with effluent ammonium and nitrite concentrations around 250 mg N L^{-1} [Fig. 1(b) and (c)]; this gave a suitable NH_4^+:NO_2^- molar ratio for the anammox process. The anammox biomass was then inoculated on day 14 to perform one-stage PNA. Results obtained during each operational period are presented in the following sections.

One-stage PNA-SBR operation in sidestream conditions (Period I)

One-stage nitritation–anammox was obtained as soon as the anammox biomass was inoculated: effluent nitrite concentration rapidly decreased from 250 mg NO_2^--N L^{-1} to values below 1 mg NO_2^--N L^{-1} and remained as low during Period I operation. In turn, nitrate production immediately started and effluent concentrations remained around 40 mg NO_3^--N·L^{-1} for all of Period I [Fig. 1(c)]. The ANR process was stable in Period I, performing at an average NRR of 0.34 ± 0.05 kg N m^{-3} d^{-1} (maximum of 0.49 kg N m^{-3} d^{-1}; day 112), corresponding to a specific removal rate of 0.190 mg N mg VSS^{-1} d^{-1} (VSS of 2.6 g L^{-1}; Fig. SI2 in File S1). The $NO_3^-{}_{produced}$: $NH_4^+{}_{removed}$ molar ratio was stable around the stoichiometric value for one-stage PNA of 0.11 [Fig. 1(a)],[7] indicating optimal ANR and absolutely negligible NOB activity during the entire sidestream operational period [Table SI3]. Due to variations in effluent ammonium concentration and pH, FA fluctuated from 0.3 to 6.2 mg NH_3-N L^{-1} [Fig. 1(b)]. The results demonstrate the feasibility of successful partial-nitritation and anammox processes in a one-stage SBR operated under strong DO limitation, by applying the oxygen transfer over ammonium loading as control strategy.

Table 3 shows the maximum SAA values obtained in batch assays carried out during Period I and the specific nitrogen conversion rates in the reactor by AOB, NOB and AnAOB for the respective operational days and calculated by nitrogen mass balance. Maximum SAA values ranged from 0.29 to 0.39 mg N mg VSS^{-1} d^{-1}, whereas nitrogen conversion by anammox in the reactor (SAA_{cycle}) ranged from 0.109 to 0.177 mg N mg VSS^{-1} d^{-1}. These values indicated that AnAOB activity was balanced with the nitrite supply rate from AOB (maximum SAA was not reached in the reactor due to nitrite limitation), whereas NOB activity was suppressed and did not contribute to nitrogen conversions.

Transition from side- to mainstream conditions (Period II)

Transition from sidestream to mainstream conditions was performed from days 133 to 173 in two steps. On day 133, the influent ammonium concentration was lowered by half, while the HRT was reduced down to 0.5 day and reactor working volume changed from 8 to 10 L to maintain the same NLR as in Period I. The total cycle time, aeration length and mixing speed were maintained, as well as the airflow rate. This hydraulic modification was followed by an unpredicted reduction in nitrogen removal and effluent ammonium concentration increased from 190 to 227 mg NH_4^+-N L^{-1} [from days 131 to 134; Fig. 1(b)], which was attributed to a decrease of the oxygen transfer caused by the modification of bulk liquid hydrodynamics. The ammonium concentration was progressively decreased down to 55 mg NH_4^+-N L^{-1} by readjusting the airflow rate from 0.18 to 0.39 L air·L^{-1} reactor·h^{-1}, to increase the OTR and improve the nitritation rate of the system once effluent ammonium stabilized. An NRR of 0.43 kg N m^{-3} d^{-1} was reached on day 145 [Fig. 1(a)].

On day 146, HRT was further reduced to 3 h and influent ammonium set to normal sewage values (around 75 mg NH_4^+-N L^{-1}),

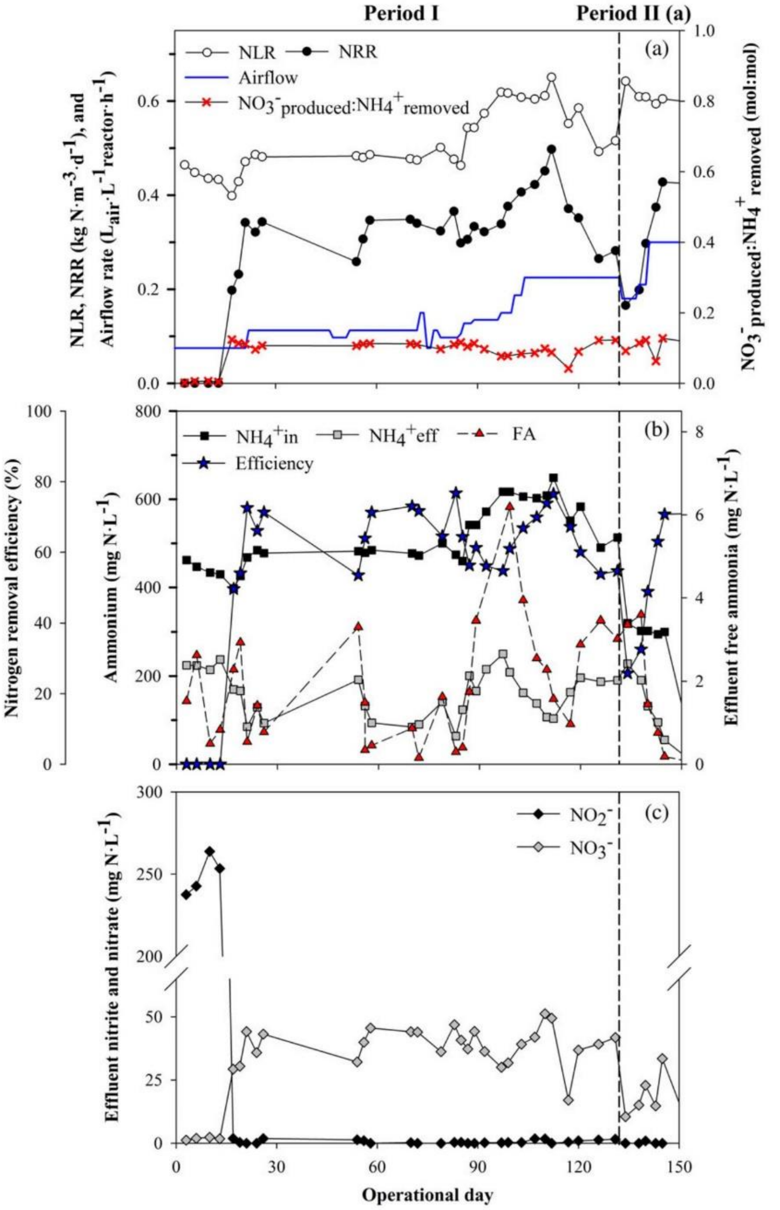

Figure 1. Continuous reactor operation at sidestream and beginning of transition to mainstream (Periods I and II, respectively): (a) Nitrogen loading and removal rates; specific airflow rate; and the molar ratio $NO_3^-{}_{produced}$:$NH_4^+{}_{removed}$; (b) Nitrogen removal efficiency, effluent ammonia and free ammonia concentrations; and (c) effluent nitrite and nitrate concentrations.

which resulted in effluent ammonium concentrations lower than 27 mg NH_4^+-N L^{-1}. Such conditions lowered FA below 0.17 mg NH_3-N L^{-1} [Fig. 1(b)]. Transition from sidestream to mainstream conditions did not impair reactor performance and nitrogen removal remained similar to Period I, ranging between 0.34 and 0.43 kg N m^{-3} d^{-1}. Furthermore, evidence for NOB activity was not detected in the period, with a stable $NO_3^-{}_{produced}$: $NH_4^+{}_{removed}$ molar ratio [Fig. 1(a)].

Mainstream one-stage PNA-SBR operation (periods III and IV)

Nitrogen removal performance during reactor operation with real wastewater (Period III) presented two distinct phases. For the initial one (days 173–194), the average NRR was 0.41 ± 0.06 kg N m^{-3} d^{-1} (corresponding to a sNRR of 0.16 ± 0.02 mg N mg VSS^{-1} d^{-1}). However, a steady decrease in NRR values was observed, whereas the $NO_3^-{}_{produced}$:$NH_4^+{}_{removed}$ ratio reached values up to 0.2 [Fig. 2(a)],

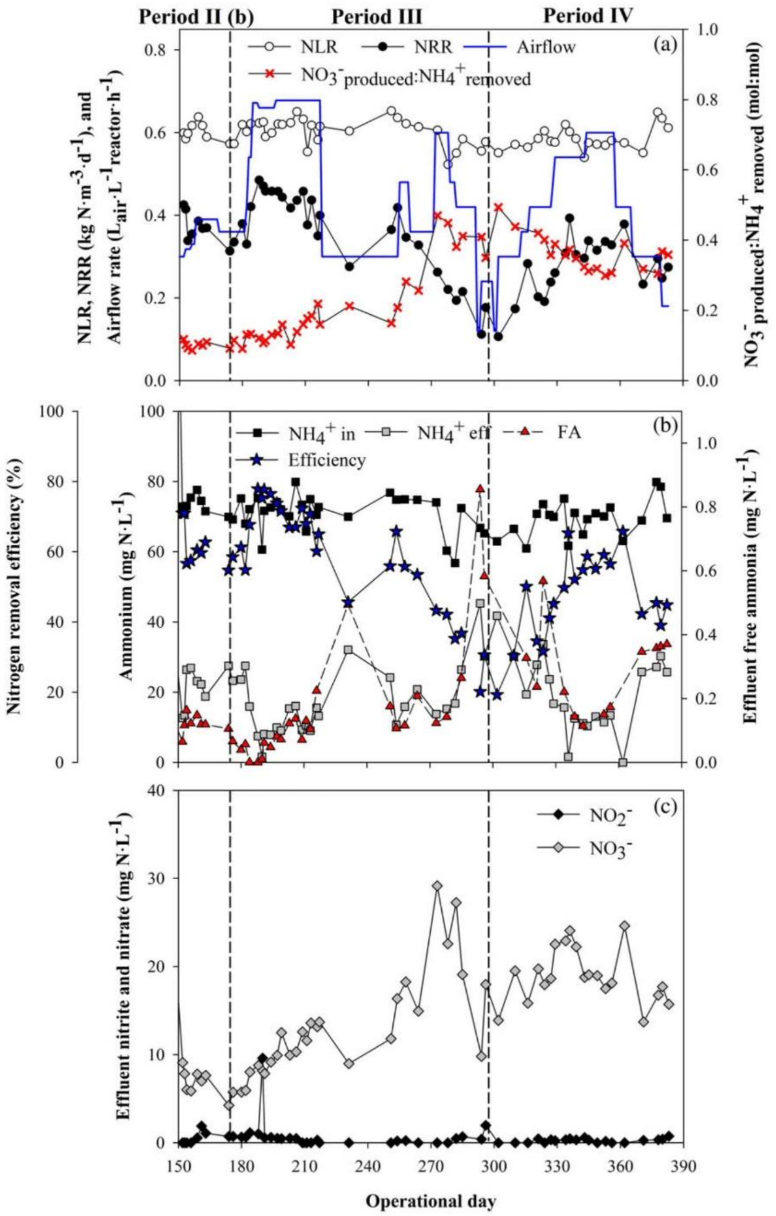

Figure 2. Reactor operation during transition period (end of Period II) and mainstream conditions (Periods III and IV): (a) Nitrogen loading and removal rates; specific airflow rate; and the molar ratio $NO_3^-{}_{produced}:NH_4^+{}_{removed}$; (b) Effluent ammonia and free ammonia concentrations; and (c) effluent nitrite and nitrate concentrations.

indicating a slight NOB activity contribution to nitrate production. NOB activation was not attributed to an excess of oxygen supply according to AOB requirements, because the effluent ammonium concentration was barely lower than 10 mg NH_4^+-N L^{-1} [Fig. 2(b)]. To test the effect of stronger OD limitation on NOB competition, the specific aeration flow was reduced from 0.68 to 0.30 L air L^{-1} reactor h^{-1}, which decreased the NRR to 0.28 kg N m^{-3} d^{-1} on day 231. NOB activity kept growing and the $NO_3^-{}_{produced}:NH_4^+{}_{removed}$ ratio increased to around 0.4, even though additional DO shortages were applied from days 250 to 285 (the airflow rate was adjusted). Consequently, the NRR was not stable and progressively decreased, reaching 0.19 kg N m^{-3} d^{-1}. Instability during Period III was partly attributed to ammonium oxidation in the influent storage tank, leading to presence of nitrite in the influent (up to 15 mg N L^{-1}; data not shown) and system unbalance.

Table 3. Specific nitrogen conversion rates

Period	Operational day	SAA	SAA_{cyle} ($rNH_4^+ + rNO_2^-$ by AnAOB)	$rNH_4^+{}_{AOB}$ mg N mg VSS^{-1} d^{-1}	$rNO_2^-{}_{NOB}$	SAA_{cycle}/SAA
I	84	0.38	0.148	0.084	0	0.39
	100	0.39	0.177	0.094	0	0.45
	128	0.29	0.109	0.064	0	0.38
III	259	0.33	0.100	0.078	0.023	0.30
IV	375	0.27	0.088	0.074	0.024	0.33

The real wastewater used to feed the reactor in Period III was effluent from an urban WWTP, which contained some remaining organic matter (average CODs of 32.7 ± 12.8 mg O_2 L^{-1}), and overall low COD removal was achieved in the PNA-SBR (effluent CODs of 31.5 ± 11.9 mg O_2 L^{-1}; Table SI4). Hence, it is acceptable to assume that the heterotrophic denitrification contribution to nitrogen removal was minimal, although some endogenous heterotrophic process might also have occurred due to the soluble microbial products (SMP).

Maximum SAA obtained in Period III batch assays was 0.33 mg N mg VSS^{-1} d^{-1} (similar values to Period I), whereas the anammox activity during reactor operation (SAA_{cycle}) was only 0.10 mg N mg VSS^{-1} d^{-1}. This shows that anammox was limited by nitrite availability. Hence, NOB activity resulted in nitrite consumption (Table 3). These results demonstrate that NOB activity was possible at extremely low DO (micromolar concentrations) and strong DO competition with AOB.

A mineral medium replaced pre-treated real wastewater from day 297 onwards (Period IV) to avoid organic matter and nitrite supply to the system by the influent. The airflow rate was raised stepwise and an improvement of the NRR up 0.39 kg N m^{-3} d^{-1} was obtained. The $NO_3^-{}_{produced}$:$NH_4^+{}_{removed}$ molar ratio was more stable and the average value was 0.36 ± 0.044. The effect of varying OTR to the system on the competition between AOB and NOB for oxygen is presented in Fig. 3(a) and (b). Neither OUR_{AOB} nor OUR_{NOB} presented a strong correlation to the airflow rate variation. Besides, results show that $rNH_4^+{}_{AOB}$ was more affected than nitrite oxidation by NOB ($rNO_2^-{}_{NOB}$) when the oxygen supply was additionally reduced (R^2 for $rNH_4^+{}_{AOB}$ and $rNO_2^-{}_{NOB}$ were 0.65 and 0.25, respectively). The competition for nitrite between NOB and AnAOB was assessed by comparing the rNH_4^+ by AOB and the conversion rates by both NOB and AnAOB (rNO_2^-) [Fig. 3 (c)]. The correlation analysis showed that the consumption of nitrite by anammox presented higher correlation ($R^2 = 0.80$) than the consumption by NOB ($R^2 = 0.61$). In other words, higher oxidation rate by AOB produced more NO_2^-, which increased availability for AnAOB, whereas NOB was less susceptible.

Microbial community composition and biomass characteristics

Several biomass samples were collected and analyzed during the whole experimental study. Figure 4 shows the relative AOB, NOB and AnAOB abundances obtained by FISH analysis. *Nitrosomonas* spp. and both anammox target bacteria, responsible for thePNA process, were the most abundant bacteria in all analyzed samples. During the sidestream operation, both *Nitrobacter* spp. and *Nitrospira* spp. accounted for about 10% of the total bacteria (samples on day 17, after anammox biomass inoculation; and day 127, at stable operation). However, *Nitrospira* spp. increased to 16 ± 1% in the sample collected after 7 months of reactor operation under mainstream conditions (day 386). The biomass cultivated in the reactor presented a mix of brown and red color [Fig. 4(f)], different from the red granules presented in the anammox inoculum (data not shown). Granular biomass was the predominant fraction, although some floccular sludge was also present [Fig. 4(g)]. The presence of these small diameter aggregates did not impact the overall biomass settling (Fig. SI2 in File S1), and the short settling time of 5 min adopted from Period II onwards was enough to retain the biomass.

Regarding the DO penetration depth in granular biomass under such strong DO limiting conditions, it was estimated at reaching average values up to 1.45 and 1.6 µm, during sidestream and mainstream conditions, respectively. By assuming that DO penetration depth is equal for all aggregates, an anoxic zone higher than 50% of the total granule volume is estimated for particles with an average diameter higher than 4 µm (SI1.5 in File S1). Consequently, a small aerobic zone is assumed to occur even with small diameter aggregates.

DISCUSSION

Oxygen transfer rate control as a strategy for ANR

Controlling the oxygen transfer rate according to the NLR was applied as a strategy to control nitritation–anammox processes in one-stage SBR in this study. Besides ANR, NOB activity repression was assessed also in both sidestream and mainstream domestic wastewater treatment. The average NRR achieved in the sidestream in this study under strong DO limitation (0.34 ± 0.05 kg N m^{-3} d^{-1}), was similar to those obtained by Cema *et al.*[18] and Vázquez-Padín *et al.*[22] at higher bulk liquid DO concentration (similar temperature). During mainstream operation and before NOB proliferation, the sNRR was 0.16 ± 0.02 mg N·mg VSS^{-1} d^{-1}, which is within the range of values reported in other granular reactors (0.08 ± 0.01 and 0.18 ± 0.01 mg N mg VSS^{-1} d^{-1}), operated at DO concentrations of 0.5–1 mg L^{-1} (15.6–31.2 µmol L^{-1} O_2),[33] and even higher than the values reported by Lotti *et al.*[3] in a reactor operated with DO of around 1.5 mg O_2·L^{-1} (46.9 µmol L^{-1} O_2), at close temperatures. This proves that satisfactory removal rates could be reached even when operating the PNA-SBR at extremely low DO levels by limiting the OTR according to the ammonium loading rate. Moreover, it also implies that aeration costs can be reduced without hampering reactor performance.

The PN level could be well-controlled, and the rates between nitritation and anammox processes were balanced throughout all

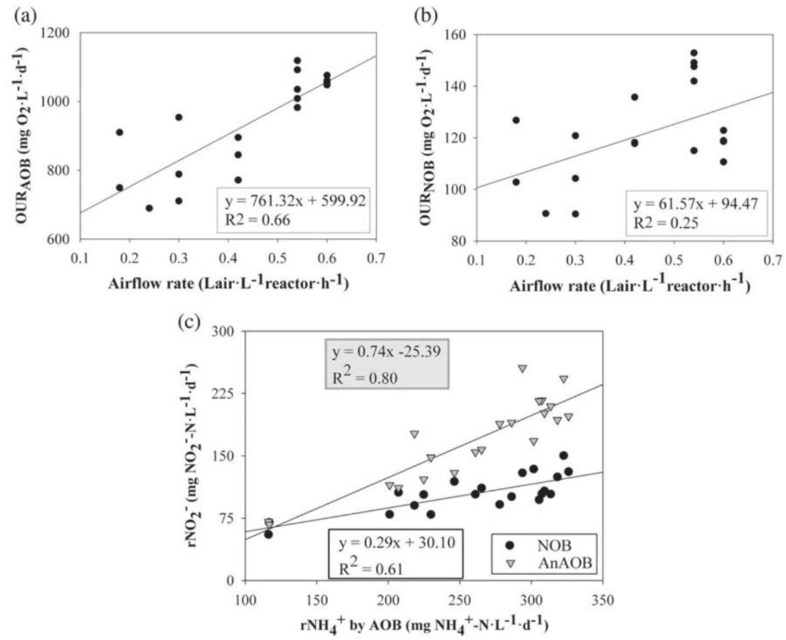

Figure 3. Correlation analysis for the reactor operation from operational day 297 onwards. (a) Correlation between the airflow rate and OUR_{AOB}; (b) correlation between the airflow rate and OUR_{NOB}; and (c) correlation between the aerobic ammonium oxidation rate by AOB ($rNH_4{}^+{}_{AOB}$) and nitrogen consumption by NOB and AnAOB.

of the experiments regardless of the alkalinity excess, resulting in low nitrite effluent concentrations [Figs 1(c) and 2(c)]. Hence, nitrite was in turn well-controlled ensuring that levels which could damage anammox activity[34] are never reached. Absence of or low concentration of nitrite in the bulk liquid indicates rapid nitrite depletion by the AnAOB as soon it is formed, preventing favorable conditions for NOB growth.[8] This contrasts with previous PNA reactors operated at a fixed DO set-point, in which unbalanced nitritation and anammox process rates experienced in sidestream[6] and mainstream applications[13] led to nitrite accumulation and rapid NOB growth.

Furthermore, the low bulk liquid DO resulted in a limited oxygen availability deep inside the granules. Based on calculations, a DO gradient occurred even in floccular biomass. The estimated values of 1.45 and 1.6 μm obtained in this study under sidestream and mainstream conditions, respectively, were lower than the 13.3 μm estimated by Winkler *et al.*[33] and 100 μm reported by Vázquez-Padín and collaborators,[35] for bulk liquid DO concentrations of 0.5 and 1 mg·L^{-1} (15.6–31.2 μmol L^{-1} O_2), respectively. In our reactor, low DO penetration in aggregates maximized the anoxic zone volume to favor AnAOB, which helped sustain SAA values between 0.27–0.39 mg N mg VSS^{-1} d^{-1} throughout the experimental period (Table 3).

Further investigation is necessary to address the impacts of the strategy assessed in this study, which had extremely low DO concentration, on nitrous oxide (N_2O) production and emission. DO has the potential of affecting N_2O production during several steps of nitrification and denitrification processes. AOB can produce N_2O by the hydroxylamine oxidation pathway, which seems to be favored under high ammonia and DO concentrations due to higher ammonium oxidation rates,[36] whereas the nitrifier denitrification pathway is stimulated under high nitrite and low DO.[37] This would suggest that low N_2O production and emission should be expected from a PNA operated at micromolar DO. However, a recent study pointed out that N_2O production by both hydroxylamine and nitrifier denitrification pathways could be stimulated under low DO concentration, regardless of nitrite concentrations.[38]

Nitritation rate potential at low (limiting) DO

Low DO concentration has always been associated with poor nitrogen removal in activated sludge reactors and DO concentration has to be set higher than 2 mg O_2 L^{-1} in order to not harm nitrification.[39] In the present study, nitritation activity was not compromised, even from reactor start-up, in spite of the low bulk liquid DO (inoculum was obtained from a PN-SBR operated at DO of 1.5 mg L^{-1}). The sNPR values (<1250 mg N g VSS^{-1} d^{-1}) were similar to the 1360 mg N g VSS^{-1} d^{-1} obtained by Wyffels *et al.*[40] at operational an DO of 2.85 mg O_2 L^{-1} (89.1 μmol L^{-1} O_2), and much higher than the value of around 430 mg N g VSS^{-1} d^{-1} obtained at DO of 2 mg O_2 L^{-1} (62.5 μmol L^{-1} O_2) by López-Palau *et al.*[41] Although in granular biomass a strong DO limitation can occur due to mass transfer, nitritation was still maintained, and it was possible to improve nitrite production for AnAOB by adjusting the airflow rate [Fig. 1(a)], always keeping DO at micromolar concentrations.

Nitrosomonas spp. were the dominant AOB and present during the entire experimental period [Fig. 4(a)]. This indicates favorable conditions for AOB growth under stringent oxygen availability, although typical $K_{O2,AOB}$ values higher than 0.07 mg O_2 L^{-1} usually are reported (Table SI2 in File S1). Our results are in concordance with Park and collaborators,[42] who proved that low DO environments are able to sustain AOB populations that exhibit high affinities for oxygen. This has been associated with high SRT which allows AOB to work under oxygen limitation.[43,44] Biomass can be retained in the case of biofilm and granular reactors, where high

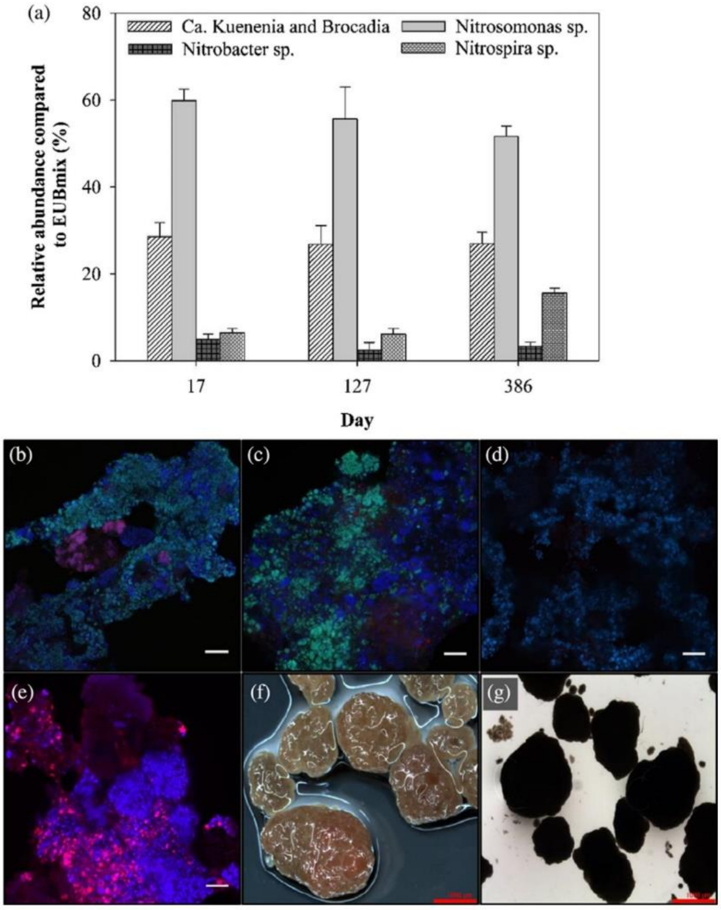

Figure 4. Microbial characterization throughout PNA-SBR operation. (a) Relative abundances obtained by FISH analysis for the samples grabbed at the beginning of reactor operation (day 17), the end of the sidestream operation (day 127), and the end of mainstream conditions (day 386); (b) and (c) fluorescence images of *Nitrosomonas* spp. (Flus-green), *Nitrobacter* spp. (Cy3-red) on days 127 and 386, respectively; (d) and (f) confocal images of *Nitrospira* spp. (Cy3-red). EUBmix was targetted with Cy5 (blue) in all the images; white scale bar: 20 μm; (g) and (h) microscopic images of the granules in the end of reactor operation; red scale bar: 1000 μm.

SRT is achieved. Besides, the presence of small diameter aggregates or even floccular biomass [Fig. 4(g)] can promote higher specific nitritation activity than in granules, because mass transfer limitation effects are softened.[45]

Effectivity of low DO for successful NOB suppression in sidestream and mainstream operations

Oxygen transfer control played an important role in limiting the nitratation process in the PNA-SBR. NOB activity was absolutely negligible in sidestream conditions, whereas it could only be limited (not totally suppressed) during mainstream operation [Figs 1(a) and 2(a)]. The nitrate production over the stoichiometric values of 0.11 at mainstream was comparable to the values obtained by other studies which operated one-stage PNA at DO concentrations higher than 1 mg O_2 L^{-1} (31.2 μmol L^{-1} O_2).[3,4] In this study, the nitratation process is attributed to *Nitrospira* spp. [Fig. 4(a)], which have been reported to be more competitive under substrate limitation (k-strategist).[46,47] As shown in Table SI2 in File S1, the DO and nitrite affinity constants reported for *Nitrospira* spp. are commonly lower than for AOB and AnAOB, which complicate the control of nitratation in the mainstream opreation. Furthermore, recent studies revealed the existence of a pathway for complete ammonia oxidation ('comammox'),[48] carried out by bacteria grouped in the genus *Nitrospira*. Their ecophysiology and niche differentiation are still not settled, but it has been shown that they can be identified in several engineering systems with low ammonium and low oxygen concentrations,[49] typical conditions obtained during the mainstream conditions. The comammox pathway was not considered in this study, but its possible impact on the AOB-AnAOB activity balance should be explored in further works at low DO conditions.

We hypothesize that the microbial stratification in the granule played an important role in the NOB competition for oxygen and nitrite. Based on previous research, NOB are located close to AOB in the biofilm–liquid interface,[33,45,50,51] whereas anammox is located in the inner biofilm.[35] Nevertheless, by limiting aerobic ammonium oxidation (rNH_4^+) a drop in the nitrite consumption rate by anammox higher than NOB was obtained [Fig. 3(c)]. Hence, NOB could have accessed nitrite before it was transferred to where AnAOB were located in the granule. Picioreanu *et al.*[52] proposed that the best structure to outcompete NOB by oxygen affinity is a granule stratification where AOB are located in the outer layer and NOB in the inner part. Nonetheless, if AnAOB is placed behind NOB, nitrite would not be available for AnAOB. Our results demonstrated that NOB activity in one-stage PNA can be limited by low DO, but not fully suppressed. However, effluent nitrate can be lowered allowing limited heterotrophic denitrification in the system, as noted in other works where the influent contained some COD or endogenous contribution due to cell lysis or decay was remarkable.[11,12]

Besides oxygen control, higher FA concentrations in sidestream than in mainstream conditions assisted full NOB suppression [Fig. 1(a)]. *Nitrospira* spp. inhibition by FA concentrations between 0.04 and 0.08 mg NH_3-L^{-1} was reported,[53] which is less than the concentrations obtained at the sidestream, but no effects of FA on NOB were obtained in Periods III and IV, where values also ranged above the inhibitory level reported. Moreover, previous studies have shown that NOB biomass can become acclimated to higher FA concentrations and no nitrite build-up can be maintained.[54,55] Thus, NOB suppression can only be reached by associating FA inhibition with DO limitation.[55]

The influent salinity of 10.16 ± 0.35 g NaCl L^{-1} also might have had positive effects on NOB repression during sidestream operations. The same salinity level proved to wash out *Nitrospira* spp. from a one-stage PNA reactor operated at a low DO set-point,[56] but was not effective in stopping nitratation and *Nitrospira* spp. where the main NOB group in the biomass of a PNA reactor operated nearly DO saturation.[57] Hence, salinity cannot prevent nitratation in a long-term operation.

CONCLUSIONS

In this study, oxygen transfer according to the ammonium loading was applied to control a one-stage nitritation–anammox SBR for sidestream and mainstream urban wastewater treatment. Results proved that stable nitritation and anammox processes could be obtained working at extremely low DO (micromolar conditions), with NRR (up to 0.49 kg N m^{-3} d^{-1}) comparable to reactors with higher bulk liquid DO. This could help decrease the energy consumption by aeration in PNA, because oxygen transfer efficiency is improved compared to DO set-point operation. *Nitrospira* spp. and *Nitrobacter* spp. were detected by FISH during all reactor operation, but only *Nitrospira* spp. abundance increased at mainstream conditions, when extremely low bulk liquid DO was not sufficient to fully suppress nitratation. In such conditions, *Nitrospira* spp. could compete for oxygen with AOB and nitrite with AnAOB. Nitrite production and consumption rates were balanced in the system, but a decrease of the NPR impacted more on SAA than nitratation, due to a better availability of nitrite for NOB which may be caused by bacterial stratification in the granule. Anammox activity was maintained higher than 0.27 mg N mg VSS^{-1} d^{-1} during the whole reactor operation. Altogether, the results reinforced the hypothesis that only DO control cannot ensure total NOB repression at mainstream, but by operating the PNA reactor at micromolar DO concentrations, a limitation in NOB activity is achieved and maintained in long-term operation.

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Supporting Information

Supporting information may be found in the online version of this article.

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Chapter 4.

Bacterial community succession in a nitritation-anammox reactor face to the transition from sidestream to mainstream conditions

This chapter has been prepared for submission:

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Abstract

This study investigated the microbial community in a partial nitritation-anammox (PNA) reactor operated at sidestream and further modified to mainstream conditions. A sequencing batch reactor (SBR), for which bulk liquid dissolved oxygen (DO) concentration was maintained at micromolar level (µM), was operated for 390 days. A rather similar nitrogen removal rate was reached at sidestream and mainstream sewage (0.34 ± 0.04 and 0.37 ± 0.07 kg $N \cdot m^{-3} \cdot d^{-1}$, respectively). Sequencing Illumina and quantitative polymerase chain reaction (qPCR) allowed identifying and following nitrifying, anammox and heterotrophic bacteria throughout all the study. Results demonstrated that the main bacteria core in sidestream conditions was similar to other PNA reactors operated at higher bulk DO and even at anoxic conditions. The transition to mainstream led to an increase in the diversity and richness of bacterial community, and it was obtained after the only period that the reactor was fed with pre-treated sewage (real wastewater). The anammox bacteria *Candidatus* Kuenenia had been the most abundant genus and presented abundance higher than 15%. Putative heterotrophic bacteria in the phyla *Chloroflexi*, *Proteobacteria*, *Bacteroidetes* and *Ignavibacteriae* were obtained in great number as well, as a result of cell decay products and external organic carbon (only at operation with pre-treated sewage). Despite the operation strategy applied to control nitratation, *Nitrospira* grew only at mainstream conditions, and only limited nitratation suppression was obtained at this operational condition.

Keywords: Deammonification; Nitrogen removal; Microbial community; High-throughput sequencing; One-stage nitritation-anammox

Chapter 5.

Assessment of operational conditions towards mainstream partial nitritation-anammox stability at moderate to low temperature: Reactor performance and bacterial community

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Assessment of operational conditions towards mainstream partial nitritation-anammox stability at moderate to low temperature: Reactor performance and bacterial community

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HIGHLIGHTS

- Balancing AOB and AnAOB rates is the key factor for mainstream PNA at μM DO.
- Stable PNA after temperature decrease from 25 to 15 °C without acclimation.
- Heterotrophs growth on dissolved organic carbon from cell lysis decreases effluent NO_3^-.
- *Nitrospira* was the only NOB and their control was assisted by AnAOB activity.
- *Candidatus* Kuenenia was the predominant AnAOB throughout the study.

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ABSTRACT

This study aimed at assessing the performance and microbial community in a granular one-stage partial nitritation-anammox sequencing batch reactor (PNA-SBR) subjected to temperature transition from 25 to 15 °C without biomass acclimation. The PNA-SBR was operated by controlling the oxygen transfer rate (OTR) according to the ammonium loading rate (ALR), which resulted in micromolar (μM) bulk dissolved oxygen (DO) concentration. The applied strategy proved to be feasible to operate the one-stage PNA-SBR at mainstream conditions because it was possible to control nitritation according to anammox rate. Nitrogen removal rate (NRR) of 330.24 ± 25.36 mg N·L^{-1}·d^{-1} was achieved at 25 °C. Nitratation control by μM bulk DO limited the $NO_{3\,production}^-$:$NH_{4\,removed}^+$ at 0.28 ± 0.04. No instability was experienced by decreasing the temperature to 15 °C, but removal rates were adapted to the resulting anammox activity, which decreased at low temperature. After temperature transition, nitratation was kept controlled and the $NO_{3\,production}^-$:$NH_{4\,removed}^+$ molar ratio remained at 0.33 ± 0.05, although anammox activity deteriorated and higher nitrate production was obtained. Sequencing analysis revealed the dominant bacterial groups in the microbial community that clustered within the phyla *Planctomycetes*, *Proteobacteria*, *Chloroflexi*, and *Bacteroidetes*. Temperature drop only affected bacterial abundance, but the main bacteria involved in nitrification and anammox processes did not change during the study. *Candidatus* Kuenenia was the main anammox genus. Moreover, the presence of bacterial groups associated with heterotrophic metabolism indicates denitrification might be supported by the release of dissolved organic carbon due to bacterial lysis, and lower nitrate effluent concentration could be reached in PNA reactors.

1. Introduction

Recent research on autotrophic nitrogen removal has been focused on partial nitritation-anammox (PNA) implementation in the mainstream line of urban wastewater treatment plants (WWTP), encouraged by the intention of achieving energy-neutral plants [1,2]. The application of mainstream anammox is profitable – when compared to conventional biological nutrient removal process – because organic matter for heterotrophic denitrification is no longer needed and can be valorized, while aeration requirements for N removal are reduced by

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60% [3]. In the attempt of addressing mainstream PNA, several studies have shown that nitrite-oxidizing bacteria (NOB) growth control is a real bottleneck, because nitrate build-up can limit nitrogen removal performance at long-term operation [4–7].

At mainstream line conditions, both low-strength nitrogen concentrations and seasonal temperature variations affect microbial activity and dynamics. Anammox bacteria (AnAOB) have lower growth rates compared to those for ammonium-oxidizing bacteria (AOB) and NOB. Typical doubling time of AnAOB is 15–30 d [8]; but low temperature operation results in doubling times above 79 d [9]. Furthermore, anammox activity reduction as consequence of temperature drop can lead to accumulation of ammonium and nitrite, which implies that in one-stage PNA reactors, both nitrogen loading rate (NLR) and nitritation rate must be controlled to avoid nitrogen accumulation. Nitrite build-up as symptom of higher nitritation than anammox rate has been described in other studies, when temperature was lowered [5,7]. To overcome this unbalance, nitritation and anammox activity should be proper regulated. This represents one of the main drawbacks to be solved in order to successfully apply one-stage PNA at low temperature [4].

Dissolved oxygen (DO) control in the bulk liquid is the main strategy used to control nitritation according to anammox rates. Indeed, it has been generally applied in full-scale sidestream PNA installations [10,11]. Successful NOB suppression in low-strength wastewater using low DO concentration (between 0.17 and 0.60 mg $O_2 \cdot L^{-1}$) has been reported in lab and pilot-scale studies [12–14]. However, failure to abate NOB activity under low DO concentration has also been described in other studies [7,15] especially at long-term operation, leading to inconclusive results regarding the efficiency of low DO on NOB suppression. Furthermore, NOB community niche differentiation also relies on oxygen availability. *Nitrospira*-like NOB have low specific growth rate compared to *Nitrobacter*-like NOB, but higher substrate affinity (for nitrite and DO), which makes them capable of taking advantage in a low DO environment [16]. Nevertheless, in one-stage PNA, both AnAOB and NOB compete for nitrite, and AnAOB could assist NOB repression [17].

Another shortcoming for PNA implementation is the large start-up time due to the slow anammox growth. Aiming at reducing start-up time requirements, the strategy applied to inoculate new reactors at mainstream is carried out by using sludge from sidestream reactors, operated at warm temperature (> 30 °C). In this sense, temperature has been stepwise decreased for several weeks in most of the mainstream one-stage PNA operation studies [5,7,18]. Although, from a practical view, acclimation time in full-scale facility is not feasible. Morales and collaborators [4] reported that granular biomass could operate without previous acclimatizing period, despite the fact granular biomass from sidestream reactor (temperature of 30 °C and 1000 mg NH_4^+-N·L^{-1}) was subjected to mainstream conditions (temperature of 15 °C and 50 mg NH_4^+-N·L^{-1}). The main downsides identified at long-term operation were the biomass washout and expressive NOB activity. The effects of exposing the biomass from a one-stage PNA operated at moderately temperature of 25 °C and low-strength nitrogen to 15 °C without acclimation have not been investigated yet. Furthermore, only few studies evaluated microbial community dynamics at mainstream conditions and low temperature [7,18–20], and most of the studies has been carried out in moving bed-biofilm reactor (MBBR), which seems to present a more stable microbial community. However, granular PNA-SBR is one of the main technologies applied at sidestream [3], which implies better availability of granular biomass for new reactors inoculation. Microbial community information under sudden temperature transition in mainstream PNA reactors is also scarce.

Aiming at gaining more features towards mainstream anammox application and stability, this study was conducted to evaluate the effects of the transition, without biomass adaptation, from moderately to low temperature on the performance and microbial community dynamics of a one-stage PNA operated at mainstream conditions. For this purpose, a lab-scale granular one-stage PNA-SBR was operated at the temperatures of 25 °C and 15 °C. The oxygen transfer rate (OTR) according to the ammonium loading rate (ALR) in order to obtain balanced AOB and AnAOB rates and suppress NOB activity was assessed as control strategy.

2. Material and methods

2.1. One-stage nitritation-anammox operation

The study was carried out in a granular PNA-SBR, which was set up in a 10 L working volume glass vessel reactor (Biostat B Plus, Sartorius) equipped with DO (InPro 6850i polarographic oxygen sensor, Mettler Toledo), pH (405-DPAS-SC-K8S/325, Mettler Toledo) and ORP (4805 DPAS-SC-K8S/325) probes. The reactor had been operated at sidestream and mainstream conditions for 390 days at 25 °C prior to this study, using a different operational cycle configuration.

This study was divided into three experimental periods: i) Period I: continuous aeration at temperature of 25 °C (until day 78); ii) Period II: continuous aeration at 15 °C (days 79–184); and iii) Period III: intermittent aeration at 15 °C (from day 185 onwards). During periods I and II, the reactor ran with an operational cycle of 1.5 h, distributed in: 5 min feeding; 5 min anoxic reaction; 60 min aerobic reaction; 15 min anoxic reaction; 5 min settling, and 5 min effluent withdrawn. In Period III, the operational cycle length was of 1.5 h until day 207, and 6 h from day 207 onwards. During the 1.5 h length, the cycle pattern was similar to periods I and II, but the aerobic reaction time was shortened to 2 min, while the anoxic one was extended to 68 min. The 6-h cycle was distributed in: 5 min feeding; 3 times aerobic and anoxic reaction of 2 and 113 min, respectively; 5 min settling; and 5 min effluent withdrawn. During all the experimental period the reactor was operated with mineral medium (see composition in SI). Ammonium was the only nitrogen source, added by $(NH_4)_2SO_4$. The mineral medium was maintained in a stirred tank at 4 °C, not deoxygenated. The bulk liquid pH was not controlled at a fixed set-point, and average values in the reactor influent and effluent were 8.45 ± 0.20 and 7.54 ± 0.20, respectively (Fig. S2).

The aeration strategy consisted in providing the stoichiometric amount of oxygen required to obtain balanced nitrite production by AOB according to AnAOB consumption rate. For this purpose, oxygen requisite was based on partial nitritation and anammox process in one-stage reactor (0.792 mol of O_2: 1 mol of NH_4^+) [21]. Thus, taking into account this value, OTR could be controlled according to ALR. By adjusting the airflow controller different oxygen transfer rates coefficient (k_La) were obtained, and as consequence, different OTR values were achieved as well. By this strategy, OTR was lower or equal to oxygen consumption rate (OTR ≤ OUR) and DO concentration remained under the detection limit of the DO probe during the aerated phases (minimum detection level of 6 ppb O_2, resulting in a DO concentration of 0.2 µmol $O_2 \cdot L^{-1}$).

2.2. Analytical procedures

Ammonium concentration was analyzed by titrimetric method (APHA 4500); solids were performed by gravimetric method (APHA 2540) [22]. Nitrite and nitrate were measured by colorimetric spectrophotometry (Hach®).

2.3. Microbial activities measured in situ

SAA in the reactor was determined in anoxic conditions. The aeration was turned off and the initial NH_4^+:NO_2^- molar ratio was set to 1 by adding $NaNO_2$. $(NH_4)_2SO_4$ was only added when ammonium concentration in the bulk liquid was lower than 30 mg N·L^{-1}. According to the substrate consumption rate by AnAOB, sampling time took 2 h and 16 h at temperatures of 25 °C and 15 °C, respectively. Nitrogen conversion rate by anammox was obtained by linear regression of the substrates consumptions (ammonium + nitrite). In all assays, SAA was obtained by dividing nitrogen conversion rates per the MLVSS concentrations.

2.4. Fractionated biomass activities

The activities of AOB (rAOB), NOB (rNOB) and anammox (SAA) in different aggregate sizes were specifically determined during Period II. The biomass collected from the reactor was sieved and separated in three mesh size groups: (i) diameter ≤ 500 µm; (ii) 500 µm ≤ diameter ≤ 1000 µm; and (iii) diameter ≥ 1000 µm. The sludge was placed in a 4 L working volume fermenter and washed 3 times with mineral medium. For the anoxic tests (SAA), the fermenter was purged with a mixture of N_2/CO_2 aiming to decrease the initial DO concentration. Biomass was acclimated for 1 h, and a pulse of $(NH_4)_2SO_4$ and $NaNO_2$ was done after acclimation period. The anoxic test lasted 3 h, and samples were collected every 1 h. For the aerobic tests, DO concentration was set at 4 mg·L^{-1} and the biomass was acclimated 1 h before adding a pulse of $(NH_4)_2SO_4$ for the determination of rAOB and $NaNO_2$ for rNOB. Initial concentrations were set at 20 mg NH_4^+-N·L^{-1} and 20 mg NO_2^--N·L^{-1}. The temperature was maintained at 25 °C by a water jacketed in all assays.

2.5. Microbial community analysis by high-throughput sequencing

Biomass samples were collected from the reactor on the operational days 32 (Period I), 172 (Period II) and 291 (Period III) to assess the microbial community composition. Granules were disintegrated and samples were then centrifuged at 8000 rpm. The resulting pellets were stored at −20 °C prior to further analysis. The DNA was extracted and purified by using FastDNA® Spin kit for soil (MP Biomedicals, Solon, Ohio, USA), according to manufacturer's protocol. DNA concentration and purity were determined in a Nanodrop ND-1000 UV–Vis spectrophotometer (Nanodrop, Wilmington, Delaware, USA). DNA samples were analyzed at MSU Genomics Core (Michigan, USA) using a 2 × 250 bp paired-end Illumina MiSeq platform [23]. The V4 region of the 16S rRNA gene of the prokaryotes was amplified using the 515F/806R primer pair [24]. The quality of raw reads was initially checked using the FastQC application (www.bioinformatics.babraham.ac.uk). In Mothur software package [25], raw sequences were demultiplexed, joined paired reads, quality-filtered (below 25% of the Q-value), chimera checked, classified with a threshold of 80% using the Silva database (version 128) and clustered into Operational Taxonomic Units (OTUs) (97% cut-off). Alpha-diversity indicators of richness (Observed richness and Chao1) and diversity (Shannon H and Simpson D) were calculated in Mothur after normalization of the number of sequences in each sample by randomly selecting a subset corresponding to the lowest amount of sequences found in a sample. Raw sequence data from this study was deposited with links to BioProject accession number PRJNA401982 in the National Center for Biotechnology Information (NCBI) BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/).

3. Results

3.1. Reactor operation

3.1.1. System response to temperature decreasing from 25 °C to 15 °C (Periods I and II)

Fig. 1A shows the nitrogen species dynamics during Periods I and II with a mean influent ammonium concentration of 74.5 ± 3.2 mg N·L^{-1}. In Period I, effluent ammonium concentration remained around 19.1 ± 8.0 mg N·L^{-1}, while nitrite was barely detectable (Fig. 1A). The reactor was operated at average NLR of 612.5 ± 25.4 mg N·L^{-1}·d^{-1} and the NRR obtained was

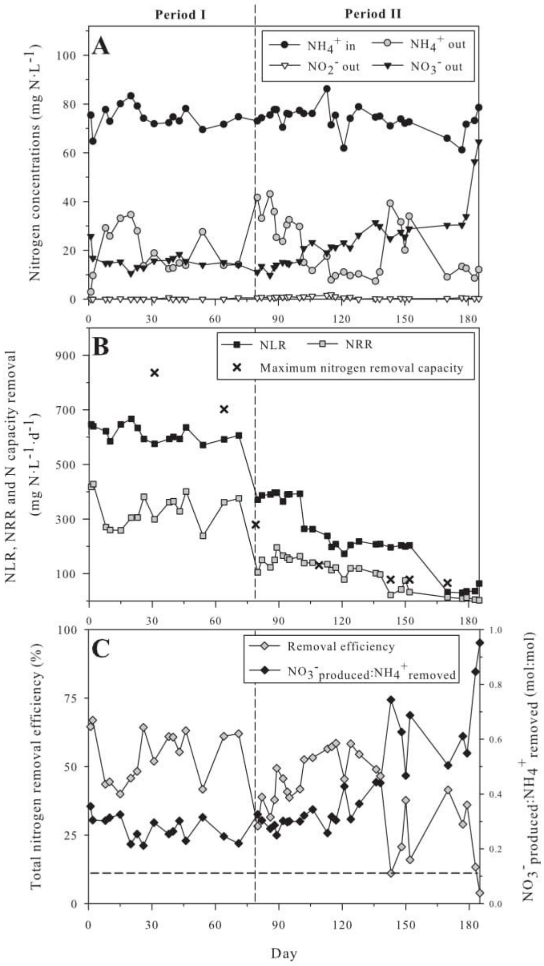

Fig. 1. Reactor performance during operation in continuous aeration at 25 °C and 15 °C (Periods I and II): A) Nitrogen species dynamics; B) Nitrogen loading and removal rates, and maximum nitrogen removal capacity of the reactor; and C) Nitrogen removal efficiency and $NO_{3\,produced}^{-}$:$NH_{4\,removed}^{+}$ molar ratio. Horizontal dashed line represents the stoichiometric $NO_{3\,produced}^{-}$:$NH_{4\,removed}^{+}$ molar ratio by nitritation-anammox processes.

330.2 ± 51.4 mg N·L^{-1}·d^{-1} (Fig. 1B). The maximum nitrogen removal capacity in the reactor was determined by SAA assays and considering the amount of biomass in the reactor. These values, presented in Fig. 1B (crossed symbols), indicate that the maximum nitrogen removal capacity was superior to NLR values. This shows that anammox activity, and consequently nitrogen removal, was restricted by the availability of nitrite. This limitation on nitrite for AnAOB was a consequence of both AOB control by limiting oxygen and NOB competition. The $NO_{3\,produced}^{-}$:$NH_{4\,removed}^{+}$ molar ratio presented an average value of 0.28 ± 0.04, which was above the 0.11 expected for one-stage PNA when nitratation is negligible and nitrate production occurs due to biomass assimilation solely [21] (Fig. 1C). These results indicated that complete NOB activity suppression was not possible to achieve, but NOB rate was successfully limited to less than 20% of the total oxidized ammonium by balancing AOB and AnAOB rates, thanks to the aeration strategy applied in this study.

In Period II (day: 79–184), the operational temperature was set at

15 °C. At the beginning of this period (day 79), SAA assay was performed to determine the maximum nitrogen removal capacity of the biomass by the anammox process at 15 °C. The result obtained at this SAA assay was only related to the short-effects of temperature reduction on anammox activity (without biomass acclimation), and revealed a nitrogen removal capacity drop to 279 mg N·L^{-1}·d^{-1}. Consequently, the NLR was adjusted and an average NLR of 386.65 ± 8.52 mg N·L^{-1}·d^{-1} was applied until day 100. These values were still superior to the initial maximum nitrogen removal capacity obtained by the SAA assay on day 79 (Fig. 1B). During this transition, airflow rate was also reduced according to NLR to achieve partial nitritation and consequently ammonium effluent concentration was in average of 32.8 ± 5.00 mg N·L^{-1} (Fig. 1A). Both oxygen supply limitation and NOB activity also affected the nitrogen removal, and an average of 151.68 ± 17.42 N·L^{-1}·d^{-1} was obtained (Fig. 1B). Furthermore, reducing temperature in the reactor did not increase nitrite concentration, which remained lower than 1 mg N·L^{-1} (Fig. 1A). The $NO_{3produced}^{-}$:$NH_{4removed}^{+}$ molar ratio remained similar to the previous period, with a mean value of 0.29 ± 0.02, showing good PNA performance (Fig. 1C).

After long-term reactor operation at 15 °C in Period II, the low temperature unfavorably affected anammox activity with a progressive decline. After day 143 (about two months at 15 °C), measured SAA decreased by an order of four times compared to the assay carried out on day 79. NRR consequently dropped (Fig. 1B). Moreover, the $NO_{3produced}^{-}$:$NH_{4removed}^{+}$ molar ratio increased up to 0.85, even though a reduction in the NLR and the airflow rate had been imposed aiming to avoid an unbalance between AOB and AnAOB activities. In order to assess the maximum SAA, obtained without substrate limitation in the PNA-SBR, and the specific nitrogen loading (sNLR) and removal rates (sNRR), being this last one the observed removal rate by the normal reactor operation, three distinct phases (one in Period I and two in Period II) were considered (Table 1). It can be verified that nitrogen removal was not the maximal during Period I and part of period II, despite anammox was the main nitrogen removal pathway during most of operational time. Although, sNRR values higher than SAA obtained in the assay done in the period (from day 102 to 114) indicate that heterotrophic denitrification due to hydrolysis of biomass might have assisted autotrophic nitrogen removal in this period, because the influent medium did not contain any organic carbon source.

3.1.2. Operation under transient anoxia (Period III)

Reactor operation under transient anoxia started on day 184. In order to adjust AOB activity according to AnAOB one, which decreased after a long-term reactor operation at 15 °C, nitritation was again controlled by shortening aerobic phase (therefore, decreasing OTR). In this way, aeration was provided to the reactor intermittently. By applying this strategy, NOB activity reduced, and the $NO_{3produced}^{-}$:$NH_{4removed}^{+}$ molar ratio dropped from 0.95 to 0.5 in the initial 20 days of Period III (Fig. 2C). The applied NLR was further adjusted because the maximum nitrogen removal capacity by anammox dropped from 57 mg N·L^{-1}·d^{-1} to 16 mg N·L^{-1}·d^{-1} (Fig. 2B). Consequently, ammonium concentration in the effluent was lowered to 20 mg N·L^{-1}. Stable nitrogen removal was maintained for about 40 days. An unexpected decrease of NRR occurred on day 242 and it resulted in ammonium and nitrite accumulation (Fig. 2A).

Table 1
Specific activities during Periods I and II.

Days	T (°C)	sNLR (mg N·g VSS^{-1}·d^{-1})	sNRR (mg N·g VSS^{-1}·d^{-1})	SAA (mg (NH_4^+ + NO_2^-)-N·g VSS^{-1}·d^{-1})
26–71	25	189.6 ± 12.3	114.4 ± 7.9	238.4 ± 37.4
102–114	15	80.9 ± 9.6	44.9 ± 6.5	30.9
170–179	15	18.7 ± 3.7	6.6 ± 1.4	22.32

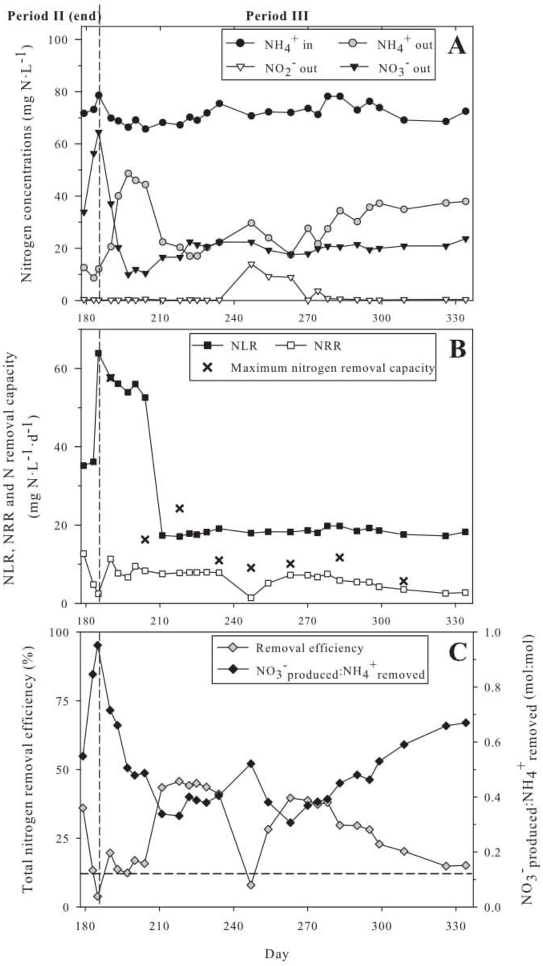

Fig. 2. Reactor performance at 15 °C during the intermittent aeration period: A) Nitrogen species dynamics; B) Nitrogen loading and removal rates, and maximum nitrogen removal capacity of the reactor; and C) Nitrogen removal efficiency and $NO_{3produced}^{-}$:$NH_{4removed}^{+}$ molar ratio. Horizontal dashed line represents the stoichiometric $NO_{3produced}^{-}$:$NH_{4removed}^{+}$ molar ratio by nitritation-anammox processes.

Unpredictably, anammox activity by SAA assay was not lost in this period. Afterwards, NRR was recovered again and the $NO_{3produced}^{-}$:$NH_{4removed}^{+}$ molar ratio remained stable at 0.4 (Fig. 2B). From day 280 onwards, a downward trend in the NRR values started to be observed. In addition, the $NO_{3produced}^{-}$:$NH_{4removed}^{+}$ molar ratio also rose. This shows that NOB community was initially affected by intermittent aeration, but might have adapted at long term.

3.2. Nitrogen conversion rates in different aggregates fractions

In Period II, the nitrogen conversion rates segregation between different aggregates fractions (diameter ≤ 500 µm, 500 µm ≤ diameter ≤ 1000 µm, and diameter ≥ 1000 µm) were characterized by means of biomass activity assays. These assays revealed different activity rates for AOB, NOB and AnAOB for the three aggregate size groups analyzed (Fig. 3). SAA results were lower than aerobic rates for all analyzed size groups, as consequence of anammox activity reduction

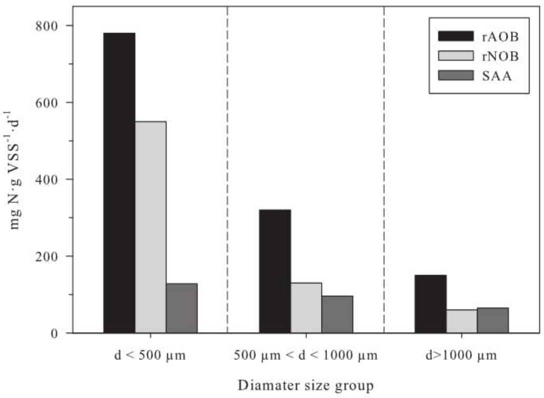

Fig. 3. Specific rates obtained from the three different aggregate size classes from activity tests performed in period II.

in the operation at 15 °C. Additionally, the aggregate biomass class with diameter lower than 500 µm presented higher aerobic (rAOB and rNOB) and anoxic (SAA) activities than the other groups. Compared to the results obtained in the aggregate class with diameter > 1000 µm, rAOB and rNOB were around 5 times and 9 times higher, respectively, in the smallest diameter size group. For SAA, the difference between the smallest and biggest size groups was only about twice higher in small aggregates. By comparing rAOB and SAA, from the small to larger aggregates, the relation rAOB/SAA decreased from 6.1 to 2.3.

3.3. Solids dynamics during long-term PNA-SBR operation

Fig. 4 shows the solids evolution throughout reactor operation. During operation at 25 °C, the mixed liquor volatile sludge (MLVSS) ranged from 2.8 to 3.7 $g{\cdot}L^{-1}$. Strong variations were due to manual purge events that were done to keep MLVSS concentrations around 3 $g{\cdot}L^{-1}$. The relation between the volatile suspended solids (VSS) and the total (TSS) was close to 0.8 and the effluent VSS concentration was lower than 10 $mg{\cdot}L^{-1}$. After temperature transition from 25° to 15 °C, MLVSS concentrations in the reactor changed with a stepwise dropping in the solids concentration in the reactor down to 1.25 $g{\cdot}L^{-1}$. Concomitantly, effluent VSS concentration increased from around 10 $mg{\cdot}L^{-1}$ to values up to 62 $mg{\cdot}L^{-1}$. Therefore, the ratio MLVSS/MLSS slowly decreased to 0.7 on day 260 and was forward recovered.

Deliberately solids washout was carried out from day 185 to 206, aiming at retaining the particles with diameter higher than 500 µm (which presented highest NOB rates). In this period, the stirrer speed

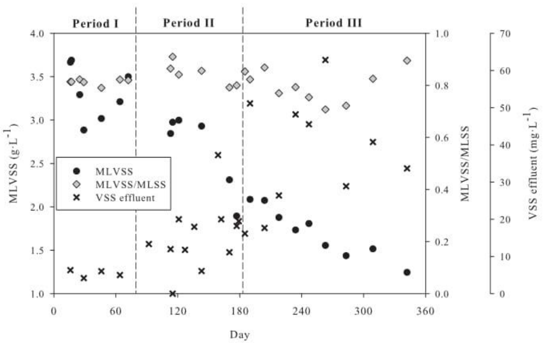

Fig. 4. Suspended solids in the reactor mixed liquor and effluent; and evolution of the MLVSS/MLSS ratio throughout operation at 25 °C and 15 °C.

rotation was set at 50 rpm just in the settling time and effluent discharge phases. This action made possible to maintain in re-suspension mainly the small diameter size aggregates, because larger granules settled normally. Effluent reactor was stored and passed daily through a sieve with a mesh size of 500 µm. The retained sludge was returned into the reactor. Average diameter size frequency distribution of the sludge in Period I and III is presented in Fig. S3.

3.4. Microbial community composition in the PNA-SBR under temperature and aeration pattern transition

After filtering for quality, the final sequence dataset consisted of 114,111 sequences that, on average, were distributed in 38,037 reads per sample (min = 32,627, max = 43,484, SD = 5429). This sampling effort was sufficient to recover most of the prokaryotic diversity as indicated by rarefaction plots (Fig. S4).

3.4.1. Diversity and richness

Table 2 shows the alpha-diversity parameters of the microbial community throughout reactor operation. The parameters were calculated after being subsampled in a total of 32,627 sequences and at a distance cutoff level of 0.03. The estimated richness given by Chao1 index varied between 320.00 and 339.67 and it showed that the microbial community richness was higher when the reactor was operated at 15 °C (Period II and III). The observed OTUs (Sobs) varied between 314 and 339 and were very similar to Chao1 index at periods II and III, confirming the low importance of rare OTUs, since Chao1 estimator relies upon the number of singletons and doubletons and also considers the number of OTUs. The Shannon H and Simpson indexes provided community diversity in samples. The results obtained showed that community diversity slightly increased in Period II by lowering the temperature from 25 °C to 15 °C, and it was followed by a slight index reduction in Period III, probably as a consequence of low nitrogen loading, and consequently low nitrogen conversions in Period III. More OTUs were found at Periods II and III, as revealed by the number of observed OTUs. The microbial analysis confirmed that the main microbiome was maintained throughout the study, because the largest fraction of OTUs (294 out of 345, 85.2%) were shared in the three periods, as indicated by the Venn diagram (Fig. 5A). Exclusively OTUs, a total of 3, was harbored only in Period III. Interestingly, the number of shared OTUs between Period I and II (15) was almost half of those shared between Period II and III (28), and in both cases higher than the shared OTUs between the distant Periods I and III (5).

3.4.2. Microbial community composition and relative abundances

The classification of the obtained OTUs into phylum level allowed revealing a total of 21 groups, but only the groups with relative abundance of at least 1% in one sample are presented in Fig. 5B (for all the groups obtained see Table S5). The most abundant phyla across the samples analyzed were *Proteobacteria* (36.29 ± 3.3%), *Planctomycetes* (26.75 ± 4.7%), *Chloroflexi* (11.51 ± 1.1%) and *Bacteroidetes* (7.60 ± 1.4%). The relative abundance of bacterial groups varied because different conditions were applied in the reactor operation. After the decrease of temperature to 15 °C in Period II, the relative abundance of *Proteobacteria* rose from 35.38 to 41.31%, whereas *Planctomycetes*, *Chloroflexi* and *Bacteroidetes* abundance dropped from 24.77%, 12.98% and 9.67%, to 21.65%, 9.87% and 7.27%, respectively. Controversially, *Nitrospirae* relative abundance increased from 4.63 to 7.23% during the same period. In Period III, when intermittent aeration and lower NLR were applied, the relative abundance of *Chloroflexi* and *Planctomycetes* rose to 11.68% and 33.81% respectively, while the abundance of other groups was reduced. The most noticeable changes were observed for *Proteobacteria* and *Nitrospirae*, with a reduction to 32.18% and 2.96%, respectively. Moreover, *Bacteroidetes* abundance was further reduced to 5.88% in Period III.

At genus level, from a total of 154 genera recovered, only 30

Table 2
Alpha-diversity values for the samples analyzed from the different periods studied. Values in the round brackets indicate the lower and upper bounds of the confidence interval.

Sample	Reactor temperature (°C)	Coverage (%)	Sobs	Chao1	Shannon H	Simpson index of diversity (1-D)
Period I	25	99.96	314	320.00 (315.55, 337.28)	4.12 (4.10, 4.14)	0.953 (0.951, 0.954)
Period II	15	99.99	339	339.67 (339.07, 345.64)	4.29 (4.27, 4.31)	0.964 (0.963, 0.965)
Period III	15	99.99	331	331.20 (331.01, 335.08)	4.06 (4.04, 4.08)	0.935 (0.933, 0.936)

presented relative abundances higher than 1% in at least one sample. These genera abundance is displayed in the heatmap (Fig. 5C). The anammox genus *Candidatus* Kuenenia was the most abundant bacteria in all the periods. It was followed by uncultured *Anaerolineaceae* (phylum *Chloroflexi), Nitrospira, Nitrosomonas* and *Woodsholea*. Interestingly, the abundance of uncultured *Anaerolineaceae* and *Candidatus* Kuenenia presented similar trend in all the samples. Within *Planctomycetes*, *SM1A02* was the second most abundant genera after *Candidatus* Kuenenia. Furthermore, *SM1A02* abundance increased about two times in Period III. The most remarkable variation in the abundance of nitrifying bacteria – *Nitrosomonas* and *Nitrospira* – during the course of this study occurred when temperature was changed to 15 °C. The increase of abundance of *Nitrospira* can be related to the higher nitrate production obtained in the reactor (Period II). Both genera abundances dropped and reached similar values in Period III (Fig. 5C), by the imposition of lower nitrogen loading rates and intermittent aeration. The phylum *Proteobacteria* was also represented consistently by the following genera: *Blfdi19*_ge, uncultured *Xanthomonadaceae*, *Denitratisoma* and *Woodsholea*. The abundance of these groups was higher in Period II, but only *Woodsholea* and *Denitratisoma* showed a higher abundance in Period III, whereas the others dropped. Several other genera abundance belonging to phylum *Bacteroidetes* had their relative abundance reduced throughout the study, such as *Ferruginibacter*, *Terrimonas* and uncultured *Saprospiraceae*. A considerable drop in their abundance was obtained in Period III, possibly as a consequence of the reduction in oxygen supply, since all are described as aerobic bacteria. In this phylum, only uncultured *Cytophagaceae* abundance remained rather constant, but with a slightly increase in Period II.

4. Discussion

4.1. Mainstream PNA-SBR performance by balancing AOB and AnAOB activities

Temperature transition from 25 °C to 15 °C without biomass acclimation was assessed in a one-stage mainstream PNA-SBR. The applied aeration strategy allowed balancing AOB and AnAOB rates and operating the PNA-SBR with bulk DO at μM concentrations. The applied oxygen conditions did not disfavor AOB activity, and thereby, nitritation-anammox processes led to average specific NRR of

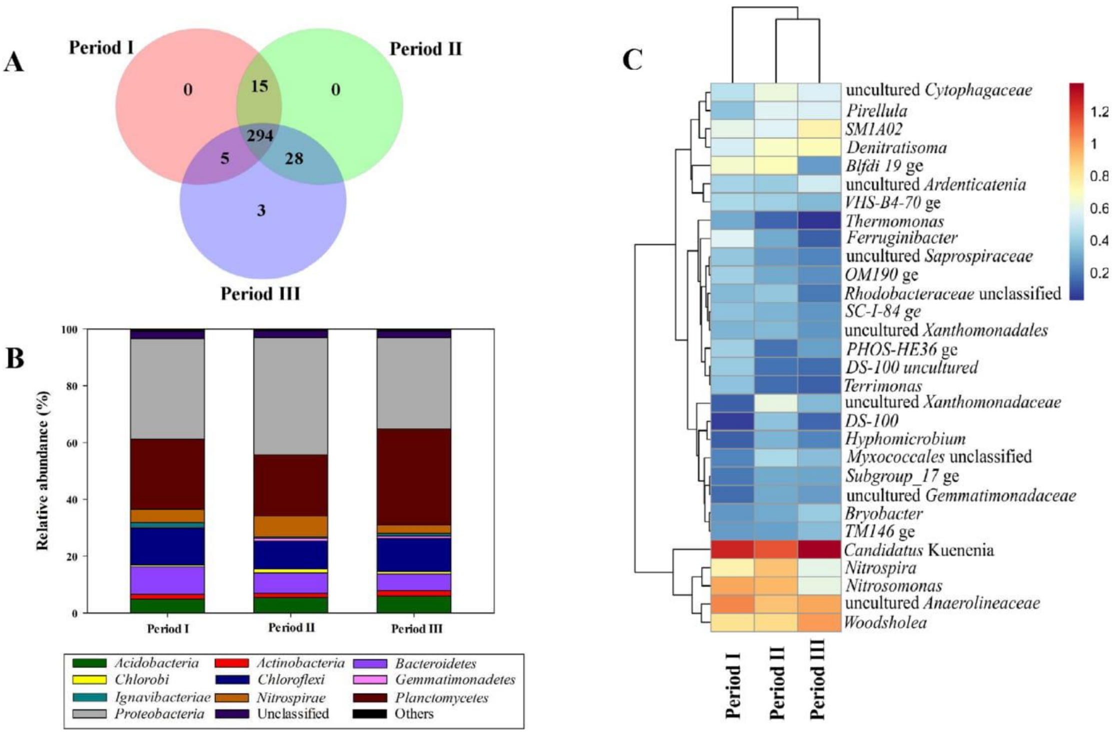

Fig. 5. Microbial community composition revealed by Miseq Illumina paired end sequencing: A) Venn diagram of the OTU distributed in the three samples analyzed; B) Relative abundance at phylum level. Groups which relative abundance were not higher than 1% in at least one sample are grouped in the label "others"; and C) Heatmap of major genera in each operational phase (relative abundance contribution > 1% at least in one of the samples). The values are calculated and plotted as log10 (sequence numbers + 1).

114.47 ± 7.95 mg N·g VSS^{-1}·d^{-1} at 25 °C (Table 1). This mean value is rather superior to other reported one-stage PNA studies at mainstream conditions (20–100 mg N·g VSS^{-1}·d^{-1}), operated at similar operational temperatures but with minimum bulk liquid DO concentration of around 0.6 mg O_2·L^{-1} [6,14,26].

The sudden temperature reduction from 25 °C to 15 °C did not cause any initial reactor instability. Nitrite did not accumulate, because AOB and AnAOB rates were well-adjusted (Fig. 1A). Controversially, nitrite accumulation due to temperature reduction has been experienced by Lotti et al. [5], with the reduction from 20 °C to 15 °C, and by Ma et al. [12], with the temperatures ranging from 11 to 17 °C. Moreover, this accumulation on both studies has been reported as consequence of unbalanced rates between nitritation and anammox. Thus, appropriate control of nitritation achieved by the control of the OTR and the adjustment of the ALR avoided nitrite accumulation during the temperature dropping with no biomass acclimation. At long-term operation at 15 °C, anammox activity experienced a deterioration while NOB activity increased. This also led to a reduction in the potential load removal at the end of Period II. The average SAA of 24.96 ± 3.53 mg N·g VSS^{-1}·d^{-1} obtained at long-operation at 15 °C (Table 1) was in the same order of the values reported by Hendrickx et al. [27] (30–44 mg N·g VSS^{-1}·d^{-1}, at 10 °C and anoxic anammox reactor) and Morales et al. [4] (30 mg N·g VSS^{-1}·d^{-1} in one-stage PNA at 15 °C). Lower SAA values also affected overall nitrogen removal in Period III. Anammox activity decreased by three times, and NRR lower than 10 mg N·L^{-1}·d^{-1} was obtained (Fig. 2B). Anammox activity deterioration under long-term operation at low temperature was also reported by Hoekstra et al. (2017) [28]. In the referred study, the diminution in the specific anammox activity was related to the long sludge retention time (SRT) required in the system, which increases the non-active and no anammox cells.

The results obtained in our study, which was carried out under μM bulk liquid DO concentration, also contribute to strengthen that energy savings can be improved, due to lower aeration cost [29]. Oxygen was provided according to the stoichiometric requirements of the process and there was no need for DO accumulation at moderately levels to ensure nitritation. Additionally, nitrogen removal rates achieved by autotrophic processes reinforce the proof of concept of energy self-sufficient WWTP, because the layout in mainstream line could be a reactor aiming at removing organic matter, such as an anaerobic process to produce methane, followed by PNA reactor to remove nitrogen and accomplish effluent guidelines [1].

4.2. NOB suppression in one-stage mainstream PNA relies on anammox activity

Bulk liquid DO is a key parameter conditioning the aerobic and anoxic layers thickness in biofilm systems. By keeping bulk liquid DO at low levels, low oxygen penetration through the biofilm is expected, and consequently a larger anoxic layer is reached, favoring AnAOB activity. Moreover, modelling studies have also indicated that low bulk liquid DO concentrations should be kept for small biomass aggregates to balance the aerobic and anaerobic activities [30]. However, the findings of this study reveal that bulk liquid μM DO alone did not allow the proper limitation of NOB activity at 15 °C, when AnAOB activity deteriorated during the course of Period II leading to unbalanced rates between AOB and AnAOB (Fig. 1B and C). The instability occurred at 15 °C resulting in higher values for the $NO^-_{3produced}$:$NH^+_{4removed}$ molar ratio in Period II (up to 0.95) compared to Period I, at 25 °C (0.28 ± 0.04; Fig. 1C). It is believed that NOB took advantage of being closer to AOB in the aerobic layer and can use the excess of nitrite produced, once AnAOB activity is low, leading to higher nitrate production at the end of Period II. The out selection of floccular biomass in order to decrease nitrate production by NOB and retain granular biomass with higher AnAOB activity as proposed in other studies [4,15], was not applicable in the current study during period II and III. Maximum SAA measured in activity tests was lower than the values obtained for NOB in all granular biomass diameter size analyzed working at 15 °C (Fig. 3).

Several studies have shown that NOB activity suppression at mainstream conditions can be accomplished by imposing an intermittent aeration strategy [6,12]. Nitratation activity suppression occurs due to different activation times presented by AOB and NOB after transient anoxic time [31]. It was also experimentally proved that after anoxic periods ranging from 15 to 60 min, the delay time for nitrite production was higher than the delay time for ammonium conversion [31]. Intermittent aeration was applied during Period III, making the adjustment of the AOB-AnAOB rates possible, which resulted in a short-term decline of the NOB activity (from day 185 to 225, Fig. 2C). However, it was experienced a gradual increase in the $NO^-_{3produced}$:$NH^+_{4removed}$ molar ratio in the course of the respective operational period, indicating a reduction of the effectiveness of intermittent aeration on NOB activity repression. Long-term NOB suppression by intermittent aeration and low DO concentration (~ 0.2 mg O_2·L^{-1}) has been reported [32], but controversially, it was shown not to be effective in others studies, where the minimum bulk DO concentration was 0.5 mg O_2·L^{-1} [6,33,34].

4.3. Key nitrifying and anammox bacterial populations unaltered after temperature transition

The overall bacterial core obtained in this study was similar to previous studies performed at mainstream conditions and was not modified despite the conditions applied in the operation [7,18,20]. Although differences in the relative abundance of the main bacterial groups were obtained. Regarding the key nitrifying bacteria responsible for nitrogen turnover, the genera identified did not change because of different conditions applied during the experimental course. *Candidatus* Kuenenia was the most abundant anammox genus present in the reactor (Fig. 5). Contrarily, *Candidatus* Brocadia was the dominant anammox genus in most of the reported studies of one-stage mainstream PNA at cold temperature [5,18,19]. It can be inferred that low temperature do not influence AnAOB niche differentiation in engineered systems.

Nitrosomonas and *Nitrospira* were the respective AOB and NOB genera which better adapted to the operational conditions applied. Gonzalez-Martinez et al. [20] obtained few more bacteria genera responsible for nitrification in a one-stage PNA operated at cold temperature. However, *Nitrosomonas* and *Nitrospira* were also the predominant genera in several studies at low temperature [7,18]. The growth and presence of *Nitrospira* at mainstream conditions agree with the K/r hypothesis [29], because DO in bulk was at μM concentrations. *Nitrobacter* abundance at low temperature mainstream PNA was only obtained by qPCR in a MBBR operated with an average DO of 0.48 ± 0.10 mg O_2·L^{-1} [19].

4.4. Nitrogen removal assisted by endogenous heterotrophic denitrification

The results obtained in this study suggest that anammox process was not the exclusively pathway responsible for nitrogen removal, but endogenous denitrification might also have contributed during the initial days in Period II (Table 1). *Denitratisomona* and *Hyphomicrobium* were among the bacterial genera for which relative abundance rose in periods II and III (Fig. 5C). Both are known for their role on heterotrophic denitrification [35,36]. It contrasts to the higher prevalence of *Thauera* obtained in a partial denitrification SBR [37]. Even though organic matter was not provided in the mineral medium, it has been shown that heterotrophic denitrification in a nitrifying and anammox reactor is supported by the production and lysis of soluble microbial products (SMP) [38], which serve as electron donor and carbon sources for heterotrophs. In addition, slowly biodegradable organic matter is also produced by biomass decay and lysis, which explains the gradual reduction of VSS in periods II and III (Fig. 4). Partial denitrification can also occur and provide nitrite to anammox bacteria [6,37]. The occurrence of endogenous denitrification might explain the controversial results regarding nitrate build-up presented by several mainstream PNA reactors operated at low DO concentration. The amount of sludge used in the start-up reactors at mainstream conditions resulted in MLVSS concentration higher than 8 mg VSS·L^{-1} [4,14]. Thus, this significant concentration could favor endogenous denitrification because of biomass lysis.

Amplicon sequencing revealed a wide diversity of heterotrophic microorganisms among *Chloroflexi*, *Ignavibacteriae*, *Bacteroidetes* and *Proteobacteria* phylum. The presence of the phylum *Chloroflexi, Ignavibacteriae* and *Bacteroidetes* have been found in other anammox reactors, and a number of studies have attempted to find their niche [39,40]. They have been frequently associated to the consumption of complex organic compounds described as SMP [41,42]. Regarding the phylum *Bacteroidetes*, a recent metagenomics study found that their genomes harbor genes capable of reducing nitrous oxide to nitrogen gas via nitrous oxide reductase [43]. This result highlights the importance of *Bacteroidetes,* not only because this group scavenges organic compounds derived from anammox bacterial cells, but to facilitate a nitrite loop with anammox bacteria or support complete denitrification. Degradation of biomass by members of *Ignavibacteriae* and *Chloroflexi*, with higher abundance obtained in our study, could support interactions in the community, by providing short-chain volatile fatty acids (VFAs) and alcohols to members of *Proteobacteria* phylum [40].

5. Conclusions

A stable one-stage mainstream PNA-SBR process was achieved in this study at temperatures of 25 °C and 15 °C and without biomass acclimation during temperature transition. The control strategy based on OTR according to ALR resulted in µM bulk DO. Average NRR of 330.24 ± 51.42 mg N·L^{-1}·d^{-1} was achieved at 25 °C. Nitratation consumed only about 20% of nitrite produced by AOB. After temperature change to 15 °C, $NO_{3\,production}^{-}$:$NH_{4\,removed}^{+}$ molar ratio was kept controlled at 0.33 ± 0.05. A high nitrate production resulted from the increase of NOB activity was obtained when AOB and AnAOB rates were unbalanced, due to anammox activity deterioration after long-term operation at 15 °C. Intermittent aeration was used to decrease the OTR and recover the balance between AOB-AnAOB rates, but results show that intermittent aeration is not effective for NOB suppression when AnAOB activity deteriorates. Microbial community analysis demonstrated that heterotrophs belonging to *Chloroflexi* and *Bacteroidetes* may be specialized in using soluble microbial products and play an important role decreasing the total effluent nitrate in mainstream PNA reactors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cej.2018.05.115.

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Chapter 6.

Mainstream one-stage partial nitritation-anammox in a plug-flow reactor at room temperature

This chapter has been prepared for submission:

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Abstract

This study shows the start-up and operation of a one-stage partial nitritation – anammox (PNA) in a plug-flow reactor with both aerobic and anaerobic chambers. The reactor was operated at room temperature (between 16 and 27 ^{o}C) and with a continuous regime of feed and aeration. The reactor was inoculated with few amount of sludge from a mainstream one-stage PNA sequencing batch reactor (SBR). It was found that anammox activity of the sludge increased about 7 times, reaching values of 200 mg N·g VSS^{-1}·d^{-1} as soon the seed sludge was added in the reactor. Despite higher specific activity, nitrogen turnover was limited at low values (close to 20 mg N·L^{-1}·d^{-1}). Such limitation occurred because (1) the reactor had low sludge concentration and (2) achieving nitration suppression, which hampered anammox growth, was difficult. Activity tests were carried out with size fractionated sludge (diameter lower and higher than 500 µm). Aerobic ammonium and nitrite oxidation rates (respective AOR and NOR) were in overall higher than specific anammox activity (SAA) for all the tests. However, NOR reduced from 440 (<500 µm) to 149 mg N·g VSS^{-1}·d^{-1} (>500 µm), indicating higher nitrite-oxidizing bacteria (NOB) activity in the small diameter size sludge.

Keywords: nitritation-anammox, NOB suppression, mainstream, diameter size

6.1. Introduction

Anammox process is an emergent and cost-effective approach to remove nitrogen from sewage at mainstream wastewater treatment plants (WWTP). This advantage is achieved by lower aeration requirements and exempted organic matter (Wett et al., 2013). Although partial nitritation-anammox (PNA) has been already stablished for sidestream wastewater (i.e. sludge reject water), the characteristics of mainstream wastewater make PNA application to these conditions considerably more complex (Lotti et al., 2015b). The implementation of anammox-based processes in the mainstream line of WWTP has been mainly limited in strategies to achieve long-term nitrite-oxidizing bacteria (NOB) activity suppression (Cao et al., 2017; Wett et al., 2013).

Most one-stage sidestream PNA reactors have been performed in sequencing batch reactors (SBR) (Lackner et al., 2014). In this kind of reactor configuration, anammox retention is frequently achieved in self-aggregate granular biomass, which also allows greater biomass retention, being suitable for anammox bacteria (AnAOB) growth (López et al., 2008). Moreover, SBR allows different operation regimes such as step-feed and intermittent aeration, aiming at controlling nitritation and anammox rates to avoid AnAOB inhibition by oxygen and nitrite. However, mainstream wastewater treatment in large-scale application is preferred to be performed in a continuous mode, because operation can be simpler and more economic (Pérez et al., 2014).

Several one-stage PNA studies, at mainstream conditions, have been carried out in systems with constant feed and aeration, by using different reactors types such as integrated fixed film activated sludge (IFAS) (Malovanyy et al., 2015a), moving-bed biofilm reactor (MBBR) (Gilbert et al., 2014b; Kouba et al., 2016), plug-flow reactors (Han et al., 2016b; Lotti et al., 2015b), and more recently, a step-feed reactor (Gu et al., 2019) In MBBR, biomass grow attached in plastic carriers, while in plug-flow reactors the biomass can grow as flocs or self-aggregate granules. Furthermore, how these different biomasses and their properties affect reactor performance, mainly due to temperature reduction, have been also investigated in previous works (Gilbert et al., 2015; Laureni et al., 2016; Morales et al., 2016). In summary, it was demonstrated that reactors with thicker biofilm in plastic carriers and larger granular biomass are more robust to deal with negative effects of temperature on microbial activity, preventing insufficient nitrogen removal. Concerning the microbial community, it was also shown that AnAOB abundance was more stable in a MBBR, whereas the analyzed biomass from SBR was less stable and decreased over the operational time (Agrawal et al., 2017; Gilbert et al., 2015).

Nitrogen removal stability in one-stage suspended biomass PNA under continuous feed and aeration has to be explored in order to potentialize its application at mainstream conditions in urban WWTP. One of the main limitations for the application of plug-flow reactors can be the NOB activity suppression. In this sense, nitritation control has been already achieved in plug-flow reactors for the conventional biologic nitrogen removal (BNR) processes (Ge et al., 2014; Ma et al., 2009). However, while dissolved oxygen (DO) concentrations at 0.4 – 0.7 $mg{\cdot}L^{-1}$ was enough to stop nitratation (Ma et al., 2009), much higher DO concentration (1.5 – 2 $mg{\cdot}L^{-1}$) was needed by Ge et al. (2014). Both studies converged to the fact that alternating anoxic/aerobic conditions influenced NOB suppression. Further studies have to be conducted to the application of plug-flow for mainstream anammox-based processes.

This study attempts to assess the feasibility of a plug-flow reactor, with continuous feed and alternating aerobic and anoxic conditions, to carry out one-stage PNA at mainstream conditions. Specifically, the start-up of the reactor with low amount of sludge, and NOB suppression by different anoxic/aerobic configurations were investigated.

6.2. Material and methods

6.2.1. Experimental setup

The study was carried out in a 37.35 L working volume reactor, which had three chambers, separated by fixed baffles, and a settling tank (Figure 6.1). Each chamber had a stirrer (200 RPM) to sustain the biomass in suspension. An external recirculation allowed reintroducing the sludge from the settling tank in the chamber A. pH (Endress

Hauser, model CPF81, Switzerland) and DO (Endress Hauser, model COS41, Switzerland) probes were placed in the aerobic chambers only. The airflow rate was controlled by mass flow (Alicat Scientific, model MC-500SCCM-D/5M, USA).

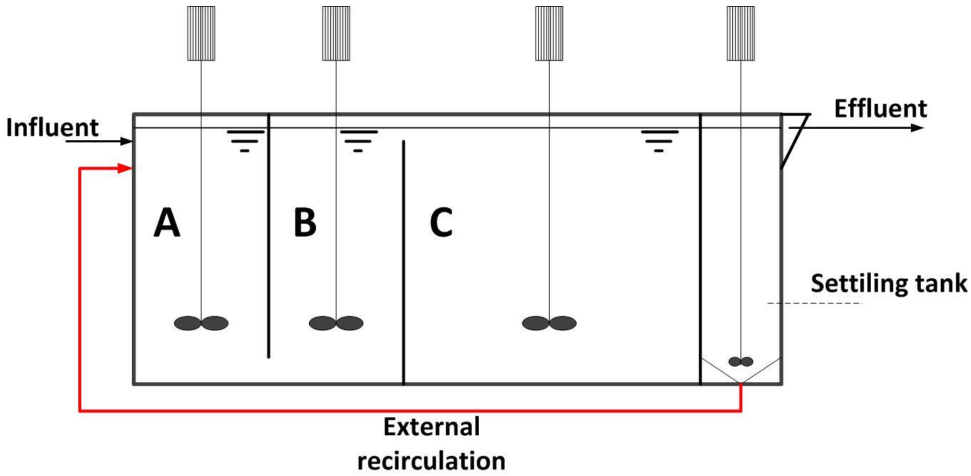

Figure 6.1. Schematic diagram of the reactor setup. The working volume of each chamber was 7.92 L (A), 10.57 L (B), and 18.86 L (C), while the volume of the settling tank was 6.7 L.

6.2.2. Experimental procedures

Reactor operation took 328 days and the study comprised four operational periods (Table 6.1). The temperature was not controlled during the study, and it followed the room temperature. The synthetic wastewater entered the reactor by the chamber A throughout all the study. Based on the air supply into the chambers, different aerobic and anoxic configurations were possible. From periods I to III, the aeration was placed only in the chambers A and B, while at period IV, aeration was provided directly to chamber C. The recirculation flow was defined to maintain biomass concentration in the reactor and avoiding its washout. Thus, different ratios between the external recirculation and influent flow rates were evaluated.

Table 6.1. Setup of aerobic and anoxic conditions in each chamber (A, B and C), inflow and recirculation flow conditions applied in each period studied

Period	Days	Configuration	R/Qin	HRT (d)
I	1-22	Aer (A), Aer (B), Ano (C)	5.13	2.69
II	23-170	Aer (A), Aer (B), Ano (C)	2.53	1.32
III	171-203	Aer (A), Aer (B), Ano (C)	10	1
IV	204-328	Ano (A), Ano (B), Aer (C)	10	1

Aer – Aerobic, Ano – Anoxic

The reactor was inoculated with 2 L of sludge from a lab-scale PNA-SBR operated at mainstream conditions and temperature of 15 ^oC. The inoculum had a concentration of 2.1 g $VSS \cdot L^{-1}$ and a specific anammox activity at 15 ^oC of 27 mg $N \cdot g\ VSS^{-1} \cdot d^{-1}$. Mineral medium was used during all the study to feed the reactor. The composition of the mineral medium per liter of tap water was: 0.0125 g $FeSO_4 \cdot 7H_2O$, 0.0125 g $EDTA \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.008 g KH_2PO_4, 0.3 g $CaCl_2 \cdot 2H_2O$, 0.354 g $(NH_4^+)_2SO_4$, 1.04 g $NaHCO_3$ and 1.25 mL of trace element solution. The trace element solution was prepared based on Graaf et al. (1996).

6.2.3. Ex situ batch aerobic assays

Ex situ aerobic activity tests were carried out in periods II and III (days 41 and 201), aiming at evaluating the specific maximum ammonium oxidation rate (AOR) and nitrite oxidation rate (NOR). Prior to the assays, the sludge was collected, washed 3 times with mineral medium and placed in a 5 L fermenter (Biostat B Plus, Sartorius). DO concentration was sustained higher than 4 mg $O_2 \cdot L^{-1}$. Thus, the biomass remained in suspension under these conditions for one hour. A pulse of $(NH_4)_2SO_4$ to obtain the equivalent of 40 mg $NH_4^+\text{-}N \cdot L^{-1}$ was then added to the reactor. Samples were collected for 3 hours and passed through a 20 µm filter paper prior to analyzing nitrogen species. These ex situ tests were performed at ambient temperature, similar to the temperature in the reactor.

6.2.4. Size fractionated biomass activities

In order to determine the AOR, NOR and specific anammox activity (SAA) in different fractionated aggregated sizes, the biomass was sieved and separated in two mesh size groups: (i) diameter size ≤ 500 µm and (ii) diameter size ≥ 500 µm. The procedures for the aerobic tests were rather similar to the exposed in item 6.2.3, excepting in the determination of NOR, since a pulse of $NaNO_2$ in a concentration of 40 mg NO_2^--N·L^{-1} was added in the reactor. For the anoxic tests, the reactor was purged with a mixture of N_2/CO_2 aiming to decrease the DO concentration. Biomass was also acclimated for 1 hour. Pulses of $(NH_4)_2SO_4$ and $NaNO_2$ were added, to obtain concentrations of 40 mg NH_4^+-N·L^{-1} and 40 mg NO_2^--N·L^{-1}. The anoxic test lasted 7 hours and samples were collected regularly. The temperature was maintained at 30 °C by a water-jacketed for the aerobic and anoxic tests.

6.2.5. Analytical procedures

Ammonium concentration was analyzed by titrimetric method (APHA 4500); solids were performed by gravimetric method (APHA 2540) (APHA, 1995). Nitrite and nitrate were measured by colorimetric spectrophotometry (Hach, USA). Calculations to obtain AOB, NOB, and AnAOB nitrogen turnover along reactor operation are presented in S1.

6.3. Results and discussion

6.3.1. Nitrogen removal performance

The feasibility of a plug-flow reactor to remove nitrogen by PNA processes at mainstream conditions, with continuous feed, as well different aerobic and anoxic configurations, was investigated (Table 6.1). Figure 6.2 shows nitrogen loading and removal rates, DO concentration, temperature, and nitrogen species concentration during the study. Ammonium influent concentrations were in average 71.1 ± 3.4 mg N·L^{-1} during the whole period.

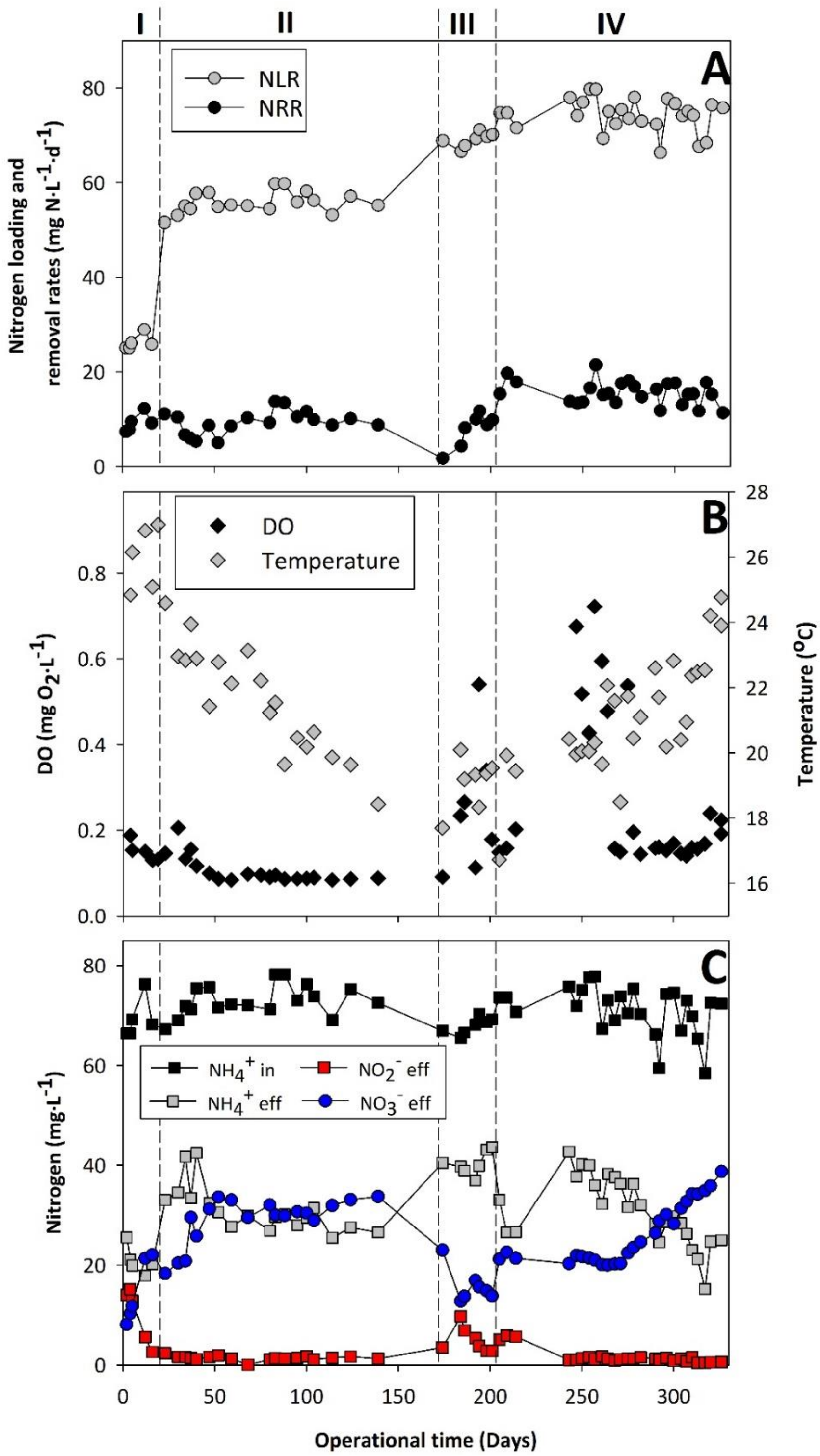

Figure 6.2. Reactor performance along the study: (A) nitrogen loading and removal rates; (B) time course DO concentration and temperature; and (C) nitrogen species concentration.

- **Period I: reactor start-up**

Reactor start-up occurred during the summer season and temperature in the reactor oscillated between 24 and 27 °C. Moreover, the sludge used to start-up the reactor presented a low anammox activity, and as consequence of the low inoculum concentration used, an average VSS of 0.052 ± 0.01 g·L^{-1} was attained in the reactor (Figure 6.3). The HRT for the start-up period was set in 2.7 d, and the reactor operated with a mean NLR of 25.92 ± 1.04 mg N·L^{-1}·d^{-1}. The conditions applied to start-up a one-stage PNA reactor are quite different to similar studies, in which PNA reactors were inoculated with sludge from sidestream PNA reactors, and commonly, at MLSS concentrations higher than 4 g VSS·L^{-1} (Li et al., 2016; Lotti et al., 2015b; Morales et al., 2016). Thus, the overall volumetric nitrogen removal is affected by the lower activity and sludge availability.

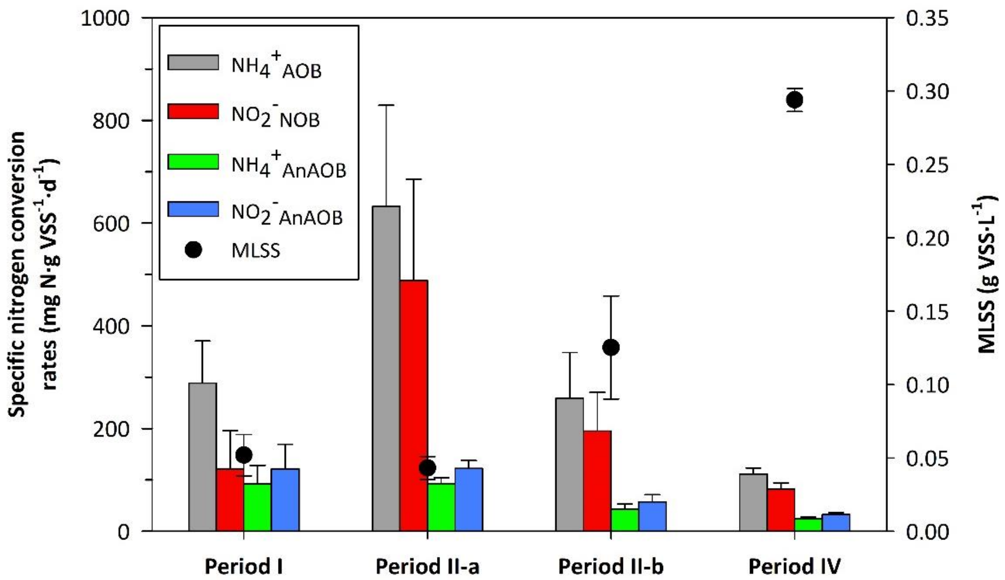

Figure 6.3. Variations in the specific nitrogen conversions by AOB, NOB and AnAOB, as well as the average MLSS concentration throughout the different periods.

The achieved NRR ranged from 4.2 to 12.2 mg N·L^{-1}·d^{-1}. Although the aeration flow was not turned on in the period I, the oxygen diffusion from atmosphere into the bulk liquid (chambers A and B were not covered), allowed DO concentration to range from 0.14 to

0.20 mg $O_2 \cdot L^{-1}$. The average ammonium concentration detected in the effluent was 23.5 ± 4.9 mg $N \cdot L^{-1}$. Interestingly, nitrite concentration above 13 mg $N \cdot L^{-1}$ was obtained until the operational day 5, but afterwards, nitrate production increased concomitantly with nitrite drop, and nitrite concentration remained lower than 5 mg $N \cdot L^{-1}$. Nitrite conversion rates by NOB and AnAOB were quite similar (Figure 6.3). Taking into account MLSS concentrations, nitrogen converted by AnAOB was higher than 200 mg N-(NH_4^++NO_2^-)·g $VSS^{-1} \cdot d^{-1}$ (for temperature between 24 and 27 °C). It indicates anammox activity rose about 7 times period I compared to the initial activity of the seed sludge at 15 °C (27 mg N·g $VSS^{-1} \cdot d^{-1}$). Moreover, average conversion by AnAOB was 4-fold higher than the values reported of 48 mg N·g $VSS^{-1} \cdot d^{-1}$ by Miao et al. (2016), in a SBR operated at 25 °C.

- **Period II: attempting to promote AnAOB growth**

In order to investigate the response of AnAOB face to more available substrate, and thereby expand nitrogen removal, the production of nitrite needed to be increased. In this way, more oxygen needed to be supplied into the reactor in order to raise nitrite production. However, the minimum airflow rate set would have provided more oxygen than necessary for the nitrogen loading applied in period II, according to the results obtained in the pre-experimental oxygen transfer coefficients (k_La) tests performed (data not included). To turn on the aeration in the aerobic chamber in period II, HRT needed to be reduced by half aiming at adjusting the NLR (Table 6.1). Thus, the mean NLR applied in this period was 55.83 ± 1.70 mg $N \cdot L^{-1} \cdot d^{-1}$. Even tough aeration was turned on in chambers A and B the mean DO concentration decreased in Period II. The modifications carried out in the operational conditions increased the ammonium oxidation, but it did not improve NRR. The achieved values ranged between 5 and 11 mg $N \cdot L^{-1} \cdot d^{-1}$, quite similar to period I. Removal capacity was limited because anammox activity did not enhance, despite low DO maintained around 0.10 mg $O_2 \cdot L^{-1}$ aiming at having larger anoxic fraction in sludge aggregates and preventing AnAOB inhibition by oxygen. The average nitrogen removal obtained in our study was inferior to previous studies, where PNA was operated in a continuous mode with granular biomass (Li et al., 2016; Lotti et al., 2015b). Despite the different operational temperature and nitrogen loading, both studies achieved NRR up to 190 mg $N\cdot L^{-1}\cdot d^{-1}$. When comparing the specific nitrogen removal, this study harbored similar or even higher activities than others did. Average activity obtained of 100 mg $N\cdot g\ VSS^{-1}\cdot d^{-1}$ (period II-a, temperature ranging from 20 - 22 ^{o}C) was higher than 46 mg $N\cdot g\ VSS^{-1}\cdot d^{-1}$, obtained at reactor operation at 19 ± 1 ^{o}C (Lotti et al., 2015b).

Nitrite concentrations in the effluent were quite low, while nitrate rose from 20 to 30 mg $N\cdot L^{-1}$ and reached stable values. Higher specific conversion by AOB (632 ± 197 mg N· g $VSS^{-1}\cdot d^{-1}$) and NOB (488 ± 197 mg N· g $VSS^{-1}\cdot d^{-1}$) were obtained, but AnAOB rates remained without any remarkable change (period II-a, Figure 6.3). Because nitrite is substrate for both NOB and AnAOB, the suppression of the first one is a pre-requisite to achieve stable autotrophic nitrogen removal. From a modelling perspective, it is expecting that low operational DO would require lower bulk ammonium concentration to suppress NOB activity (Pérez et al., 2014). Nevertheless, concentration between 25 and 39 mg $N\cdot L^{-1}$ (Figure 6.2C) was not enough to achieve full nitratation repression. Controversially, with a residual ammonium concentration of 3 mg $N\cdot L^{-1}$, nitrate production by NOB decreased considerably in an IFAS reactor (Yang et al., 2017). The failure in abating NOB in our study might be related to the low DO environment created which can promote the growth of k-strategist *Nitrospira*, with higher affinity at low substrates concentrations (Liu and Wang, 2013). Another influence might be the sludge composition that presented also floccular biomass, which commonly harbor higher NOB activity, as it is presented in section 6.3.2.

Regarding the biomass retention, average MLSS slightly reduced from period I to II-a. This reduction was experienced due to sludge wash-out in the settling tank. Stored anammox sludge had to be added in the reactor on day 69, but no improvement in NRR was achieved. Specific nitrogen conversions at period II-b (after sludge addition) was lower than at period II-a, indicating that higher solids concentration in the Period II-b did not increase the removal performance. The growth of anammox under the stringent conditions obtained at mainstream has been studied and a notably increase in the solids concentration was achieved (Hendrickx et al., 2014; Laureni et al., 2015; T. Lotti et al., 2014b), however, those studies were carried out under anoxic conditions in anammox reactors. Anammox doubling of 35 days have been reported at temperature of 20 ^oC, but it increased to 132 days at 10 ^oC (Lotti et al., 2014b).

- **Periods III and IV**

HRT of 1 d was set in period III. Additionally, new inoculation with stored anammox sludge was also carried out on day 171 to improve reactor performance. The airflow rate was raised and thus higher DO concentration than the previous period was achieved, reaching up to 0.54 mg $O_2 \cdot L^{-1}$. Nitrite accumulation was observed and concentrations up 10 mg $N \cdot L^{-1}$ were measured in the effluent. Initially, NRR was reduced due to oxygen inhibition on AnAOB, however after some operational days it was improved and NRR up to 12 mg $N \cdot L^{-1} \cdot d^{-1}$ was achieved. The volume settling index (VSI) performed in the reactor during period III is presented in Table 6.2. The VSI at 30 minutes slightly decreased from day 178 to 194, however the main improvement in the sludge settling was obtained for the VSI at 5 minutes. This indicates that part of the floccular sludge was washed out from the reactor and only granular sludge was retained, since average MLSS decreased in the referred period.

Table 6.2. Biomass volume settling index after the third addition of sludge

Day	VSI_5 (mL·g TSS^{-1})	VSI_{30} (mL·g TSS^{-1})	VSI_5/VSI_{30}	MLSS (g $VSS \cdot L^{-1}$)
178	92.8	60.4	0.65	0.80 ± 0.06
194	60.7	52.6	0.87	0.21 ± 0.004

Samples grabbed in all the chambers throughout the study showed that nitrogen concentrations were similar in aerobic and anoxic chambers (data not showed). The aeration was changed from chamber A and B to C in period IV, expecting to reduce the circulation of oxygen from the aerobic to the anoxic chamber. Afterwards, the airflow rate was manually increased to promote ammonium oxidation. DO values fluctuations occurred until day 250 probably due to the contact of the air bubbles with the DO sensor, but stabilized under 0.2 mg $O_2 \cdot L^{-1}$. NRR values stabilized in 15.60 ± 1.97 mg $N \cdot L^{-1} \cdot d^{-1}$, though, nitrate concentrations rose steadily. By analyzing the relation between the conversions of ammonium by AOB and nitrite consumed by NOB and AnAOB, it was observed an increase in NOB activity only when the aerobic ammonium oxidation rose, showing higher competitiveness of NOB over AnAOB (Figure 6.4). Once higher MLSS concentrations were obtained in the reactor at period IV, low specific nitrogen conversions were achieved compared to the previous periods.

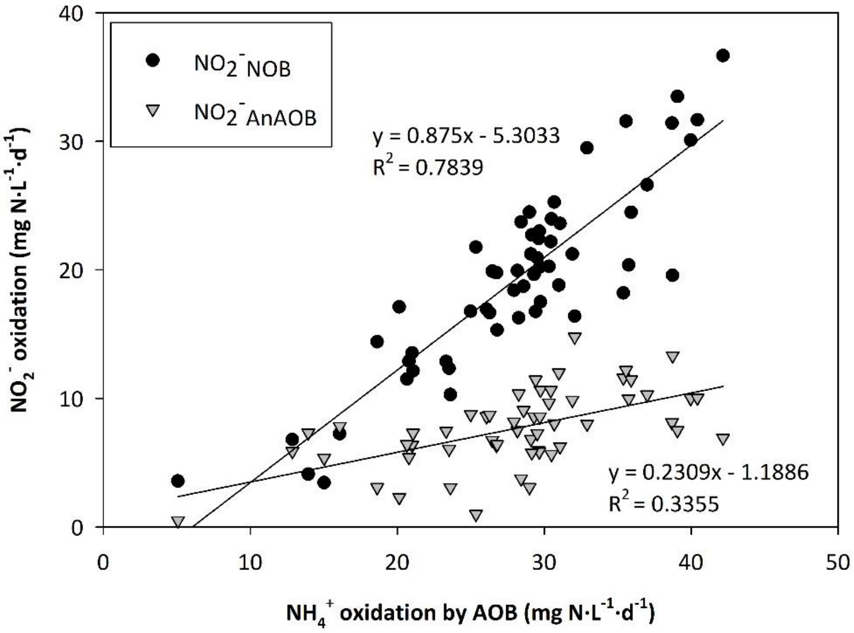

Figure 6.4. Time course of nitrite oxidation during stepwise increase of ammonium oxidation.

6.3.2. Microbial activities

The maximum conversion of ammonium and nitrite under aerobic conditions were analyzed in batch tests (Figure 6.5A) at the beginning of period II (day 41) and end of period III (day 201). It was obtained superior AOR than NOR on both days analyzed. One possible reason for lower NOR than AOR is that nitrite was not readily available at the beginning of the test, because AOB had to produce nitrite first, and NOB activity was quite delayed by this first step. The tests performed also displayed that AOR reduced from 1173 to 61 mg N·g VSS^{-1}·d^{-1}, whereas NOR declined from 800 to 26 mg N·g VSS^{-1}·d^{-1}. This decreasing of almost 20 and 30-fold in AOR and NOR, respectively, might have occurred mainly as a result from temperature variation from 23 to 16 ^{o}C, once lower temperature reduces microbial activity, which impacts substrates conversions rates.

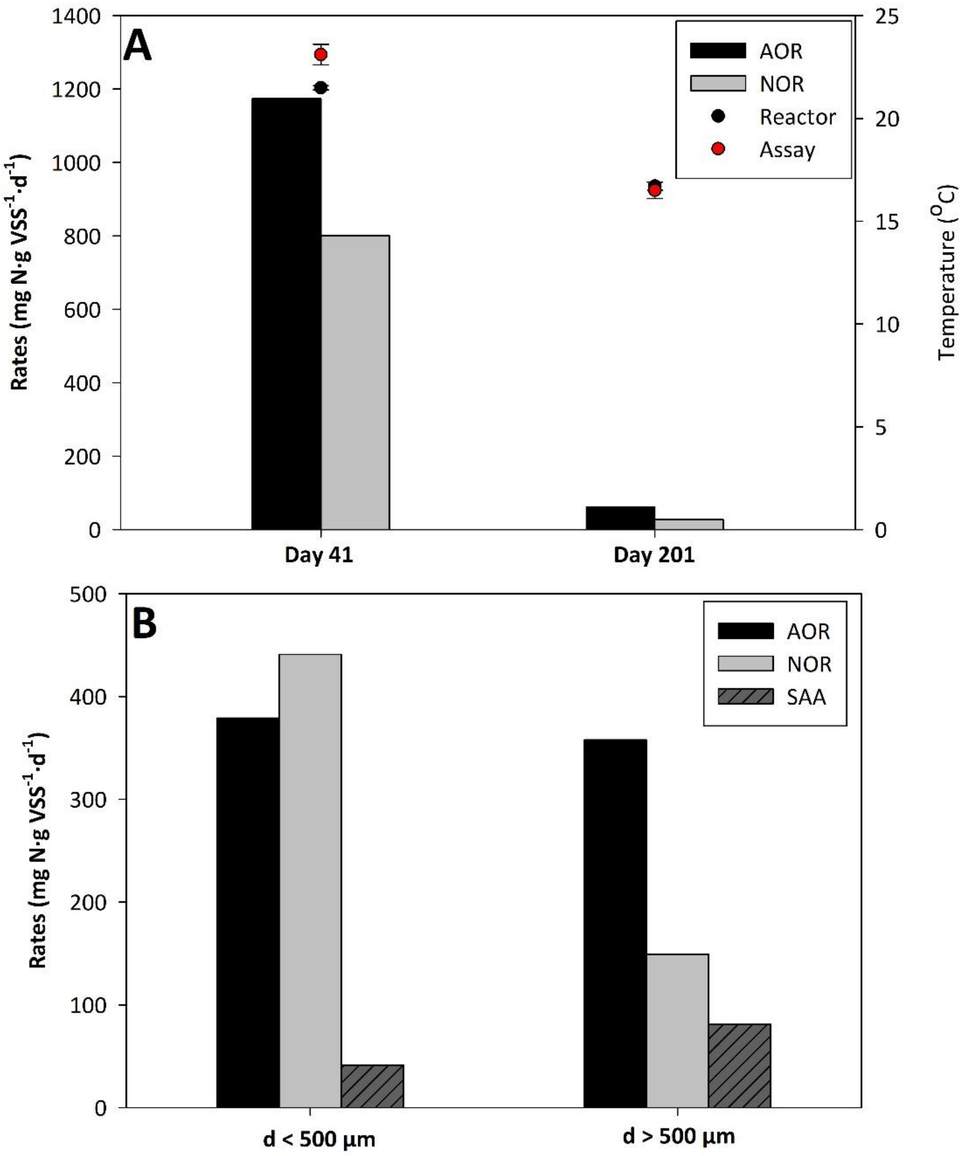

Figure 6.5. Nitrogen conversion rates obtained by the activity tests (A) AOR and NOR from reactor biomass in aerobic conditions; and (B) AOR, NOR and SAA from the tests to different size fractionated sludge (lower and higher than 500 µm).

By assessing the activity rates in the size fractionated sludge (Figure 6.5B), the results showed that aerobic rates were superior to anoxic rates in both small and large diameter size sludge. In the small diameter size fraction NOR was slightly higher than AOR (NOR of 440 mg N·g VSS^{-1}·d^{-1} and AOR of 379 mg N·g VSS^{-1}·d^{-1}). AnAOB activity was almost 11-fold lower than NOB, with a conversion rate of 41 mg N·g VSS^{-1}·d^{-1}. In the sludge fraction with diameter higher than 500 µm, AnAOB almost doubled with respect to small aggregates, and it was obtained SAA of 81 mg N·g VSS^{-1}·d^{-1}. In the other hand, NOR reduced to 149 mg N·g VSS^{-1}·d^{-1}, whereas AOR was 357 mg N·g VSS^{-1}·d^{-1}. In this assay, it was possible to detect that AnAOB have grown better in the higher diameter sludge, while higher AOB, and mainly NOB, were more active in small aggregates.

The higher NOR obtained, mainly in the small diameter size aggregates, also explains the higher nitrate production (Figure 6.5B). These results confirms previous studies which also investigated the impact of aggregate size on the activities of bacterial key-groups, with higher NOB activity in the small diameter sludge (Morales et al., 2016; Shi et al., 2016). The oxygen limitation on floccular biomass tends to be minimized, even at lower DO concentrations, which explains the higher aerobic activities in the sludge with diameter lower than 500 µm (Figure 6.5B). To balance aerobic and anoxic activities, this small diameter size biomass could be out-selected from the reactor. In this study, the aggregates' size was not analyzed throughout the operation, but it could indicate the amount of each fraction and understand the real contribution of small aggregates to the higher nitratation rates. The wastage of small diameter sludge have improved reactor's performance, and this wastage have been obtained by using either hydrocyclones classifier (Wett et al., 2013) or screens (Han et al., 2016b). Particularly in our study, as it was also showed by the rates obtained for the particles with diameter higher than 500 µm, nitratation activity would be probably not totally suppressed in case of applying small sludge out-selection, because SAA was still lower than NOR (Figure 6.5B).

6.3.3. Reactor configuration and operation implications

While reactors with continuous feed and aeration used in previous studies comprised only an aerobic chamber (Laureni et al., 2016; Lotti et al., 2015b; Malovanyy et al., 2015a), in our study, a plug-flow reactor with anoxic and aerobic chambers was used. By having an anoxic chamber, it was tried to increase anammox activity in such conditions. However, no difference in substrates bulk concentration (DO, ammonium and nitrite) was observed between the aerobic and anoxic chambers.

Regarding reactor's configuration, some shortcomings were experienced. The first one was the difficult in keeping the sludge in the aerobic and anoxic chambers, because part of it was being compacted in the settling chamber. Consequently, small agitation had to be maintained to suspend the sludge and ensure its recirculation. This action also helped to wash-out small aggregates in the sludge, but larger granules with higher AnAOB were washed-out from the reactor as well. It would be recommended the use of screen (Han et al., 2016b) or hydrocyclones (Wett et al., 2013) to select the desired fractionated sludge. In comparison with MBBR or IFAS reactor, the possibility to prevent AnAOB to leave the reactor by growing the biofilm attached to carriers shows those reactors have more advantages than the system used in this study. Gu and collaborators (2019) have obtained satisfactory nitrogen removal in a step-feed reactor with aerobic and anoxic chambers, where carriers with AnAOB biofilm was placed in the anoxic chambers, and floccular biomass with nitrifiers were subject to aerobic-anoxic spatial conditions.

Targeting at decreasing reactor's start-up time, granular sludge or biofilm carrier from sidestream reactors have been used to inoculate new reactors. However, unsatisfactory reactor performance due to poor nitrogen removal had also required reactor re-inoculation (Hoekstra et al., 2018; Morales et al., 2016), which is not feasible at full-scale operation. Thus, systems with suspended granular biomass have the challenge of retaining larger granules with higher AnAOB activity, while washing-out small aggregates that generally have higher NOB activity (Han et al., 2016b).

Once biomass retention was limited, anammox growth was difficult in the system as well. During all the operation of the reactor AnAOB unfavorable conditions was not only due to the sludge wash-out, but also due to the competition with NOB. NOB was far more competitive during all the study, leading less nitrite available for AnAOB.

In sum, for practical applications, the hints derived from this study are (i) higher amount of AnAOB sludge might be necessary in order to obtain better nitrogen removal rates; (ii) start-up time can take longer if only small amount of sludge is available; and (iii) NOB outcompeting has to be the first step to not hamper AnAOB growth.

6.4. Conclusions

- A reactor with aerobic and anoxic chambers was investigated as a proposal reactor configuration to be implemented at mainstream conditions for one-stage PNA processes;
- By operating the reactor at room temperatures (between 16 and 27 ^{o}C) and low DO concentration, NRR of around 20 mg N·L^{-1}·d^{-1} was obtained;
- Nitrogen removal conversions were quite low due to the few amounts of sludge used to start-up the reactor. However, with temperature ranging from 20-22 ^{o}C, the mean specific removal rates achieved values higher than 100 mg N·g VSS^{-1}·d^{-1}, which is comparable to other studies;
- AOR reduced from 1173 to 61 mg N·g VSS^{-1}·d^{-1}, while NOR declined from 800 to 26 mg N·g VSS^{-1}·d^{-1} with temperature operation of 23 and 16 ^{o}C;
- Activity tests carried out with size fractionated sludge showed that AOR and NOR were, in overall, higher than SAA for all the tests. However, while NOR of 440 mg N·g VSS^{-1}·d^{-1} was obtained for the sludge with diameter lower than 500 µm, the fraction with higher diameter (>500 µm) had a NOR of 149 mg N·g VSS^{-1}·d^{-1}.

Chapter 7.

Limitation of inorganic carbon suppresses nitrite-oxidizing bacteria activity in a granular SBR at mainstream conditions

This chapter has been submitted for Chemosphere:

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Abstract

This study investigated the effects of inorganic carbon (IC) availability on nitrifying microbial interaction of a sequencing batch reactor (SBR) operated for partial-nitritation (PN) at mainstream conditions (temperature of 15 ^oC and low influent N concentration). Nitrite accumulation could not reached during reactor operation at IC availability excess for nitrification, with the oxidized ammonium being fully nitrified. Limiting influent IC (influent NH_4^+:IC of 0.73 ± 0.03) favored nitrite-oxidizing bacteria (NOB) suppression while nitritation was maintained, leading to nitrite accumulation over 80%. PN control was also favored by keeping low bulk oxygen concentration (below 0.5 mg $O_2 \cdot L^{-1}$). The ratio between maximum nitratation over maximum nitritation rates ($rNO_2^-{}_{max}/rNH_4^+{}_{max}$) dropped from 0.92 (without IC limitation) to 0.04 (with IC limitation). The results obtained highlight the critical IC role in the microbial interactions of partial-nitritation process and present new insights in the direction of obtaining NH_4^+:NO_2^- for mainstream nitrite short-cut nitrogen removal process.

Keywords: Mainstream deammonification; Partial nitritation; NOB outcompeting; Inorganic carbon; Low temperature; Short-cut nitrogen

7.1. Introduction

Implementing autotrophic nitrogen removal (ANR) processes in the mainstream line of urban wastewater treatment plants (WWTP) will allow to maximize organic matter valorization (through biogas production), savings in aeration, less chemicals dosing requirements and sludge minimization leading to energy-balanced WWTP. Regardless of the configuration used for partial-nitritation/anammox (PNA) process (either one stage: partial-nitritation (PN) and anammox in the same reactor; or two-stage: separate reactors for PN and anammox), successful N removal relies on nitrite-oxidizing bacteria (NOB) activity suppression to obtain stable (partial) nitritation. NOB can compete for oxygen and nitrite to produce nitrate, and, therefore, their activity compromises the N removal. Several control strategies for NOB suppression have been evaluated and widely applied for high N-strength wastewater, mainly based on the different ammonium-oxidizing bacteria (AOB) and NOB activity response to free ammonia (FA) and/or free nitrous acid (FNA) inhibitions, and to high (mesophilic) temperature (Ganigué et al., 2008; Hellinga et al., 1998; Scaglione et al., 2013). However, such strategies are not feasible when dealing with mainstream wastewater treatment, due to low N content and seasonal temperature variations (Gilbert et al., 2014b).

Most approaches proposed so far to achieve NOB suppression in one or two-stage mainstream PNA biofilm reactors, are based on the different AOB and NOB affinities for oxygen and focus on dissolved oxygen (DO) set-point control. Hence, literature reports several studies on the impact of DO on successful mainstream PN, either at low bulk-liquid DO concentration (< 0.5 mg $O_2 \cdot L^{-1}$) (Akaboci et al., 2018; Gilbert et al., 2014b; Li et al., 2016) or higher concentration (between 1 and 2 mg $O_2 \cdot L^{-1}$) (Bian et al., 2017; Isanta et al., 2015; Morales et al., 2016). Since NOB present longer delay time than AOB after transient anoxia, intermittent aeration has also been exploited (Gilbert et al., 2014a; Regmi et al., 2015). However, diverse outcomes have been reported from those studies regarding the effectiveness of the strategy applied, and the efficiency of NOB suppression was generally low or did not last at long-term operation (Han et al., 2016a; Trojanowicz et al., 2016).

Inorganic carbon (IC) is the only carbon source for both AOB and NOB and represents the main alkalinity source in urban wastewater. In common pH and temperature ranges in urban wastewater, bicarbonate (HCO_3^-) is the prevalent IC specie. The effects of limited IC availability on nitrification have been studied for sidestream wastewater (high N-strength) wastewater (Guisasola et al., 2007; Wett and Rauch, 2003), and it was shown that IC limitation has adverse effects on nitritation, by reducing AOB activity, whereas no negative effects on NOB were observed. Few studies have explored the impact of IC scarcity on microbial activities in PNA reactors, where influent ammonium was maintained from 100 to 500 mg NH_4^+-N·L^{-1} and operational temperature above 25 ^oC (Chen et al., 2012; Y. Ma et al., 2015; Zhang et al., 2016). Those studies reported negative effects on AOB and anaerobic ammonium-oxidizing bacteria (AnAOB) activities when IC was limited, resulting in a deterioration of N removal performance. Moreover, NOB was favored by the IC limitation, being suppressed only after influent IC increase. At mainstream conditions, the low temperature decreases AnAOB activity (Jin et al., 2013), and reduces nitrogen removal rate. Besides, AOB have a lower growth rate than NOB (Hellinga et al., 1998).

IC availability is a critical parameter affecting the N-conversion rates and the microbial interactions in nitrifying reactors, and could, therefore, play a key role in the mainstream implementation of PNA in urban WWTP. But the impact of IC on PN has never been explored so far under mainstream conditions. Hence, the main objective of this study was to assess the effects of influent IC limitation on the performance of a granular sequencing batch reactor (SBR), operated at long term treating urban wastewater under mainstream conditions (15 ^oC, low influent N concentration; after organic matter removal step).

7.2. Material and Methods

7.2.1. Reactor setup

This study was conducted in a lab-scale SBR with working volume of 10 L (Biostat B Plus, Sartorius). An external water cooler allowed maintaining the reactor temperature at ±15 ^oC, by recirculating the water through a water-jacket. The pH (405-DPAS-SC-K8S/325, Mettler Toledo), ORP (4805 DPAS-SC-K8S/325), and DO (InPro 6850i polarographic oxygen sensor, Mettler Toledo) were on-line monitored. The volume exchange ratio (VER) was kept at 50% in each operational cycle.

7.2.2. Operational conditions

Before the experimental period described in this study, the SBR was running continuously as one-stage mainstream granular PNA for approximately one year at temperature of ±15 ^oC (Akaboci et al., 2018). Under such stringent conditions, specific AnAOB activity (SAA) remained extremely low (SAA of 7.5 mg N·g $VSS^{-1}\cdot d^{-1}$), as reported in other works in similar conditions, and NOB population increased. Amplicon sequencing Illumina revealed that *Nitrosomonas* and *Nitrospira* were the main bacteria within AOB and NOB groups, respectively.

This study comprised five periods, according to the influent NH_4^+:IC molar ratio (Table 7.1). Based on the nitritation stoichiometry, a ratio NH_4^+:IC of 0.5 is required for full nitritation while the molar ratio to obtain the partial-nitritation level for anammox process is 0.88 NH_4^+:IC (Ganigué et al., 2009). During periods I and II, the reactor was operated without IC limitation for full nitritation, and different airflow rates were set, aiming at obtaining different bulk-liquid DO concentrations. In the subsequent periods, the reactor operation was switched to IC limitation for full nitritation. Furthermore, in period V, pH was controlled at a minimum value of 6.8 during the aeration period by adding 1 M NaOH, in order to compensate the proton consumption by nitrification. Influent wastewater was prepared with mineral medium, containing per liter of tap water: 0.0125 g $FeSO_4\cdot 7H_2O$, 0.0125 g $EDTA\cdot 2H_2O$, 0.2 g $MgSO_4\cdot 7H_2O$, 0.008 g KH_2PO_4, 0.3 g $CaCl_2 \cdot 2H_2O$, 0.354 g $(NH_4)_2SO_4$ and 1.25 mL of trace element solution. The trace element solution was prepared based on Graaf et al. (1996). Ammonium was provided by $(NH_4)_2SO_4$ to obtain a concentration around 70 mg $N \cdot L^{-1}$, while IC was obtained by $NaHCO_3^-$ addition, with the concentration changed according to the NH_4^+:IC influent ratios assessed.

Table 7.1. Operational period's characteristics

Period	Operational day	Airflow rate ($L \cdot min^{-1}$)	Influent NH_4^+:IC ratio (mol:mol)	Bulk-liquid maximum pH*	Bulk-liquid minimum pH*
P-I	0-40	0.010	0.49±0.06	8.02±0.14	7.38±0.09
P-II	40-82	0.020 to 0.070	0.40±0.01	7.74±0.16	7.27±0.14
P-III	82-147	0.020	0.73±0.03	7.70±0.14	5.81±0.30
P-IV	147-190	0.020	0.74±0.03	7.61±0.06	6.84±0.06
P-V	190-210	0.020	1.39±0.03	7.31±0.03	6.59±0.04

*The maximum pH in the reactor was obtained after the filling phase, while the minimum in the end of operational cycle.

The SBR was operated with operational cycles of 6 h (periods I to III) and of 4 h (periods IV to V) (Figure 7.1). The hydraulic retention time (HRT) was maintained in 12 h (periods I to III) and 8 h (periods IV to V).

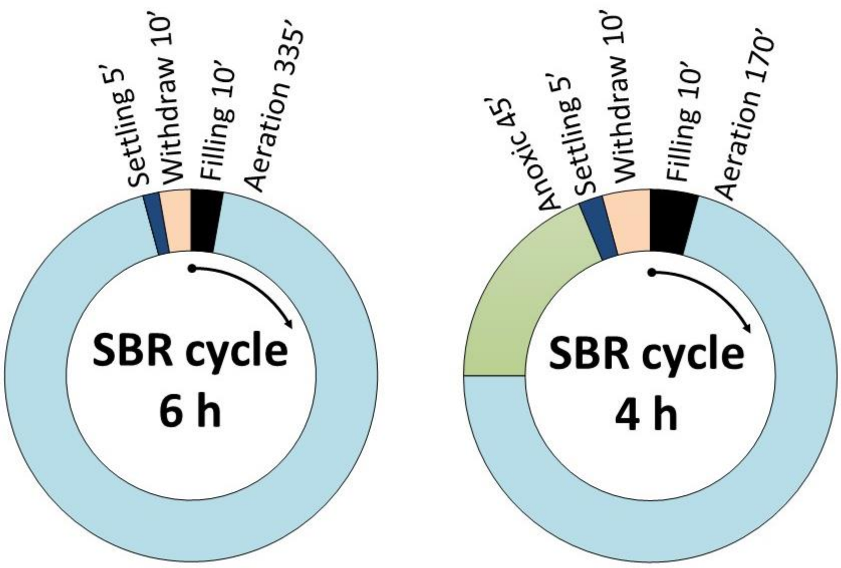

Figure 7.1. SBR cycle distribution.

7.2.3. Biomass activity assays

In situ activity assays of the SBR sludge were carried out to determine the maximum ammonium ($rNH_4^+{}_{max}$) and nitrite ($rNO_2^-{}_{max}$) oxidation rates of the biomass in aerobic conditions, during the different periods. The operational cycle was switched off, sludge was allowed to settle and the supernatant liquor was withdrawn. Afterwards, the sludge was washed three times with mineral medium without nitrogen to remove all possible remaining substrate present in the bulk liquid. Then, the sludge was acclimated for 1 h by stirring the bulk liquid at 150 rpm and by adding fresh air, which ensured aerobic conditions (DO concentration around 4 mg·L^{-1}, maintained during all the assay). Additionally, the temperature was controlled at 15 ºC. A pulse of 40 mg NH_4^+-N·L^{-1} was dosed by the addition of $(NH_4)_2SO_4$. Samples were collected every 30 minutes for 3 hours to analyze ammonium, nitrite, and nitrate evolution throughout the assay. Linear regression over data was used to calculate ammonium and nitrite oxidation rates (respectively $rNH_4^+{}_{max}$ and $rNO_2^-{}_{max}$). Specific activities were obtained by dividing nitrogen conversion rates by MLSS. The mineral medium used to perform the activity assays had the same composition as described above, but only ammonium and bicarbonate content changed. In this case, $NaHCO_3$ was added to provide at least a molar ratio of 0.5 NH_4^+:IC and avoid limiting inorganic carbon conditions for nitrification.

7.2.4. Analytical procedures

Ammonium concentration was analyzed by titrimetric method (APHA 4500); volatile suspended solids determined by (APHA 2540E) (APHA, 1995). Nitrite and nitrate were measured by ion chromatography (DIONEX). Inorganic carbon concentrations were obtained by combustion method (TOC-VCSH, Shimadzu, Japan).

7.2.5. Calculations

FA and FNA concentrations were calculated according to Anthonisen et al. (1976).

Nitrite accumulation ratio (%) was calculated according to Equation 7.1:

$$NO_2^- \text{accumulation} = \frac{NO_2^- \text{effluent}}{(NO_2^- + NO_3^-) \text{ effluent}} \times 100 \qquad \text{(Eq. 7.1)}$$

7.3. Results

Figure 7.2 shows the trends of nitrogen species, ammonium loading and oxidation rates, and nitrite accumulation ratio during the SBR operation treating mainstream urban wastewater at 15 ^oC at different influent IC conditions.

7.3.1. Reactor operation under full IC availability

The reactor ran without limiting IC conditions for 82 days (periods I and II, Figure 7.2). The bicarbonate added in the feeding medium during those periods was enough to provide alkalinity for full nitrification, with average NH_4^+:IC molar ratio of 0.49 ± 0.06 and 0.40 ± 0.01 in period I and II, respectively (Figure 7.2A). In period I, air flow rate was adjusted to keep low oxygen supply (Table 7.1), which resulted in a bulk-liquid DO maintained under the minimum detection level of the probe. It allowed only partial ammonium oxidation (mean conversion of 60 ± 0.06 %). Moreover, most of the ammonium oxidized was completely nitrified, occasioning an effluent with nitrate concentration between 35 and 44 mg NO_3^--N·L^{-1}, whereas nitrite concentration was practically negligible (Figure 7.2B).

To deeply evaluate the nitrogen conversion during an operational cycle in period I, a typical SBR cycle was analyzed. Results are presented in Figure 7.3A, -C and -E. Ammonium and IC increased during the filling phase (0 - 10 min) while nitrate concentration decreased due to the dilution. During the aeration phase, ammonium concentrations dropped from 44 to 22 mg NH_4^+-N·L^{-1} and it was oxidized to nitrate. Nitrite did not accumulate and consequently the concentrations were almost null during

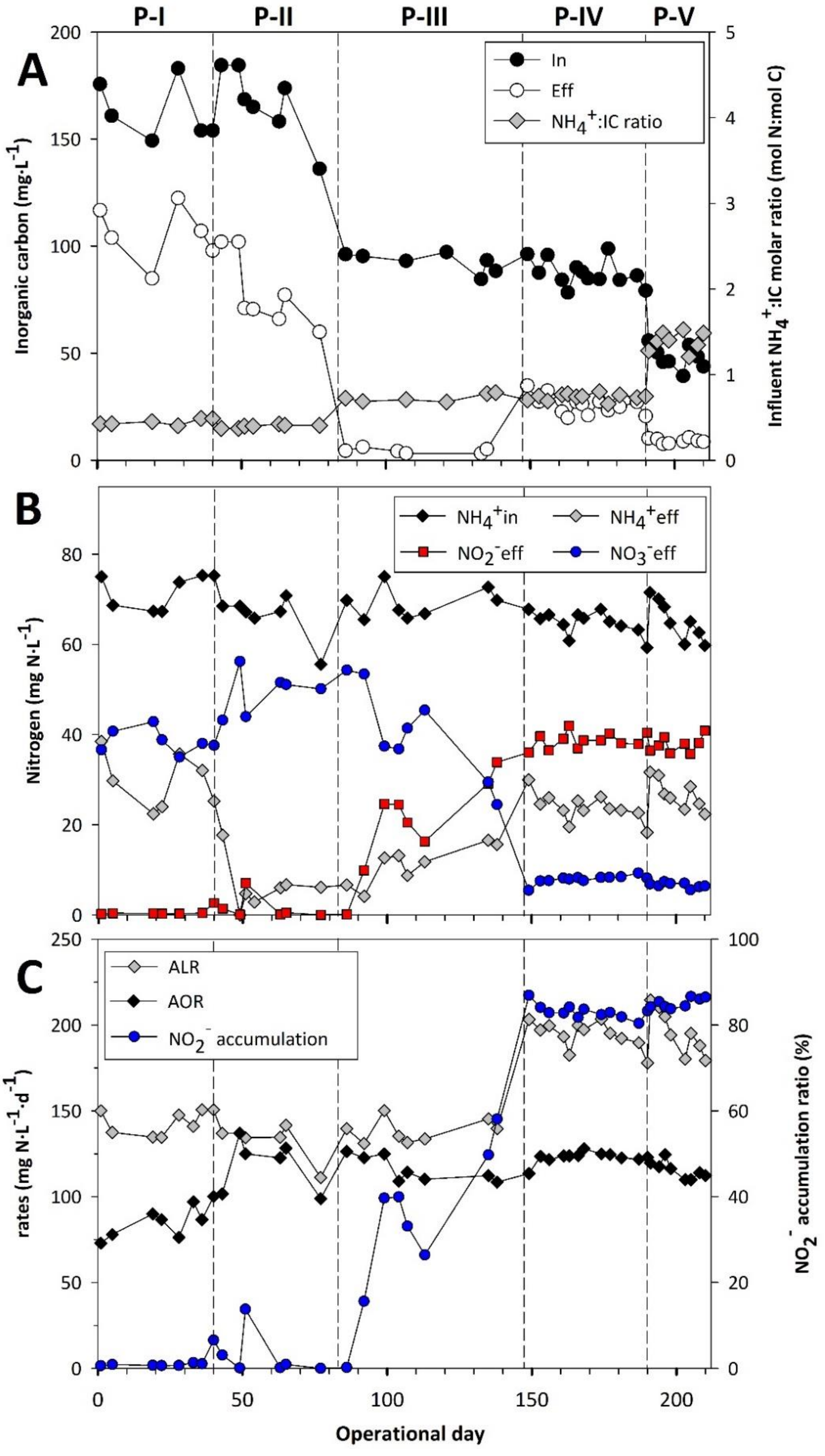

Figure 7.2. (a) Time course inorganic carbon concentrations and NH_4^+:IC molar ratio in the influent; (b) Nitrogen species; and (c) ammonium loading (ALR) and ammonium oxidation rate (AOR); and nitrite accumulation ratio.

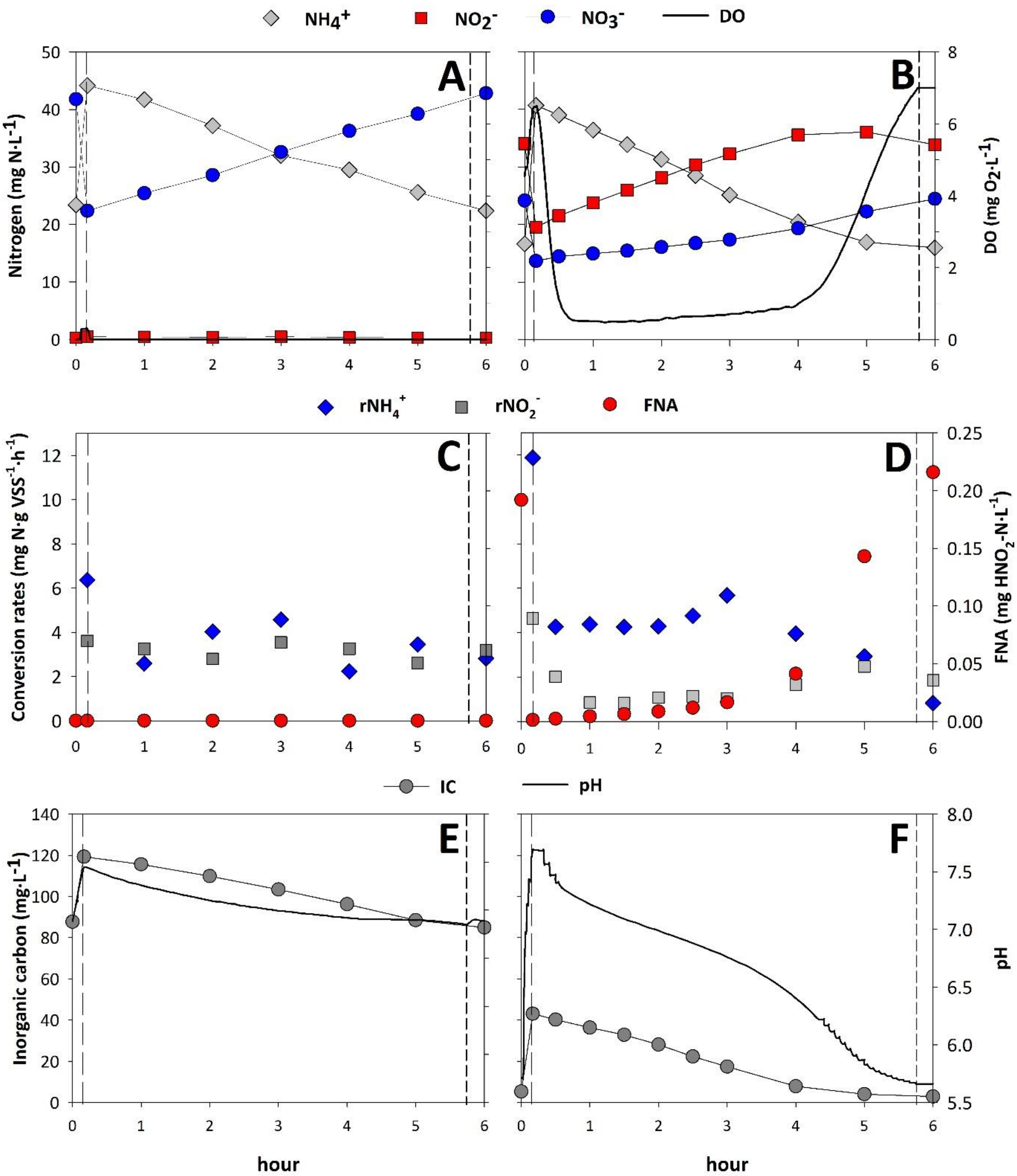

Figure 7.3. Operational cycles analyzed in Period I (day 19, left plots) and Period III (day 138, right plots). A-B: Nitrogen species and DO concencentrations; C-D: Ammonium and nitrite conversion rates, together with FNA concentration; and E-F: IC concentration and pH. In each plot, vertical lines indicate: the end of filling/beginning of aerobic phase (left long-dashed); and the end of aerobic phase/beginning of settling time and effluent withdraw (right short-dashed).

all the cycle (Figure 7.3A). Despite air flow being off, conversion rates of ammonium (rNH_4^+) and nitrite (rNO_2^-) were of 6.3 and 3.6 mg N·gVSS^{-1}·h^{-1}, respectively, during the filling phase. The feed medium was not deoxygenated, and both OD from the medium and air diffusion into the bulk liquid might have contributed to the oxidation of ammonium and nitrite during the initial phase of the cycle. In the course of the cycle, rNH_4^+ and rNO_2^- values were comparatively close and oscillated between 2.2 and 4.5 mg N·gVSS^{-1}·h^{-1} (Figure 7.3C). Because alkalinity was not limited, pH was kept higher than 7.40, and IC concentration dropped from 119 to 84 mg C·L^{-1}, as a consequence of alkalinity consumption for nitrification (Figure 7.3E).

Oxygen supply was manually increased in period II by rising the airflow rate to adjust the bulk liquid DO concentration around 1 mg O_2·L^{-1} . After an initial period of adjustment, the airflow rate was set at 0.020 L·min^{-1} and kept at this value for the rest of the study (Table 7.1). DO concentration remained below 2 mg O_2·L^{-1} during almost all the aeration phase in the cycle (Figure S1), but it tended to rise at the end of the aeration indicating a possible reduction in the oxygen consumption as a consequence of ammonium depletion. This higher oxygen supply led to an ammonium oxidation improvement, and the AOR improved from 85.9 ± 7.6 mg N·L^{-1}·d^{-1}, period I, to 120.0 ± 9.9 mg N·L^{-1}·d^{-1} at period II (Figure 7.2C). Effluent ammonium concentration around 6 mg NH_4^+-N·L^{-1} was reached during this period. At moderately bulk DO achieved in period II (between 0.5 and 2 mg O_2·L^{-1}), nitratation suppression was not achieved and nitrate was still the main product in the effluent, with only punctual nitrite accumulation (Figure 7.2C). Furthermore, the results obtained by the aerobic activity assays showed that higher oxygen supply improved both $rNH_4^+{}_{max}$ and $rNO_2^-{}_{max}$, and the rates doubled from day 55 to day 71 (Figure 7.4). Furthermore, both $rNH_4^+{}_{max}$ and $rNO_2^-{}_{max}$ presented quite similar results.

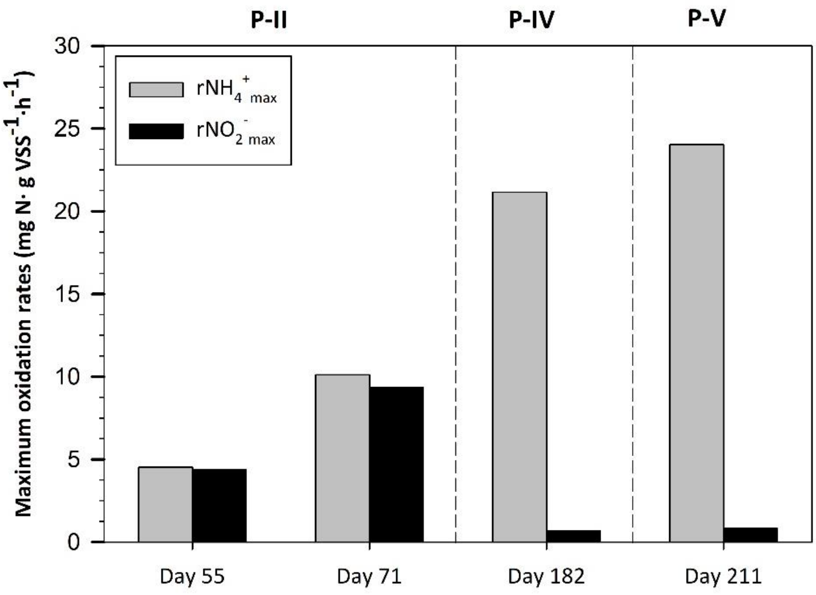

Figure 7.4. Maximum ammonium and nitrite oxidation rates measured during in-situ activities assays performed.

7.3.2. Reactor operation under IC limitation

IC limitation to achieve partial nitritation started being assessed in period III (day: 82 - 147). Sodium bicarbonate concentration was reduced in the influent synthetic medium. Theoretically, at a NH_4^+:IC molar ratio of 0.73 ± 0.03 around 68% of the ammonium could be oxidized to nitrite. At this condition, AOR slightly dropped in comparison to period II and an average of 111.6 ± 7.7 mg N·L^{-1}·d^{-1} was achieved (Figure 7.2C), with effluent ammonium concentration ranging between 4 and 16 mg NH_4^+-N·L^{-1} (Figure 7.2B). IC was almost all consumed, and concentrations below 6 mg C·L^{-1} were obtained in the effluent withdrawn (Figure 7.2A). Due to the lack of enough alkalinity to buffer the system, average pH in the effluent was 5.81 ± 0.30 (Table 7.1). Under these conditions nitrite accumulation started, resulting in effluent concentration higher than 20 mg NO_2^--N·L^{-1}. In turn, due to NOB activity limitation, effluent nitrate concentration dropped and the nitrite accumulation reached up to 80% of the ammonium oxidized at the end of this period (Figure 7.2C).

In the cycle analyzed on day 138 (Figure 7.3B, -D and -F), a different behavior regarding nitrogen species evolution was obtained compared to the cycle analyzed in period I. Ammonium was oxidized from 41 to 16 mg NH_4^+-N·L^{-1} during the aeration time, while nitrite concentration went from 19 to 36 mg NO_2^--N·L^{-1} until time 5 h, with a slight decline to 34 mg NO_2^--N·L^{-1} in the last hour. Concomitantly, nitrate concentrations rose from 14 to 24 mg NO_3^--N·L^{-1} (Figure 7.3B). With respect to the oxidation rates, values of 11.8 mg N·$gVSS^{-1}$·d^{-1} for rNH_4^+ and 4.6 mg N·$gVSS^{-1}$·d^{-1} for rNO_2^- were obtained after the end of filling phase, and both were the highest compared to all the operational cycle (Figure 7.3D). These rates might have been increased because DO in the reactor reached 6.5 mg O_2·L^{-1} at the same time and the effects of substrates limitation were softened (Figure 7.3B). rNH_4^+ was overall twice higher than rNO_2^-, but a drop of rNH_4^+ values in the second half of the operational cycle was experienced and might be linked to the lack of IC and low pH (Figure 7.3D). Controversially, rNO_2^- was not impaired, reaching 2.4 mg N·$gVSS^{-1}$·d^{-1} at time 5 h, and the increase of OD concentration at the end of the cycle probably minimized low IC availability effects on the nitratation process. Due to the achieved nitrite concentration and abrupt pH drop at the end of the cycle (Figure 7.3F), FNA considerably improved from approximately 0.04 to 0.21 mg HNO_2-N·L^{-1}. It is shown that despite this FNA concentration, nitrate growing occurred at the end of the cycle (Figure 7.3D). IC concentrations were lower than the previous analyzed cycle and limited the ammonium oxidation after time 5 h (Figure 7.3F).

The total cycle time was reduced to 4 hours in period IV (day: 147 - 190). From this total cycle time, aeration took place only 75% of the time, while an anoxic time of 55 minutes was set after aeration length aiming at obtaining favorable anoxic conditions which could activate anammox activity. HRT reduction increased the ALR (Figure 7.2C). It was possible to reach higher ammonium effluent concentration than in the previous period, while nitrite oscillated between 36 and 40 mg NO_2^--·N·L^{-1} and nitrate was lower than 9 mg NO_3^--·N·L^{-1} (Figure 7.2B). The average effluent NO_2^-: NH_4^+ ratio was 1.53 ± 0.14, slightly higher than required according to anammox stoichiometry of (NH_4^+:1.32 NO_2^-; Strous et al., 1998). Nitrite accumulation around 80% was obtained. In this period,

$rNH_4^+{}_{max}$ doubled in comparison to period II, despite low influent IC, and reached 21.1 mg N·g VSS^{-1}·h^{-1}, while $rNO_2^-{}_{max}$ fell to 0.7 mg N·g VSS^{-1}·h^{-1} (Figure 7.4).

The results obtained from an operational cycle analyzed in period IV (day 156) are depicted in Figure 7.5. The behavior of nitrogen species was rather similar to the cycle analyzed in period III. Once again, the highest oxidation rates were obtained at the end of the filling phase, with rNH_4^+ and rNO_2^- values of 14.6 mg N·g VSS^{-1}·h^{-1} and 11.2 mg N·g VSS^{-1}·h^{-1}, respectively (Figure 7.5B). Moreover, this rNO_2^- was higher than the $rNO_2^-{}_{max}$ obtained in period IV ($rNO_2^-/rNO_2^-{}_{max}$ ≈16.7). Despite the DO value achieved during the filling phase, this might have been also influenced by higher nitrite concentration in the bulk liquid (≈21 mg N·L^{-1}), once in the aerobic assays only ammonium was spiked and NOB could be limited by the available nitrite produced by AOB. During the aeration phase, rNH_4^+ remained around of 5 mg N·g VSS^{-1}·h^{-1}, and rNO_2^- oscillated below 0.3 mg N·g VSS^{-1}·h^{-1}. IC was not fully consumed during the cycle and the alkalinity allowed buffering the system, resulting in higher pH values and FNA lower than 0.014 mg HNO_2-N·L^{-1} (Figure 7.5-B and -C). This lower FNA concentration did not result in an increase of NOB activity (Figure 7.5B).

IC was even more decreased in Period V (day: 190 - 210). Nitrite accumulation did not change, and stable values similar to the ones obtained in Period IV were observed (Figure 7.2B), while a slight increase in ammonium concentration is observed in the effluent. The mean NO_2^-: NH_4^+ obtained was at 1.34 ± 0.13. The results obtained from the biomass activity assays proved that NOB suppression was maintained, resulting in $rNO_2^-{}_{max}$ as low as 0.8 mg N·g VSS^{-1}·h^{-1} (Figure 7.4).

7.4. Discussion

7.4.1. Restoration of nitrite accumulation by limiting IC

This study investigated whether the imposition of IC limitation would affect the activity of the microorganisms in a nitrifying granular SBR operated at mainstream conditions and 15 °C. The reactor was operated without IC limitation for two periods (I and II). With

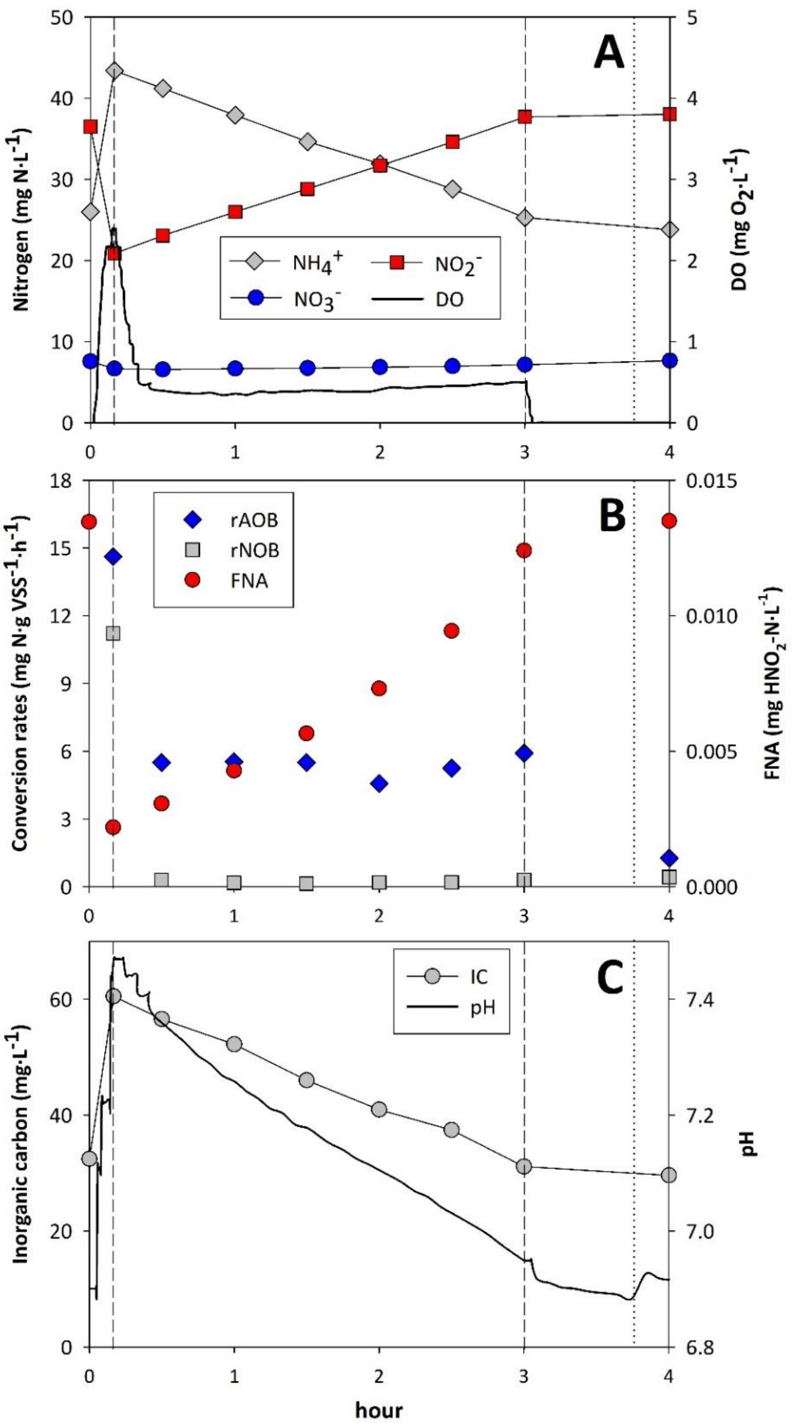

Figure 7.5. Operational cycles analyzed in Period IV (day 156). A: Nitrogen species and DO concencentrations; B: Ammonium and nitrite conversion rates, together with FNA concentration; and C: IC concentration and pH. In each plot, vertical lines indicate: the end of filling/beginning of aerobic phase (left long-dashed); the end of aerobic/beginning of anoxic phase (right long-dashed); and the end of anoxic phase/beginning of settling time and effluent withdraw (right short-dashed).

the IC and DO conditions imposed during these periods, stable nitrite accumulation could not be obtained (Figure 7.2C). Besides, the ammonium and nitrite conversion rates ($rNH_4^+{}_{max}$ and $rNO_2^-{}_{max}$) presented very similar values (Figure 7.4). The imposition of IC limitation after period III, by reducing the bicarbonate content in the influent medium (average NH_4^+:IC of 0.73±0.03), led to nitrite build-up in the system (Figure 7.2C) proving that NOB activity suppression (partial) was obtained. These results, with IC limitation promoting NOB activity suppression in mainstream operation, differ from an earlier study carried out at sidestream conditions (Guisasola et al., 2007), in which limiting IC did not show any negative effect on NOB activity. According to these authors, NOB either consume little amount of CO_2, which can be provided by heterotrophic biomass during the consumption of organic matter from biomass lysis, or they could also grow mixotrophically using traces of organic matter. Sidestream PN is commonly operated at mesophilic temperatures (>30°C), and higher hydrolysis rates are a significant source of CO_2 for autotrophic biomass. AOB was also shown more sensitive than NOB to IC limitation in a full-scale reactor at mainstream water line (Seuntjens et al., 2018). However, IC kinetic parameters for AOB and NOB were determined by add-on mechanistic model, with parameters for 20 °C. In our experimental study, the temperature was always maintained at 15 °C, and therefore, temperature could impact the rates obtained.

Nitrite accumulation up to 80% was obtained and sustained during 60 days by low IC availability (periods IV and V), demonstrating that NOB activity was almost totally suppressed (Figure 7.2C). NOB activity repression was confirmed by the strong reduction in $rNO_2^-{}_{max}$ (Figure 7.4). In contrast, only short-term nitrite accumulation as a consequence of IC limitation has been achieved elsewhere (Bae et al., 2015; Ma et al., 2015). Ma et al. (2015) investigated IC limitation in a one-stage nitritation-anammox operated at sidestream conditions (influent ammonium 500 mg $N \cdot L^{-1}$). The authors have experienced nitrite accumulation for approximately 15 days, but NOB recovered their activity later. Bae and collaborators (2015) also reported nitrite accumulation in a nitrifying reactor operated at a 35 °C temperature and under IC limited conditions related to ammonium concentrations (influent ammonium around 1000 mg $N \cdot L^{-1}$). But this accumulation was not maintained longer (less than 10 days), showing controversial outcomes about the impact of IC limitation on nitrite accumulation.

7.4.2. Elucidating the factors linked to NOB activity repression

By limiting IC in period III, the buffer capacity of the reactor was affected and it led to lower pH values. The average minimum pH values dropped from 7.27 ± 0.14 (period II) to 5.81 ± 0.30 (period III) (Table 7.1). pH is known to influence microbial activity and can be the critical factor triggering nitratation under limited IC availability. While minimum pH was controlled at 7.5 ± 0.03 (Ma et al., 2015) and at 8 (Bae et al., 2015) in previous works, in our study the values reached under IC limitation in period III are under the optimal pH reported for *Nitrospira* -between 7.6 and 8.3 (Blackburne et al., 2007; Ehrich et al., 1995)- and *Nitrobacter* -between 7.5 and 8.2 (Grunditz and Dalhammar, 2001). Bacterial community composition was not followed in this study, but the reactor previously showed prevalence of *Nitrospira* as the main NOB group (Akaboci et al., 2018). Thus, at mainstream conditions, where *Nitrospira* is more competitive in the presence of low oxygen and nitrite concentration, pH could act as key selecting factor.

Besides low pH, the results also point towards IC as niche differentiation between *Nitrospira* and *Nitrobacter*. It was previously reported *Nitrospira* growth at higher IC availability, whereas *Nitrobacter* was experienced at low IC conditions (Bae et al., 2015; Fukushima et al., 2013). Thus, it is believed that the limited IC imposed in our study contributed to the repression of *Nitrospira,* which was the NOB presented in the SBR biomass at the beginning of this study. The preference of *Nitrospira* under higher IC conditions is also confirmed by the study carried out by Ma and collaborators (2015). In their work, neither *Nitrobacter* nor *Nitrospira* concentration increased under the imposition of IC limitation (pH at 7.5 ± 0.03), but after a recovering strategy that increased influent IC concentrations, only *Nitrospira* abundance rose. This IC niche differentiation might be related to the different metabolic ways of both cited genera to fixate CO_2. Whereas *Nitrobacter* fix carbon dioxide by the Calvin-Benson-Bassham pathway (Kim et al., 2012; Pérez et al., 2015), enzymes found by metagenomic studies indicated that *Nitrospira* perform the reductive tricarboxylic acid cycle (TCA) pathway (Koch et al., 2015). Further studies are needed to address this issue.

Regarding FNA effects on nitrifying community, it has been stated that NOB is more sensitive to FNA effects than AOB (Anthonisen et al., 1976; Vadivelu et al., 2007). Once nitrite accumulation started in this study, higher FNA concentration was achieved as well (Figure 7.3C and D). Complete NOB activity inhibition was reported at 0.026 – 0.22 mg HNO_2- N·L^{-1} (Zhou et al., 2011), but *Nitrospira* and *Nitrobacter* inhibition threshold is also different. *Nitrospira* is far more sensitive to FNA concentration lower than 0.03 mg HNO_2- N·L^{-1}, while *Nitrobacter* inhibition occurs at higher FNA levels (Blackburne et al., 2007). NOB repression in SBR was attributed to FNA concentration in the range of 0.02 – 0.06 mg HNO_2- N·L^{-1} (Pedrouso et al., 2017) and from 0.011 to 0.07 mg HNO_2- N·L^{-1} (Wei et al., 2014), SBR operated at mainstream (16 °C and influent ammonium of 50 mg NH_4^+-N·L^{-1}) and sidestream conditions (24 – 28 °C and influent ammonium of 600 mg NH_4^+-N·L^{-1}), respectively. However, FNA effects on nitratation were minor in our study. Nitrate production and respective decrease in the nitrite accumulation rate were observed when FNA higher than 0.014 mg HNO_2- N·L^{-1} was reached in the cycle monitored in period III (Figure 7.3D), but it is important to highlight that DO concentrations also rose and might have weakened FNA effects. Nevertheless, long-term operation would be required to elucidate if NOB could be recovered by controlling pH at higher set-point and maintaining lower FNA, but keeping IC limited.

7.4.3. Low DO facilitated stable NOB suppression under IC limitation

Nitrite accumulation was quite unstable in period III (Figure 7.2C), and therefore, rNO_2^- was enhanced when DO tended to higher values towards the end of the operational cycle (period III, Figure 7.3). However, the reduction of aeration phase and the increase of ALR allowed controlling DO at lower levels and it adjusted rNH_4^+ and rNO_2^-, which became rather constant (Figure 7.5B). By controlling bulk liquid DO at lower levels, oxygen limitation is achieved in the granule, and it influences the competition between AOB and NOB due to their affinity for substrates. Moreover, AOB may have benefited from the residual ammonium and low DO kept in the bulk liquid, which resulted in a ratio DO:NH_4^+ that potentiated ammonium oxidation rates in the outer layer of the granule, as it was previously described in other works (Isanta et al., 2015; Poot et al., 2016). The maintenance of ammonium concentration between 10 and 20 mg NH_4^+-N·L^{-1}, together with sludge retention time of 4.2 d, allowed to enhanced the growth of fast growing r-AOB, while NOB activity was repressed (Wu et al., 2016). It is important to highlight that low DO was effective in controlling nitratation only by imposing IC limitation. In periods I and II, without IC restriction, regardless of the ammonium concentration in the bulk liquid, oxygen limitation had a worthless role on suppressing NOB activity (Figure 7.2). Indeed, these results also reinforce previous reports that have pointed out the drawback in achieving NOB suppression based exclusively on oxygen kinetics (Akaboci et al., 2018; Gilbert et al., 2014b; Miao et al., 2016).

7.5. Conclusions

The effects of IC availability were studied in a granular SBR operated for PN at mainstream conditions. By decreasing the influent IC concentration to a NH_4^+:IC ratio of 0.73 ± 0.03, nitrite build-up was reached, while AOB activity was not negatively affected. The buffer capacity of the system was consequently lowered and resulted in pH values of 5.81 ± 0.30, which appears to be a key factor leading to NOB activity suppression. DO values (below 0.5 mg O_2·L^{-1} for almost all the aerated phase), also favored rNO_2^- control under IC limitation. The ratio $rNO_2^-{}_{max}$/$rNH_4^+{}_{max}$ dropped from 0.92 to 0.04.

Chapter 8.

General discussion

The need for improving resources sustainability is driving a change in the concept of wastewater treatment processes from residues removal to resource recovery. In this context, anammox process has arisen as an alternative to decrease WWTP energy consumption close to energy-neutral or even net energy-producing facilities. This would be achieved by implementing anammox in the mainstream line for nitrogen removal, allowing a complete valorization of the sewage organic matter through biogas (Larsen, 2015; Xu et al., 2015). As the interest on anammox at mainstream conditions (mainly characterized by low N concentration and seasonal temperature variations) increases, knowledge on how to deal with the challenges presented is needed. In this thesis, operational strategies for mainstream PNA have been explored aiming at obtaining key operational parameters that could strengthen PNA operation stability in such conditions. The research focused on dealing with NOB activity abatement and stable nitrogen removal under temperature variation. A summary of the conditions studied in the framework of this thesis is presented in Table 8.1.

Table 8.1. Brief of the conditions assessed in this study

	Chapters 3 and 4	Chapter 5	Chapter 6	Chapter 7
Reactor configuration	SBR	SBR	Plug-flow	SBR
Mixed liquor DO concentration	No accumulation of DO in the bulk liquid	No accumulation of DO in the bulk liquid	0.10 to 0.15 mg $O_2 \cdot L^{-1}$	From no accumulated DO in the bulk liquid to 7.5 mg $O_2 \cdot L^{-1}$
Temperature	25 ºC	25 and 15 ºC	Between 17 and 27 ºC	15 ºC
Wastewater	Synthetic and real sewage	Synthetic	Synthetic	Synthetic
Limitation of IC	No	No	No	Yes

Each result chapter already contains a discussion of the results obtained. In this general discussion, we present the main contribution of this Thesis with respect to operational strategies for NOB control, reactor stability at low temperature and the main characteristics of the microbial community subjected to several operating conditions.

8.1. Discussing key parameters for stable PNA in side and mainstream application

8.1.1. Dissolved oxygen as a strategy to control NOB activity

Due to the difficulty in applying the NOB control strategies that are commonly used in sidestream to mainstream, nitratation repression is one of the major challenges to be assessed for successful mainstream PNA performance in urban wastewater treatment. So far, DO has been the most frequently used key parameter. Earlier studies at mainstream PNA (published at the beginning of this PhD study) had indicated different outcomes about the role of DO on the suppression of nitratation. By one side, low bulk DO was showed suitable to control nitratation (Lotti et al., 2014a; Pérez et al., 2014), but on the other side several studies reported that NOB could adapt to low oxygen availability and become competitive at these conditions, impeding achieving efficient nitrate production suppression (Gilbert et al., 2014b; Wett et al., 2013). This study attempted to answer whether bulk liquid DO concentration could be in fact used as nitratation control strategy at mainstream conditions, by pushing DO availability limitation a step forward and operate the system at extremely low bulk liquid DO (DO concentration under the minimum detection level of the probe, except in chapter 6) and assess the microbial community response from low to higher DO concentrations (Chapters 6 and 7).

The main strategy explored, based on controlling the ratio between oxygen transfer rate according to ammonium loading rate, was assessed under both sidestream and mainstream conditions, at 25 ^{o}C and 15 ^{o}C. Total NOB suppression was sustained during the whole operational period at sidestream (more than 100 days; Figure 1 in Chapter 3), being the nitrite consumed by AnAOB. This strategy would be feasible in full-scale PNA installations, where nitrate production by NOB is still a common problem faced by operators (Lackner et al., 2014). While operating with high-strength nitrogen wastewater, total NOB activity suppression by low DO availability is assisted by the resulting FA levels (FA up to 6.2 mg NH_3-N·L^{-1} during sidestream operation, average concentration of 2.01 ± 1.19 mg NH_3-N·L^{-1}; Figure 1B in Chapter 3). Once FA support on NOB suppression was removed by decreasing influent ammonium concentration to sewage levels, absolute NOB suppression could not be maintained by DO alone. However, only negligible NOB activity was observed for about 80 days (microbial community adaptation period), and NOB activity remained limited afterwards (Figure 2 in Chapter 3). The results prove that bulk liquid DO is an effective parameter to control NOB activity and prevent nitrate build up in granular PNA-SBR processes, when utilizing either side or mainstream operation.

Low bulk liquid residual DO impact was further investigated in a plug-flow reactor with aerobic and anoxic chambers, operated at mainstream conditions and with continuous regime of feed and aeration (Figure 6.2B). This reactor configuration reflects the common current activated sludge processes in urban WWTP, and therefore, can be used as an assessment for the possibility of converting the current nitrogen removal into autotrophic nitrogen removal. The results showed that stable partial NOB repression, similar to the results showed by the SBR, could also be obtained in a plug-flow reactor by applying stringent DO conditions.

The results obtained by this Thesis reinforce that NOB activity control at mainstream conditions cannot rely only on the bulk DO. The maintenance of micromolar DO did not hinder the oxidation of nitrite to nitrate, because limited nitrate production occurred. Nonetheless, the amount of nitrate produced by NOB, expressed as $NO_3^-{}_{produced}$:$NH_4^+{}_{removed}$ molar ratio, was around 0.4, while it is expected a ratio of 0.11 in case NOB activity is negligible (Figure 2A in Chapter 3). Those values were in the same range reported by earlier studies (Laureni et al., 2016; Lotti et al., 2015b; Malovanyy et al., 2015a). On the other hand, NOB activity limitation was not guaranteed at low temperature for the very long term in both SBR (Figure 1C in Chapter 5) and plug-flow reactor (Figure 6.3). These results contrast with modelling studies that have linked effective NOB suppression directly to low bulk DO (Hubaux et al., 2015; Pérez et al., 2014; Volcke et al., 2010). In practice, once *Nitrospira* sp. have low half-saturation constants for oxygen and nitrite, they are favored under low DO and nitrite availability (Liu and Wang, 2013), while in those models it assumed that AOB is more competitive under low DO.

Under extremely low bulk liquid DO concentration, DO gradient in granules and biofilm is minimized since oxygen is rapidly consumed in the surface layer. At low temperature, the low microbial activities result in low nitrite production rate by AOB and the nitrite diffusion into the inner parts of the biofilm slows down. In such conditions, even a very limited NOB population growing close to AOB represents a base-line nitrate production that has a significant impact on the total N-conversion balance, regardless of the volume ratio between aerobic/anoxic layers in biofilm. At higher temperatures (>20 ^{o}C) nitrite production rate increases and nitrite diffusion favors the balance of the anoxic zone (dominated by AnAOB) into the N-conversion balance in detriment of nitratation.

The specific N-conversion rates (i.e. mgN/gVSS) in the different biomass size aggregates is another key factor which influenced the overall reactor performance. The higher specific nitrite conversion rates by NOB were observed in small diameter aggregates (Figure 3 in Chapter 5, and Figure 6.5B) as it was expected, since the aerobic layer volume-fraction is higher compared to larger aggregates . Similar results were reported in other studies (Morales et al., 2016; Shi et al., 2016; Vlaeminck et al., 2010) and the use of sieving and cyclones have been proposed to out select the sludge with higher NOB activity (Han et al., 2016b; Wett et al., 2013). However, after long-term reactor operation at low temperature, both biomass from SBR and plug-flow reactor had low AnAOB activity in all the aggregates size analyzed and the application of a driven pressure to washout small aggregates would not probably bring any remarkable improvement in the suppression of NOB but biomass washout.

Regarding the aeration regime, intermittent aeration was applied to the SBR during the operation at 15 ^{o}C (Figure 2 in Chapter 5), and was effective to limit the high NOB activity achieved before. It took only 20 days to reduce the $NO_3^-{}_{produced}$:$NH_4^+{}_{removed}$ from 0.95 to

0.5. This achievement is comparable with other studies where intermittent aeration also decreased nitrate production by NOB (Ma et al., 2015; Miao et al., 2016; Trojanowicz et al., 2016). The low AnAOB rates achieved after prolonged operation at cold temperature required lower aerobic activity. As a result, SBR operational cycle had a longer anoxic phase than aerobic one. The ratio between the aerated and anoxic phase times was 1/56. Comparatively, in literature studies this ratio ranged from 1/3 to 1/6 (see Table 1.8). This reduced aerated phase might have affected AOB activity as well, because the relative abundance of *Nitrosomonas* followed the same trend of *Nitrospira*, being both decreased after intermittent aeration (Figure 5C in Chapter 5). Further studies would be necessary to assess the long anoxic phase impacts on AOB growth at mainstream and cold temperature conditions.

8.1.2. Low temperature impacts on mainstream PNA performance

The second challenge explored by this Thesis was how to achieve stable nitrogen removal in reactor operation at low temperature. It is known that cold temperature strongly affects microbial activity in nitritation-anammox systems (Gilbert et al., 2015; Lotti et al., 2015c). Temperature also controls nitrogen species equilibrium (Anthonisen et al., 1976) and all nitrogen species are below the inhibition threshold for the microbial populations involved in PNA when operating at mainstream conditions (Jin et al., 2012). Effective N removal in PNA systems is basically driven by a good equilibrium between AOB and AnAOB activities and the decrease of temperature due to seasonal variations could result in loosing this balance in favor to nitrite availability for NOB growth.

Several studies have reported one-stage PNA reactor operation at cold temperatures, down to 10 ^{o}C (Table 8.2)(Gilbert et al., 2014b; Laureni et al., 2016; Lotti et al., 2014a; Morales et al., 2016; Trojanowicz et al., 2016). In this Thesis, we assessed the effects of temperature reduction in a SBR, from 25 ^{o}C to 15 ^{o}C without biomass adaptation (Chapter 5), and also in a plug-flow reactor, with (uncontrolled) temperature variation ranging from 28 ^{o}C to 16 ^{o}C (Chapter 6).

Table 8.2. Summary of one-stage PNA performance under low temperature

Reactor configuration	Temperature (ºC)	NRR (mg N·L^{-1} d^{-1})	Reference
IFAS-MBBR	17.1	013.25	Trojanowicz et al. (2016)
MBBR	15	30	Laureni et al. (2016)
MBBR	10	15	Gilbert et al. (2014b)
SBR	10	100	Lotti et al. (2014a)
SBR	15	80 ± 20	Morales et al. (2016)
Plug-flow	13.4 ± 1.1	97 ± 0.016	Hoekstra et al. (2018)
SBR	15 (Period II) 15 (Period III)	151 ± 17.42 7.2 ± 1.7	Chapter 03
Plug-flow	16 - 20	≤ 12	Chapter 06

Analogous to previous studies, besides the overgrowth of NOB, AnAOB activity was also the limiting factor to achieve stable nitrogen removal. The nitrogen removal rate dramatically decreases at cold temperature (below 20 ºC). The volumetric removal rates achieved in our study were in line with the results obtained in other studies (Table 8.2). The long-term SBR operation at 15 ºC derived in a strong reduction of AnAOB activity (Chapter 5). Specific NRR of 44.9 ± 6.5 mg N·g VSS^{-1}·d^{-1} was obtained during the days following the temperature reduction to 15 ºC (from day 102 to 114), but after three-month operation at low temperature, the sNRR measured was 6.6 ± 1.4 mg N·g VSS^{-1}·d^{-1} (from day 170 to 179). After this long-term operation at 15 ºC, we experienced lower volumetric nitrogen turnover and efficiency removal, as well a reduction of volatile solids concentration. Similar results were also produced by operating the plug-flow reactor (Chapter 6), despite of seeding twice extra anammox sludge in the reactor during the experimental period.

All these results emphasize that during one-stage PNA operation in winter/cold conditions, higher anammox biomass concentration would be necessary to sustain the required N removal rates. Due to the difficulty in getting NOB suppression, less substrate becomes available for AnAOB to grow. At lab and pilot-scale, reactor re-inoculation with new seeding sludge has been carried out to sustain the N conversion rates (Han et al., 2016a; Hoekstra et al., 2018). However in a scenario where highly active AnAOB sludge is not available, the reduction of AnAOB biomass in the reactor is a concern that makes tough the accomplishment of effluent thresholds.

8.1.3. PNA microbial community adaptation to sidestream and mainstream conditions

The PNA microbial community was deeply studied in the SBR using FISH, qPCR and 16S rRNA sequencing (Chapter 3 to 5). It allowed to identify the taxa in the floc/granule-based biomass, and detect the changes in the main functional groups according to the operational conditions applied.

Concerning AOB populations, several lineages of betaproteobacterial ammonia-oxidizers are reported in literature studies on wastewater treatment processes, being *Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrosomonas marina* and *Nitrosococcus mobilis*-lineage the most common bacteria clusters identified in WWTP (Wagner et al., 2002). During the whole PNA-SBR operation, *Nitrosomonas* was the dominating AOB (Chapter 3 to 5). Despite the different operational conditions applied in this study during sidestream and mainstream operational periods (high and low strength N content, respectively), *Nitrosomonas* was not outcompeted by any other AOB group. *Nitrosomonas* has been also identified in full-scale sidestream PNA reactor (Gonzalez-Martinez et al., 2015b) and also in mainstream PNA operated at low temperature (Agrawal et al., 2017; Gilbert et al., 2014b). Moreover, the sudden temperature reduction from 25 ^{o}C to 15 ^{o}C did not cause any disturbance in nitritation, which resulted in nitrogen removal efficiency breakdown (Figure 1B in Chapter 5).

As mentioned, the bulk liquid DO limitation totally repressed NOB activity only at sidestream operation. During mainstream operating conditions, the results obtained on NOB population analysis were different for both reactors studied. All the techniques applied identified *Nitrospira* as the most competitive NOB under mainstream (Chapter 4), while a low *Nitrobacter* abundance could only be detected by FISH (Figure 4A in Chapter 3). The higher affinity for oxygen and nitrite of *Nitrospira* contributed for their selection instead of *Nitrobacter*. The better *Nitrospira* adaptation to limited substrate conditions is attributed to the fact that they might have evolved from microaerophilic or even from anaerobic ancestors (Lücker et al., 2010), which is in line with our operational strategy. Furthermore, *Nitrospira* activity was not influenced by FA, because concentration values were lower than the reported inhibition levels (Blackburne et al., 2007).

Five anammox candidates taxa have been identified so far in the variety of studies. In this work the anammox community was always dominated by *Ca.* Kuenenia, both in sidestream and mainstream operational periods, although a negligible abundance of *Ca.* Brocadia was detected by 16S rRNA (Figure 4.4A, and Figure 5C in Chapter 5). The prevalence by *Ca.* Kuenenia is related to their capability to grow under high salinity environment, because the sludge initially used to seed the reactors came from pilot scale reactors fed with synthetic mineral medium or landfill leachates, both containing more than 10 g NaCl·L^{-1} (Ruscalleda, 2011). *Ca.* Kuenenia had been reported to be more adapted to saline wastewater and tolerate salt concentrations around 29 g NaCl·L^{-1} (Kartal et al., 2006).

It is not clear how temperature determines the niche differentiation on anammox predominance. *Ca.* Brocadia have been the genus often reported at mainstream conditions and low temperature (Gilbert et al., 2014b; Laureni et al., 2016; T. Lotti et al., 2014a; Persson et al., 2016), but is also the most common genus in lab-scale studies. In our case, the temperature change from 25 ^{o}C to 15 ^{o}C did not switch the dominant anammox taxon and *Ca.* Kuenenia remained with higher abundance in this study, even though a minimal *Ca.* Brocadia presence was detected by 16S rRNA analysis showing that they could be enriched if they were more adapted to low temperature. Interestingly, another study reported the switch of anammox dominance from *Ca.*

Brocadia to *Ca.* Kuenenia by reducing gradually the temperature from 30 ^{o}C to 10 ^{o}C (De Cocker et al., 2018).

There is a lack of knowledge regarding the selection of the anammox genus driven by temperature. A recent study used metaproteomics to examine proteome modulation patterns of different anammox genera at different temperatures and evidenced that at 15 ^{o}C *Ca.* Kuenenia suffer energy limitation and severe stress to a higher extent than *Ca.* Brocadia and Jettenia (Lin et al., 2018). It was suggested that those alterations may explain lower conversion rates, which might result in lower growth rate. This points to important issues to be assessed which could help to overcome the low temperature limitations for anammox application at mainstream.

The bacterial community revealed in this Thesis was not limited to nitrifying and anammox taxa, but several other groups classified in the phyla *Chloroflexi*, *Bacteroidetes*, *Proteobacteria*, *Ignavibacteriae* and *Acidobacteria*. It is important to highlight that those phyla are usually reported in anammox-based reactors (Agrawal et al., 2017; Cao et al., 2016; Persson et al., 2016). The phylum *Proteobacteria* involves numerous denitrifying bacteria. In our study, sequences could be classified to the genus *Denitratisoma*, with an increase in the abundance during the operation at mainstream conditions (Figure 4.4C). Others sequences were able to be classified only at order level in *Xanthomonadales*, *Rhodobacterales* and *Rhizobiales*, which also have bacteria genera able to denitrify.

With respect to the potential role of the HB within *Chloroflexi*, *Bacteroidetes*, and *Ignavibacteriae*, studies have used metagenomics aiming at exploring their symbiotic interactions with AnAOB (Bhattacharjee et al., 2017; Lawson et al., 2017; Speth et al., 2016; Zhao et al., 2018b). It was found out that bacteria affiliated in those phyla are potentially implicated in nitrate reduction to nitrite, by using organic matter from influent wastewater, dead organic matter or even consuming extracellular products excreted by autotrophs (Lawson et al., 2017; Speth et al., 2016). Consequently, the nitrite produced by dissimilatory nitrate reduction (DNRA) processes could be further used by AnAOB, increasing the nitrogen removal (Zhao et al., 2018b). Furthermore, in granular-based system such as anammox reactors, higher sludge retention is achieved, which supports higher degree of bacterial decay. Hence, it contributes to obtaining higher abundance of *Chloroflexi* and *Ignavibacteriae* (Bhattacharjee et al., 2017). Regarding to *Bacteroidetes* phylum, it has also been shown that they grow attached to the outer layer of sludge granules, and this characteristics assists granule structure (Fernandez-Gomez et al., 2013).

Once activity in a granule is determined by the spatial distribution of microorganisms within the aggregate and the diffusion of substrates from the bulk liquid towards the inner core (or vice versa), the impact of extremely low bulk DO and temperature influence on microbial distribution, mainly nitrifiers and AnAOB, should be further investigated. Here, we could assess only overall microbial community composition, but not a deep analysis of its structure. However, activity assays indicated a proportional prevalence of nitrifiers in small aggregates, while AnAOB dominated (in terms of relative abundance) thicker granules. Moreover, special attention should be paid to investigate the distribution of NOB and AnAOB in the granule, how their location influences the accessibility to the nitrite produced by AOB, and what is the resulting impact to the competition between both AnAOB and NOB populations. The presence of ammonia-oxidizing archaea (AOA) under low bulk DO in one-stage PNA and how it contributed to ammonium oxidation needs to be elucidated as well.

8.1.4. Potential of inorganic carbon availability to assist NOB suppression

Nitrogen removal during the operation at mainstream conditions was impaired mainly by NOB activity. As stringent DO conditions were not sufficient to prevent NOB growth in the SBR after long-term operation at 15 ^oC, AnAOB activity was strongly affected and reduced to values around 7.5 mg $N \cdot gVSS^{-1} \cdot d^{-1}$ (Chapter 5). In order to explore alternative parameters to assist NOB out-competition in mainstream (as FA in sidestream), the effect of IC availability on microbial dynamics was assessed in Chapter 7.

By lowering influent IC concentration down to a NH_4^+:IC ratio of 0.73 ± 0.03, NOB activity was affected and nitrite accumulation was restored. An operational cycle monitored during period III, allowed to verify that in the end of the aerated phase, the rNH_4^+ dropped, whereas the rNO_2^- started been enhanced. Simultaneously, DO increased up to 7 mg $O_2 \cdot L^{-1}$. The operational cycle was modified, by reducing the aerated phase, and stable nitrite accumulation ratio over 80% was reached, while DO values ranged below 0.5 mg $O_2 \cdot L^{-1}$. The feasibility of achieving stable nitratation suppression assisted by IC limitation was showed for the first time.

It is known that the lack of IC in the reject water causes a decrease of the AOB activity in sidestream treatment (Guisasola et al., 2007; Wett and Rauch, 2003). Reject wastewater presents a molar ratio HCO_3^-:NH_4^+ around 1 (Dosta et al., 2015) and, therefore, full nitrification requires the addition of bicarbonate to provide enough IC for growth of autotrophs and to neutralize the hydrogen ions produced. However, in the mainstream line, typical sewage contains enough IC for full nitrification, with an average HCO_3^-:NH_4^+ ratio of around 2 or extrapolated NH_4^+:IC of 0.5 (Von Sperling, 2015). Hence, this strategy could be feasible in scenarios where sewage contains low IC (limited bicarbonate availability), or punctually applied (lowering influent IC) to recover the system after NOB overgrowth.

8.2. One versus two-stage PNA at mainstream: an open question

Several operational conditions were investigated in the Thesis with the main goal of providing new insights for the implementation of anammox-based N removal at the mainstream line of urban wastewater treatment. The outcomes observed in both reactor configurations (SBR and plug-flow) can be discussed to assess whether one or two-stage PNA would be the preferable scheme for mainstream anammox implementation.

The first issue to be analyzed is related to the NOB suppression, which can be referred by the molar ratio $NO_{3\,produced}^-$:$NH_{4\,removed}^+$. From the obtained results presented above, it has been proved that the strategy for operating one-stage at (extremely) low bulk liquid DO can limit NOB activity at a reasonable extent allowing sufficient N removal efficiency, but not totally suppress it. As it was already discussed above in the section 8.1.1, the similar molar ratio $NO_3^-{}_{produced}$:$NH_4^+{}_{removed}$ obtained between our study and the reported data in literature for one-stage PNA was not strongly dependent on the operated DO set-point. In comparison to the two-stage PNA, where nitritation is carried out in the first stage, the amount of nitrate produced by NOB in our study was in the same range of the molar ratio calculated based on data reported in previous studies (Pedrouso et al., 2017; Poot et al., 2016). However, when it comes to long-term operation at lower temperatures, one-stage PNA was not able to sustain NOB activity repression, while temperatures lower than 15 ^oC did not prejudice nitritation control in other studies (Isanta et al., 2015; Reino et al., 2016). Furthermore, specifically for PN in granular systems, the maintenance of residual ammonium in the bulk liquid facilitates the creation of a stratification in the granule, favoring AOB to grow in the outer surface, and therefore increasing ammonium oxidation rates (Poot et al., 2016). It was showed in that study that the consumption of oxygen in the external layer limited NOB in the inner granule. On the other hand this Thesis showed that stable nitrite accumulation when the SBR was operated by limiting the influent IC, and AOB activity was enhanced at 15 ^oC, reaching values for $rNH_4^+{}_{max}$ higher than 24 mg N·g $VSS^{-1}·d^{-1}$ (Figure 7.4).

The second issue is related to AnAOB at low temperature. While AnAOB activity could be maintained at 25 ^oC, a strong activity reduction occurred at 15 ^oC, with a drastic reduction of the biomass growth and resulted in a decrease of VSS concentration in both SBR and plug-flow. The main drawback obtained by this study (in concordance with literature, see Table 8.2) is the considerably low removal rates during cold temperature operation. These rates were mainly due to low SAA. Some studies present higher SAA at cold temperature, basically sustained on new reactor inoculation with active biomass (Hoekstra et al., 2018). Comparatively, slightly better removal rates have been obtained when AnAOB is cultivated in anoxic reactors, applied as the second-stage (Laureni et al., 2015; Lotti et al., 2014b; Reino et al., 2018). The better conversion rates might be related to the optimal anoxic condition which can be achieved for anammox. An upflow anammox sludge bed reactor (UAnSB) operated at 11 ^{o}C, showed removal rates of 1.2 ± 0.5 g $N \cdot L^{-1} \cdot d^{-1}$ at long term (Reino et al., 2018). Lotti et al. (2014b) proved the feasibility of growing AnAOB at 10 ^{o}C in an upflow fluidized granular sludge reactor fed on pre-treated sewage (post COD removal), obtaining an NRR and biomass specific NRR of 0.43 g N L^{-1} d^{-1} and 0.08 g N g VSS^{-1} d^{-1}, respectively. Interestingly, the biomass concentration increased from 3.6 to 6.7 g $VSS \cdot L^{-1}$ during their work. Hence, our results combined with literature reports show that results are similar in one or two stage PNA configurations in terms of specific biomass activity, but reactor designs promoting high biomass retention will be necessary to avoid biomass loss during low activity periods (winter). This implies that current conventional WWTP will require important modifications in reactor configuration to implement mainstream PNA.

By having nitritation and anammox processes in separated reactors, the operation could be performed in order to optimize both processes according to their requirements, as well as preventing process failures. In the case of the anammox process, AnAOB would be protected in the anoxic reactor, from failures in the A-stage (organic matter removal), as well NOB would not compete for nitrite. Thus, in the presence of both ammonium and nitrite, AnAOB would have better conditions to grow, enhancing the anammox process.

Chapter 9.

Conclusions

Operational strategies for anammox-based processes applied in the mainstream line of urban WWTP were investigated in this PhD Thesis.

The main conclusions can be summarized as it follows:

- *Establishment of an operational strategy for operating one-stage PNA reactors*: The strategy based on controlling the oxygen transfer over ammonium loading rate (OTR/ALR) was assessed in a one-stage PNA-SBR operated at sidestream and mainstream conditions. Although it achieved extremely low bulk-liquid DO (µM concentration) the nitritation potential was demonstrated, reaching an average NRR of 0.34 ± 0.05 and 0.37 ± 0.07 kg $N \cdot m^{-3} \cdot d^{-1}$ at sidestream (period I) and mainstream conditions (period II), respectively (Chapter 3). Furthermore, those removal rates were in the same range of values reported in literature, obtained by PNA reactors operated at higher DO levels. This fine control of the supplied oxygen was also an important feature to match nitritation and anammox activities. It avoided nitrite accumulation, mainly at sidestream conditions because higher nitrite concentration could lead to reactor instability due to anammox inhibition;
- *Improvements in understanding low DO role on NOB activity repression*: low DO was effective in suppressing NOB activity at sidestream conditions, but limited suppression was achieved during mainstream conditions. At sidestream conditions, the molar ratio $NO_3^-{}_{produced}$:$NH_4^+{}_{removed}$ was 0.10 ± 0.02, while at mainstream conditions (25 ^oC) it was increased to values higher than 0.20 (Chapter 3). The values of nitrate production rose even more in the PNA-SBR at temperature of 15 ^oC (Chapter 5). These results showed that NOB repression could be robust when it was assisted by: (i) FA concentration, because of an average of 2.01 ± 1.19 mg NH_3-$N \cdot L^{-1}$ during sidestream making *Nitrospira* growth difficult; and (ii) AnAOB activity that was maintained at 25 ^oC, with nitrite consumption rate by AnAOB higher than by NOB. As the temperature was reduced and AnAOB activity was negatively impacted by this low temperature of 15 ^oC, NOB presented an overgrowth in the PNA-SBR. Low DO and intermittent

aeration were also feasible to control NOB rates at low-temperature, however an adaptation of NOB was experienced on long-term operation;

- *Identifying process parameters that could support NOB repression at mainstream conditions*: Activity tests carried out at mainstream conditions pointed towards granule size contribution to obtaining higher nitrate production by NOB, mainly at low temperature operation. In the tests performed for biomass from both PNA-SBR (Chapter 5) and plug-flow reactor (Chapter 6), NOB oxidation rates were expressively superior to AnAOB rates for biomass with average diameter lower than 500 µm. On the other hand, despite increasing average diameter to higher than 500 µm, NOB also had higher rates, but the difference to AnAOB was reduced, indicating smaller aggregates were able to harbor higher nitrate production, which dropped nitrogen removal capacity. In summary, besides low DO, kinetic out selection of floccular and smaller granules would impact favorably NOB suppression. The second parameter which showed important influence on NOB activity was the IC (Chapter 7). Nitrite short-cut was achieved by operating the reactor with influent NH_4^+:IC ratios of 0.73 ± 0.03. After long operation with limiting IC, the maximum conversion rates of NOB dropped, while AOB activity was enhanced. These results showed the potential of achieving NOB suppression for wastewater with low alkalinity;
- *Anammox activity enhancement at low temperature:* Low temperature effects on AnAOB activity were studied in two reactors (Chapters 5 and 6). In the PNA-SBR, the temperature change from 25 °C to 15 °C without acclimating the biomass did not cause any preliminary operation instability, and it was possible to adjust the NLR according to the new removal capacity. However a strong AnAOB activity reduction at long-term operation until reaching an activity lower than 10 mg N·g VSS^{-1}·d^{-1} was experienced. This lower AnAOB activity along MLVSS reduction negatively impacted the removal rates, and lower values were achieved. In the plug-flow reactor, the removal rates were also limited by the low AnAOB activity. In both reactors, it was difficult to enhance AnAOB activity

due to better adaptation of NOB to the conditions obtained at low-temperature mainstream;

- *Microbial community characterization:* The effects of the operation conditions on microbial community were investigated by using high-throughput techniques (Chapters 4 and 5). The bacterial community presented higher diversity and richness at mainstream conditions. The microbial core harbored higher abundance of *Planctomycetes*, *Proteobacteria*, *Chloroflexi* and *Bacteroidetes* being similar to other one-stage PNA reactors. Some of the taxa identified has been exhaustively studied in recent years due to their heterotrophic metabolism and syntrophic link to AnAOB. *Nitrosomonas* and *Ca*. Kuenenia were the taxa related to AOB and AnAOB throughout all the study. The temperature reduction did not influence on AnAOB niche, because the main AnAOB was not shifted. Lower oxygen and nitrite concentrations achieved at mainstream conditions favored *Nitrospira* growth, in accordance to previous studies that have related this NOB genus preference and better affinity to low substrates availability.

Regarding the strategies investigated in this Thesis, further studies should be conducted:

- To assess the potential of N_2O emissions due to extremely low DO and IC limitation, once both factors are known to affect the N_2O production rates. This would allow a deeper understanding of the carbon footprint of PNA systems;
- To analyze microbial community (nitrifying and anammox) stratification achieved in the granule due to the maintenance of oxygen at µM concentrations aiming to obtain a better understanding of the strategy for NOB suppression;
- To explore archaea and comammox participation in nitrogen turnover;
- To investigate microbial communities and ecology by using metagenomic and metabolomics, in order to provide deeper insights to the bacterial response to IC limitation, low DO and temperature.

Chapter 10.

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Appendixes

The following pages contain support information to preceding chapters.

Appendix A: Supporting information for Chapter 3 – Effects of extremely low bull liquid DO on autotrophic nitrogen removal performance and NOB suppression in side- and mainstream one-stage PNA.

Appendix B: Supporting information for Chapter 4 – Bacterial community succession in a nitritation-anammox reactor face to the transition from sidestream to mainstream conditions.

Appendix C: Supporting information for Chapter 5 – Assessment of operational conditions towards mainstream partial nitritation-anammox stability at moderate to low temperature: Reactor performance and bacterial community.

Appendix D: Supporting information for Chapter 7 – Limitation of inorganic carbon suppresses nitrite-oxidizing bacteria activity in a granular SBR at mainstream conditions.

Appendix A

SUPPORTING INFORMATION

Effects of extremely low bulk liquid DO on autotrophic nitrogen removal performance and NOB suppression in side- and mainstream one-stage PNA

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SI1. Calculation procedures

SI1.1. Nitrogen consumption rates

The nitrogen consumption rates were calculated by nitrogen mass balance according to reactor performance. The following mass balance equations were obtained for the nitrogen species:

$$\frac{\Delta NH_4^+}{HRT} + \alpha = -NH_{4\,AMX}^+ - NH_{4\,AOB}^+$$

$$\frac{\Delta NO_2^-}{HRT} + \beta = -NO_{2\,AMX}^- + NH_{4\,AOB}^+ - NO_{2\,NOB}^-$$

$$\frac{\Delta NO_3^-}{HRT} + \delta = NO_{3\,AMX}^- + NO_{2\,NOB}^-$$

where $\Delta NH_4^+, \Delta NO_2^-$ and ΔNO_3^- are the increment (EFF-INF) of NH_4^+, NO_2^- and NO_3^-, respectively (mg $N \cdot L^{-1}$); α, β and δ are the accumulation of NH_4^+, NO_2^- and NO_3^-, respectively (mg $N \cdot L^{-1} \cdot d^{-1}$); HRT is the hydraulic retention time (d); $NH_{4\,AMX}^+$ ammonium consumption rate by anammox (mg $N \cdot L^{-1} \cdot d^{-1}$); $NH_{4\,AOB}^+$ ammonium consumption rate by AOB (mg $N \cdot L^{-1} \cdot d^{-1}$); $NO_{2\,NOB}^-$ nitrite consumption rate by NOB (mg $N \cdot L^{-1} \cdot d^{-1}$); $NO_{2\,AMX}^-$ nitrite consumption rate by anammox (mg $N \cdot L^{-1} \cdot d^{-1}$); $NO_{3\,AMX}^-$ nitrate production rate by anammox (mg $N \cdot L^{-1} \cdot d^{-1}$).

From anammox process stoichiometry (Strous et al., 1998) is known that:

$$NO_{2\,AMX}^- = 1.32 \cdot NH_{4\,AMX}^+$$

$$NO_{3\,AMX}^- = 0.26 \cdot NH_{4\,AMX}^+$$

Changing in the variables above in the mass balance equations:

$$\frac{\Delta NH_4^+}{HRT} + \alpha = -NH_{4\,AMX}^+ - NH_{4\,AOB}^+$$

$$\frac{\Delta NO_2^-}{HRT} + \beta = -1.32 \cdot NH_{4\,AMX}^+ + NH_{4\,AOB}^+ - NO_{2\,NOB}^-$$

$$\frac{\Delta NO_3^-}{HRT} + \delta = 0.26 \cdot NH_{4\,AMX}^+ + NO_{2\,NOB}^-$$

Solving the mass balance equations, nitrogen consumption rates are obtained by:

$$NH_{4\,AOB}^+ = \frac{-1.06\left(\frac{\Delta NH_4^+}{HRT} + \alpha\right) + \left(\frac{\Delta NO_2^-}{HRT} + \beta\right) + \left(\frac{\Delta NO_3^-}{HRT} + \delta\right)}{2.06}$$

$$NO_{2\,NOB}^- = 1.32\left(\frac{\Delta NH_4^+}{HRT} + \alpha\right) - \left(\frac{\Delta NO_2^-}{HRT} + \beta\right) + 2.32\, NH_{4\,AOB}^+$$

$$NH_{4\,AMX}^+ = -\left(\frac{\Delta NH_4^+}{HRT} + \alpha\right) - NH_{4\,AOB}^+$$

$$NO_{2\,AMX}^- = 1.32\, NH_{4\,AMX}^+$$

$$NO_{3\,AMX}^- = 0.26\, NH_{4\,AMX}^+$$

SI1.2. Oxygen transfer rate coefficient

The oxygen transfer rate coefficient (k_La) was obtained through the mass balance for the DO in the bulk liquid reactor. The tests were performed for different air flow rates. The DO mass balance aerobic bioreactors can be represented by the following equation.

$$\frac{dDO}{dt} = k_L a_{O_2}(DO^* - DO) - OUR$$

where DO is the dissolved oxygen concentration (mg $O_2 \cdot L^{-1}$); DO^* is the saturation dissolved oxygen (mg $O_2 \cdot L^{-1}$); and OUR is the oxygen uptake rate (mg $O_2 \cdot L^{-1} \cdot h^{-1}$), which is obtained from the slope of the plot of DO concentration versus time, when air flow is stopped. The k_La tests were performed before reactor inoculation, with tap water and mineral medium, and after reactor inoculation. This was done to correct the effects of the biomass in the oxygen transfer, for the both working volume: 8 and 10 L.

SI1.3. Volumetric oxygen loading rate

The oxygen volumetric loading rate (L_{O_2}, kg·m^{-3}d^{-1}) was calculated by:

$$L_{O_2} = k_L a_{O_2} (DO^* - DO) R$$

where $k_L a_{O_2}$ is the volumetric oxygen transfer coefficient (d^{-1}); DO is the dissolved oxygen concentration (kg O_2·m^{-3}); DO^* is the saturation dissolved oxygen (kg O_2·m^{-3}) ; and R is the ratio between the time with the aeration in mode on and time with aeration in mode off.

SI1.4. Oxygen penetration depth

The oxygen penetration depth in the granules was calculated according to equation bellow, assuming zero-order substrate removal rates (Morgenroth, 2008):

$$\delta_{pd} = \sqrt{\frac{2 \times D_F \times C_{LF}}{k_{0.F} \times X_F}}$$

where δ_{pd} is the oxygen penetration depth (µm); D_F is the diffusivity coefficient for oxygen in water at 25 ºC, 2.1·10^{-4} m^2·d^{-1} (Morgenroth, 2008); X_F is the concentration of biomass in the granules, 93,333 g COD·m^{-3} (Volcke et al., 2010a); and $C_{LF}$is the oxygen concentration at the surface of the granule (as DO concentration in the bulk liquid was maintained below DO probe's detection level, DO was assumed as 6 µg $O_2 \cdot L^{-1}$). The total oxygen consumption ($k_{0.F}$, g $O_2 \cdot$g COD^{-1}) was determined considering the biomass composition as presented in the following equation:

$$k_{0.F} \cdot X_F = k_{0,O_2H} \cdot X_{H,F} + k_{0,O_2N} \cdot X_{N,F}$$

where k_{0,O_2H} is the zero-order constant of the heterotrophic biomass for oxygen, 7.2 g $O_2 \cdot$g $COD^{-1} \cdot d^{-1}$, and k_{0,O_2N} is the zero-order constant for the nitrifying biomass for oxygen, 18.8 g $O_2 \cdot$g $COD^{-1} \cdot d^{-1}$. $X_{H,F}$ and $X_{N,F}$ are the heterotrophic and nitrifying biomass concentrations, respectively (Morgenroth, 2008). The percentage of heterotrophic and nitrifying biomass was quantified by FISH analysis. It was fixed a percentage of 65% and 8% of nitrifying and heterotrophic biomass, respectively for sidestream conditions ($X_{N,F} = 60{,}666\ g\ COD \cdot m^{-3}\ and\ X_{H,F} = 7{,}466\ g\ COD \cdot m^{-3}$). At mainstream conditions, nitrifying and heterotrophic biomass represented 56% and 16%, respectively ($X_{N,F} = 52{,}266\ g\ COD \cdot m^{-3} and\ X_{H,F} = 14{,}933\ g\ COD \cdot m^{-3}$).

SI1.5. Anoxic and aerobic fraction of the granule

Assuming the granular biomass as form of sphere, the total volume is obtained by:

$$V_T = \frac{3}{4}\pi r^3$$

where V_T is the total volume (µm^3) and r is the radius of the granule (µm).

Assuming that oxygen penetration depth (δ_{pd}) is equal for all the surface of the granule:

$$r_{anx} = r - \delta_{pd}$$

$$V_t = V_{anx} + V_{aer}$$

where r_{anx} is the anoxic radius (µm), V_{anx} is the anoxic volume fraction (µm^3) and V_{aer} the aerobic volume fraction (µm^3).

Table SI1. Probes used for FISH analysis with different markers (Cy5, Cy3 and Fluos)

Probes	Target organism	Sequence (5'→3')	Marker
EUB338	Eubacteria	GCT GCC TCC CGT AGG AGT	Cy5
EUB338II	*Planctomyces* branch	GCA GCC ACC CGT AGG TGT	Cy5
EUB338III	*Verrucomicrobia*	GCT GCC ACC CGT AGG TGT	Cy5
NSO190	Ammonia oxidising β-proteobacteria	CGA TCC CCT GCT TTT CTC C	Fluos
NSO1225	Ammonia oxidising β-proteobacteria	CGC CAT TGT ATT ACG TGT GA	Fluos
NIT3	*Nitrobacter*	CCT GTG CTC CAT GCT CCG	Cy3
Ntspa662	*Nitrospira*-like organism	GGA ATT CCG CGC TCC TCT	Cy3
compNIT3	*Nitrobacter* competitors	CCT GTG CTC CAG GCT CCG	-
compNtspa663	*Nitrospira*-like organism competitors	GGA ATT CCG CTC TCC TCT	-
Amx-0820-a	Ca. Brocadia + Ca. Kuenenia	AAA ACC CCT CTA CTT AGT GCC	Cy3
Kst-0157-a	Ca. Kuenenia stuttgartiensis	GTT CCG ATT GCT CGA AAC	Fluos
Amx-0223-a	Ca. Brocadia anammoxidans	GAC ATT GAC CCC TCT CTG	Cy3

Table SI2. Summary of half-saturation constants values for substrate and oxygen

Group	K_S (mg N·L^{-1}) NH_4^+	K_S (mg N·L^{-1}) NO_2^-	K_O (mg O_2·L^{-1})	Type of system/biomass	Temperature (°C)	Reference
AOB						
Nitrosomonas	5.11±0.79	-	1.21±0.3	Suspended biomass	20	Park et al. (2004)[a]
	12.95±4.82	-	0.98±0.42	Suspended biomass	20	Park et al. (2004)[b]
	-	-	0.29	Suspended biomass	20	Liu and Wang (2015)
	1.4	-	0.0736	Granular biomass	30	Sliekers et al. (2005)[c]
NOB						
Nitrobacter	-	1.2±0.05	0.43±0.08	Suspended biomass	22±1	Blackburne et al. (2007)
	-	0.686 – 7.61	-	Suspended biomass	28	Nowka et al. (2015)
Nitrospira	-	0.14	-	Biofilm-fluidized biofilm reactor	30	Schramm et al. (1999)
	-	0.9±0.07	0.54±0.14	Suspended biomass	22±1	Blackburne et al. (2007)
	-		0.08	Suspended biomass	20	Liu and Wang (2015)
	-	0.21	0.0416	Granular biomass	30	Sliekers et al. (2005)[c]
	-	0.126 – 0.37	-	Suspended biomass	37	Nowka et al. (2015)
Anammox						
Brocadia	-	0.035	-	Suspended biomass	30	Lotti et al. (2014)
	8.96±1.82 -	4.9±1.26	-	Granular biomass	30±0.1	Puyol et al. (2013)
	7.42±0.7	5.18±0.56	-	Floccular biomass	30±0.1	
Kuenenia	36.75	0.657	-	Granular biomass	35	Chen et al. (2011)
	-	0.0028-0.0042	-	Suspended biomass	38	van der Star et al. (2008)

[a]Low DO enrichement

[b]High DO enrichement

[c]NOB population was named as a mixed culture, however *Nitrospira* sp. was 5 times higher than *Nitrobacter* sp.

Table SI3. Nitritation and nitratation rates determination

	mg N·L^{-1}·min^{-1}	mg N·L^{-1}·d^{-1}	mg N· gVSS^{-1}·d^{-1}
rNH_4^+	0.2720	31.86	764.71
rNO_2^-	0.0023	0.27	6.47

The assay was performed on the operational day 100 (Sidestream condition). The biomass was collected from the reactor and washed 3 in order to remove substrates. After, it was placed in the 4 L fermenter. The assay was done by using synthetic medium, which the composition of macro and micronutrients were the similar as the normal operation. Oxygen was controlled around 4 mg·L^{-1}. Previous to substrate pulse feed, the biomass stayed under aerobic conditions for 1 hour. 100 mg N-NH_4^+ and 1.2 g $NaHCO_3$·L^{-1} were added (NH_4^+:2HCO_3) later. pH was not controlled and it decreased from 7.4 to 7.15 during the assay.

Table SI4. Average COD and TKN of real wastewater used in Phase III

	COD_T (mg O_2·L^{-1})	COD_S (mg O_2·L^{-1})	TKN (mg N·L^{-1})
Influent	53.7±15.5	32.8±12.8	79.9±7.6
Effluent	42.6±14.3	31.5±11.9	24.9±9.5

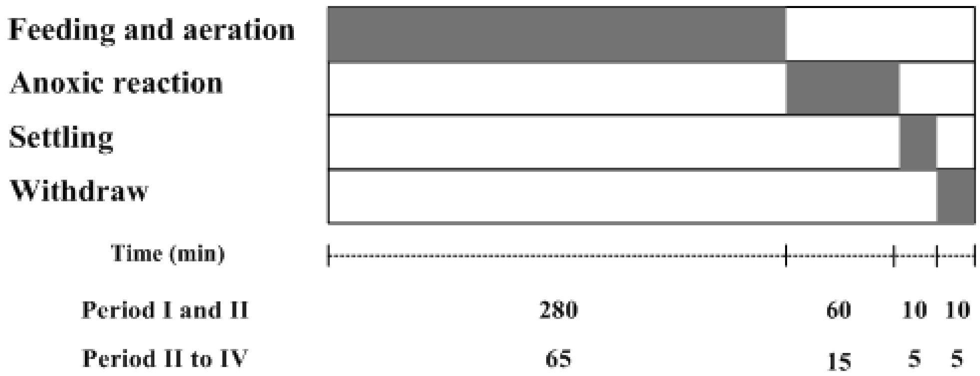

Figure SI1. Cycle phases configuration at both sidestream and mainstream conditions.

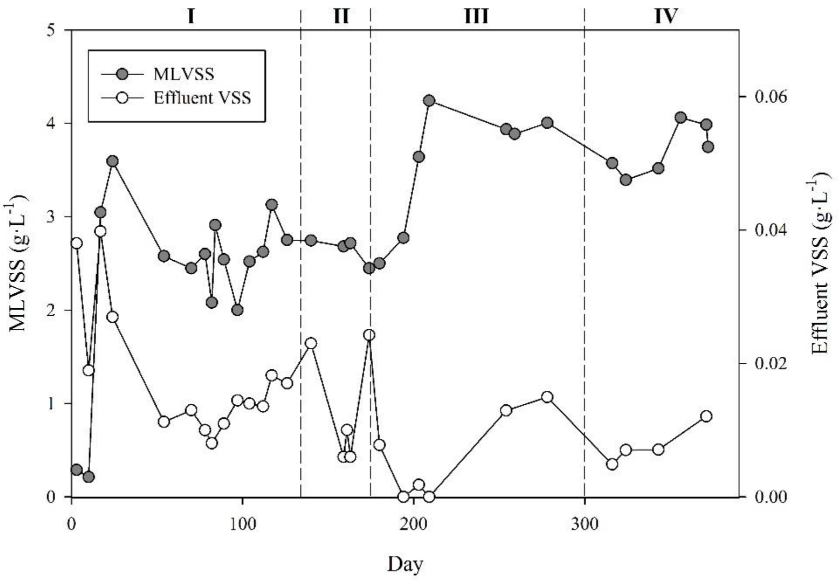

Figure SI2. Volatile solids concentration in the mixed liquor and effluent.

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Appendix B

SUPPORTING INFORMATION

Changes in bacterial community in a nitritation-anammox reactor face to the transition from sidestream to mainstream conditions

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Table S1. Synthetic medium composition

Compound	Concentration (per liter of tap water)
$FeSO_4 \cdot 7H_2O$	0.0125 $g \cdot L^{-1}$
$EDTA \cdot 2H_2O$	0.0125 $g \cdot L^{-1}$
$MgSO_4 \cdot 7H_2O$	0.2 $g \cdot L^{-1}$
KH_2PO_4	0.008 $g \cdot L^{-1}$
$CaCl_2 \cdot 2H_2O$	0.3 $g \cdot L^{-1}$
$(NH_4^+)_2SO_4$	Variable
$NaHCO_3$	Variable
Trace element solution*	1.25 $mL \cdot L^{-1}$

* The trace element solution was prepared according to (Graaf et al., 1996).

Table S2. qPCR probes and thermal profile

Gene	Primer pair	Sequence (5'-3')	Fragment (bp)	Thermal profile	Reference
16s rRNA	341f 534r	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGGCA	194	95 °C; 10 sec, denaturation 95 °C; 10 sec, annealing 72 °C; 10 sec, extension and fluorescence	Hallin et al. (2009)
amoA (AOB)	amoA-1F/ amoA-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	491	**94 °C; 30 sec, denaturation** **55 °C; 45 sec, annealing** 72 °C; 35 sec extension and fluorescence	Hallin et al. (2009)
16s rRNA Anammox (All anammox bacteria)	Amx 694F/ Amx 960R	GGGGAGAGTGGAACTTCGG GCTCGCACAAGCGGTGGAGC	266	95 °C; 30 sec, denaturation 56 °C; 15 sec, annealing 72 °C; 35 sec extension and fluorescence	Ni et al. (2010)
16s RNA gente (*Nitrospira* - NOB)	Nspra 675f/ Nspra 746r	GCGGTGAAATGCGTAGAKATCG TCAGCGTCAGRWAYGTTCCAGAG	71	94 °C; 30 sec, denaturation 50 °C; 45 sec, annealing 72 °C; 60 sec extension and fluorescence	Pellicer-Nàcher et al. (2014)
16S rRNA gene (*Nitrobacter* - NOB)	FGPS872f/ FGPS1269r	CTAAAACTCAAAGGAATTGA TTTTTTGAGATTTGCTAG	397	94 °C; 30 sec, denaturation 50 °C; 45 sec, annealing 72 °C; 60 sec extension and fluorescence	Cao et al. (2016)

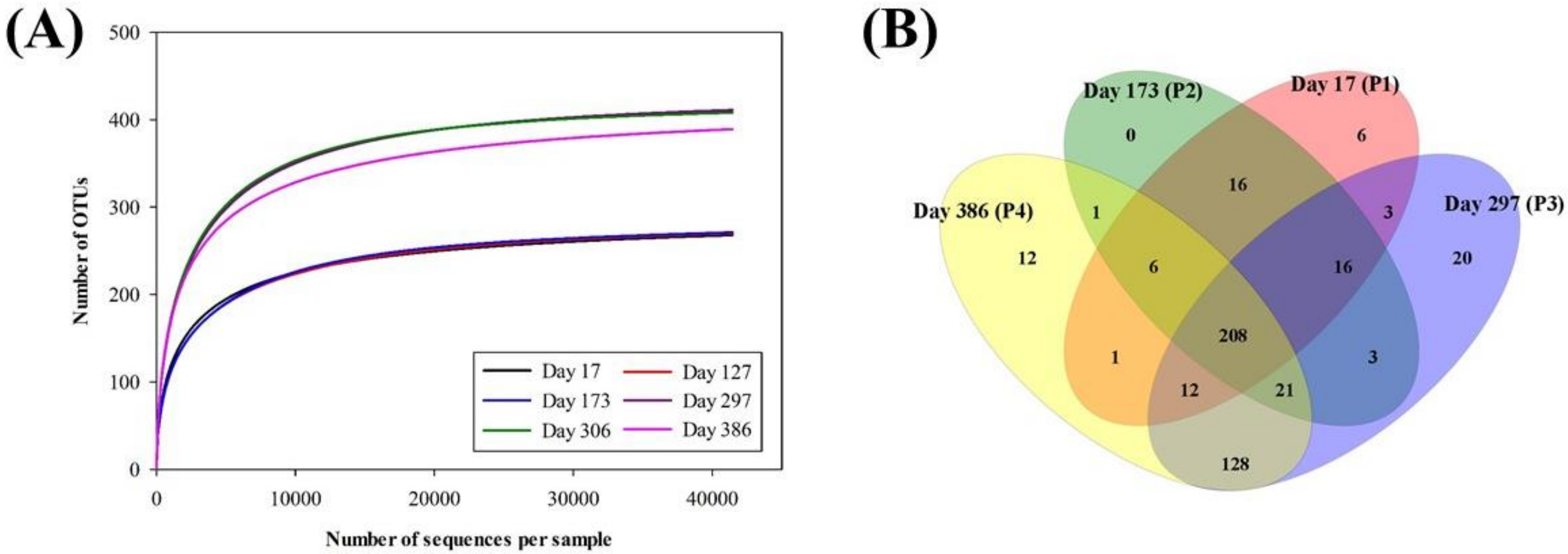

Figure S1. (A) Rarefaction analysis of the samples throughout reactor operation. Rarefaction curves of OTUs with a threshold of 97 % sequence similarity; (B) Venn diagram of the OTUs distributed among operational days 17 (Period 1, P1), 173 (Period 2, P2), 297 (Period 3, P3) and 386 (Period 4; P4).

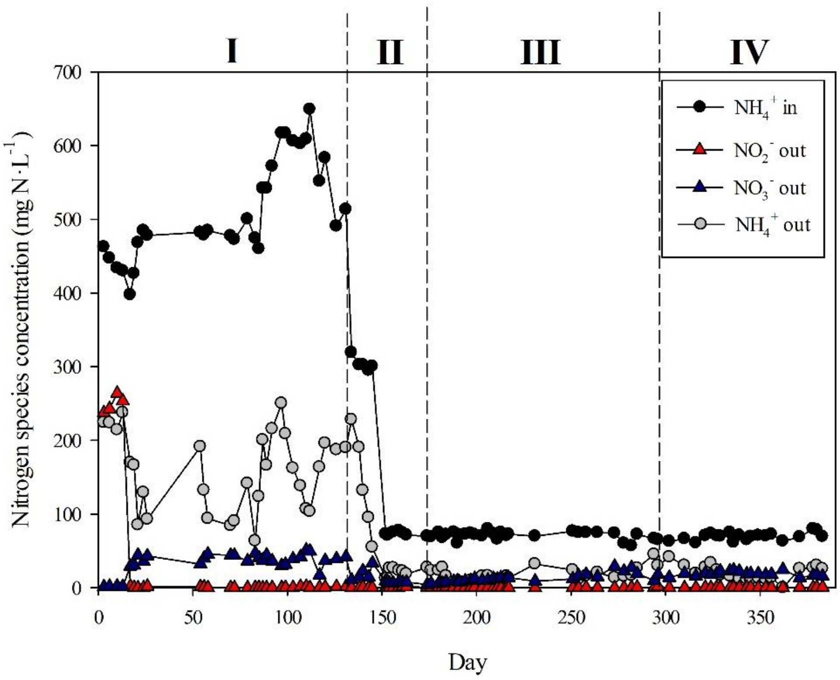

Figure S2. Nitrogen compound influent and effluent reactor throughout reactor operation

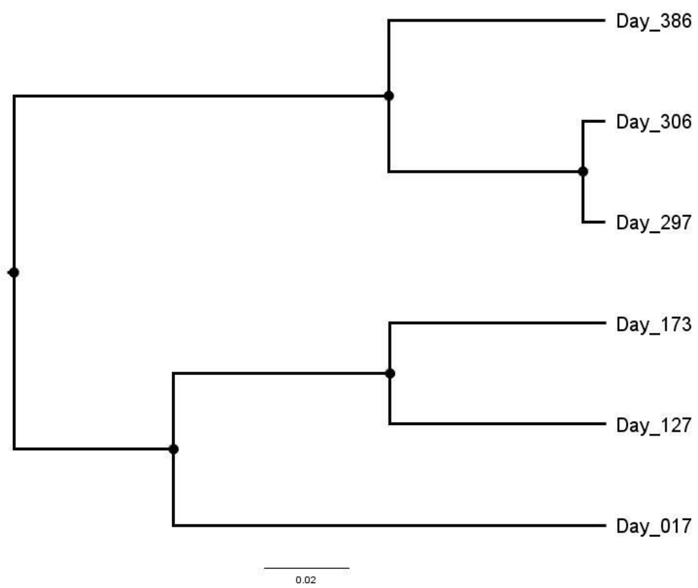

Figure S3. Dendrogram

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Appendix C

SUPPORTING INFORMATION

Assessment of operational conditions towards mainstream partial nitritation-anammox stability at moderate to low temperature: reactor performance and bacterial community

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S1. Mineral medium composition

Mineral medium used in the study contained per liter of tap water: 0.0125 g $FeSO_4 \cdot 7H_2O$, 0.0125 g EDTA$\cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.008 g KH_2PO_4, 0.3 g $CaCl_2 \cdot 2H_2O$, 0.354 g $(NH_4^+)_2SO_4$, 1.04 g $NaHCO_3$ and 1.25 mL of trace element solution addapted from Graaf et al. (1996).

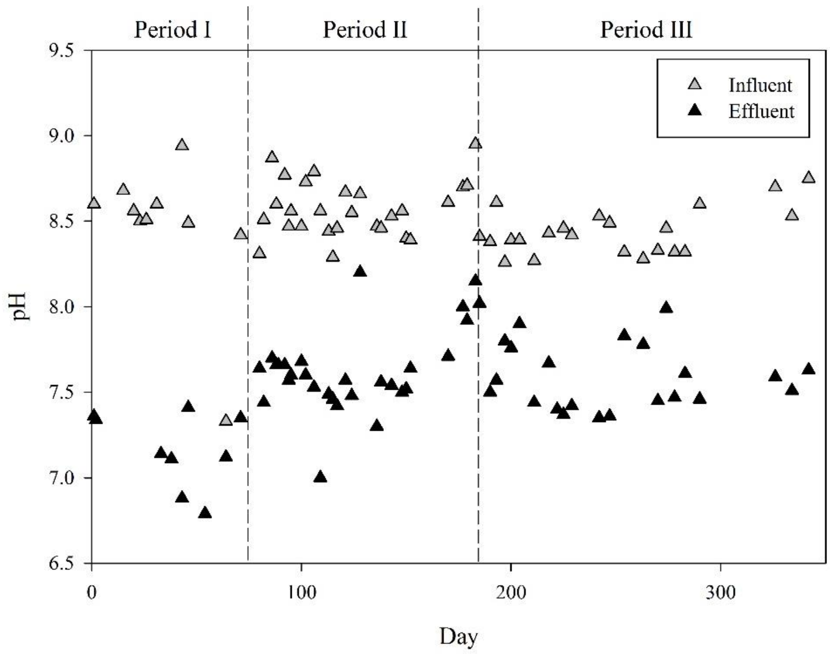

Figure S2. pH dynamics during reactor operation.

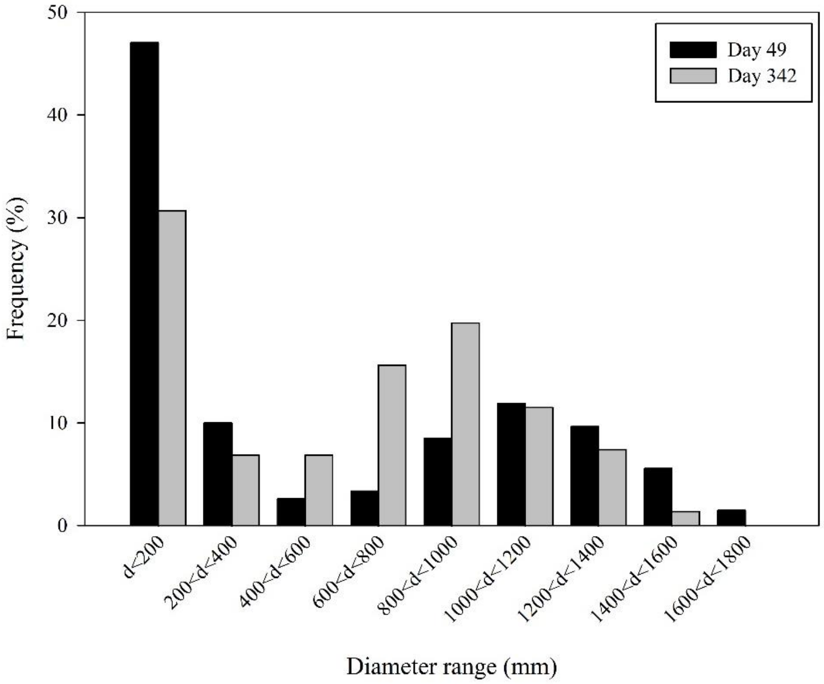

Figure S3. Average diameter size frequency distribution from day 49, reactor operation at 25 °C, and day 342, reactor operation at 15 °C. Biomass aggregate size was determined by acquiring samples pictures in stereomicroscope and analyzing on ImageJ software. At least 250 aggregates were measured.

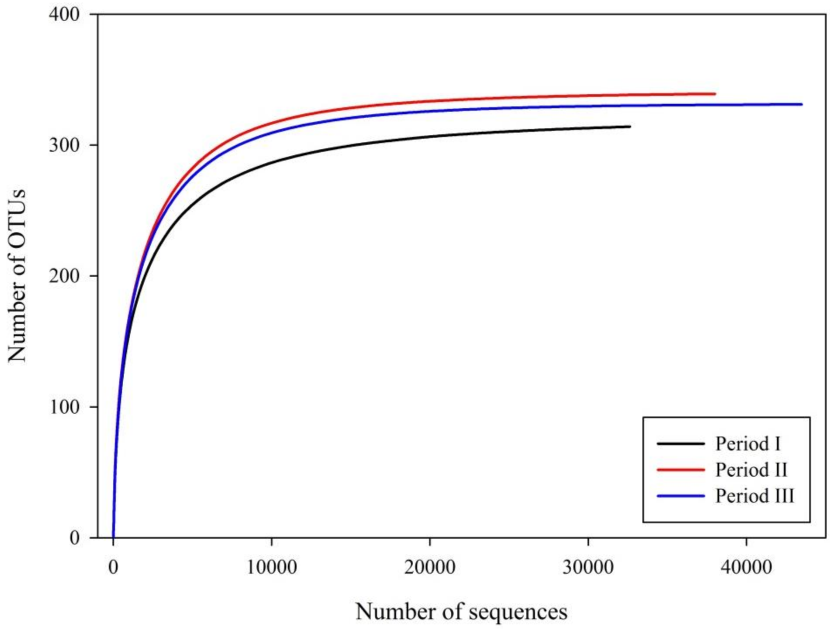

Figure S4. Rarefaction curves for the three samples at an OTU threshold of 97 % sequence similarity.

Table S5. Bacterial relative abundance (%) at phylum level

taxon	Period I	Period II	Period III
Acidobacteria	4.90	5.40	5.90
Actinobacteria	1.67	1.44	1.91
Armatimonadetes	0.11	0.13	0.15
BRC1	0.19	0.06	0.01
Bacteria_unclassified	2.42	2.24	2.21
Bacteroidetes	9.67	7.27	5.88
Chlorobi	0.65	1.31	0.83
Chloroflexi	12.98	9.87	11.68
Cyanobacteria	0.05	0.09	0.19
Deinococcus-Thermus	0.04	0.01	0.02
Fibrobacteres	0.01	0.05	0.04
Gemmatimonadetes	0.43	1.03	0.86
Hydrogenedentes	0.07	0.05	0.06
Ignavibacteriae	1.55	0.48	0.92
Microgenomates	0.10	0.08	0.05
Nitrospirae	4.63	7.23	2.96
Planctomycetes	24.77	21.65	33.81
Proteobacteria	35.38	41.31	32.18
SBR1093	0.02	0.04	0.05
Spirochaetae	0.03	0.02	0.00
Verrucomicrobia	0.35	0.27	0.29

References used in this Supporting Information

Graaf, A.A. van De, Bruijn, P. de, Robertson, L.A., Jetten, M.S.M., Kuenen, J.G., 1996. Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. Microbiology 142, 2187–2196.

Appendix D

SUPPORTING INFORMATION

Limitation of inorganic carbon suppresses nitrite-oxidizing bacteria activity in a granular SBR at mainstream conditions

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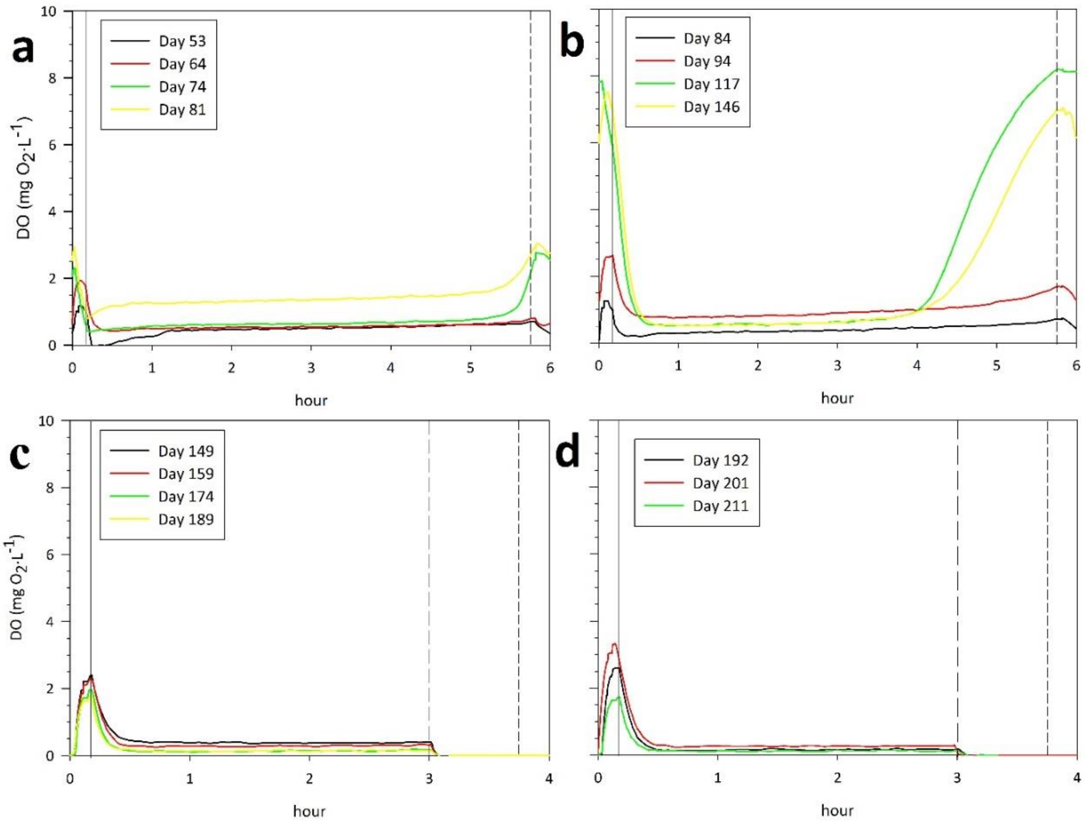

Figure S1. Dissolved oxygen profiles from radomly chosen operational cycles trhoughout reactor operation: (a) Period II; (b) Period III; (c) Period IV; and (d) Period V. All profiles presented were obtained during the operation with airflow of 0.020 L·min^{-1}. Continous vertical lines and short-dashed lines indicate the end of filling phase/beginning of aeration and beginning of settling and effluent withdrawn, respectively. In C and D, long-dashed vertical lines indicate the beginning of the anoxic phase.

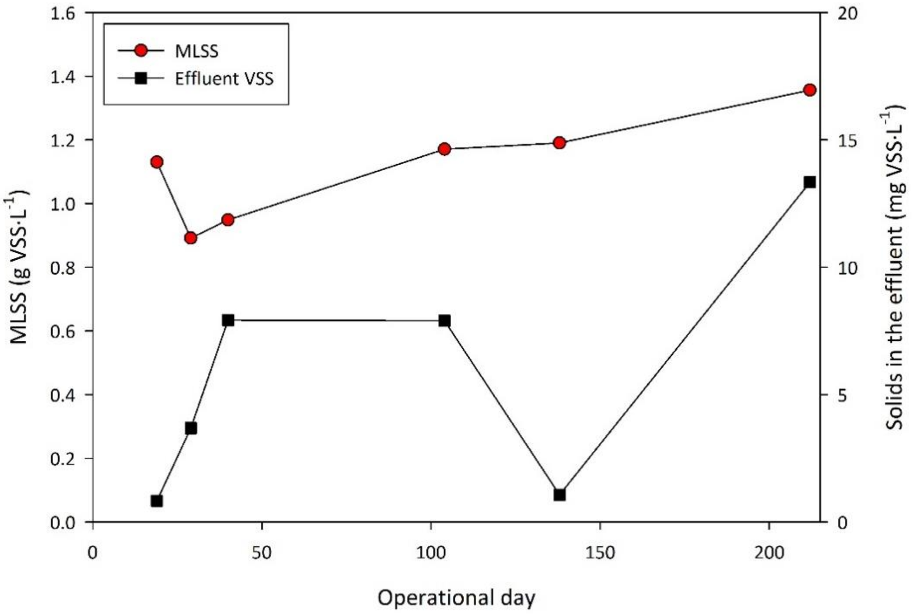

Figure S2. Solids concentration