Universitat Autònoma de Barcelona

AUTOTROPHIC NITROGEN REMOVAL FOR URBAN WASTEWATER TREATMENT BASED ON ANAMMOX

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Certifiquem

Que l'ambientòloga **Xènia Juan Diaz** ha realitzat sota la nostra direcció el treball titulat "**Autotrophic nitrogen removal for urban wastewater treatment based on anammox**" que es presenta en aquesta memòria i que constitueix la seva Tesi per optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

I perquè en prengueu coneixement i consti als efectes oportuns, presentem a l'Escola d'Enginyeria de la Universitat Autònoma de Barcelona l'esmentada Tesi, signant el present certificat a

Bellaterra, 20 de juliol de 2021

Dr. Julián Carrera Muyo Dr. Julio Pérez Cañestro

Thank the Ocean

With every drop of water you drink,
every breath you take,
you're connected to the sea.
No matter where on Earth you live.
Most of the oxygen in the atmosphere
is generated by the sea.

Sylvia Earle

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Summary

Wastewater needs to be correctly treated to avoid the associated public health-risks and pollution of natural ecosystems. Nowadays, wastewater treatment plants (WWTPs) with conventional activated sludge (CAS) systems remove nitrogen through nitrification/denitrification. Despite current facilities are robust and provide a successful effluent quality, they require for a high energy consumption and operating costs. Research efforts have been recently focused on achieving energy sustainable and self-sufficient facilities. The implementation of anammox bacteria in the main water line of an urban WWTP (known as “mainstream”) allows the uncoupling of the organic carbon (COD) and nitrogen removal. In the first stage, COD is concentrated and redirected to anaerobic digestion to maximize biogas production. The effluent of this stage is treated autotrophically for nitrogen removal in two-consecutive reactions. Firstly, partial nitritation takes places, where roughly half of the ammonium is oxidized into nitrite by ammonium oxidizing bacteria (AOB) under aerobic conditions. The remaining ammonium and the obtained nitrite are subsequently converted into nitrogen gas (N_2) by anammox bacteria under anoxic conditions. The partial nitritation/anammox (PN/AMX) process has been successfully implemented to treat ammonium concentrated streams at mesophilic temperatures. However, its implementation at mainstream conditions is limited by the cold, diluted and variable concentrations of the urban wastewater, by the strict discharge limits and by the need to be competitive with conventional treatments.

As a first approach, the PN/AMX process at mainstream conditions was tested in one-stage reactor configuration, although destabilization occurred at low temperatures. Two-stage systems were recognized for a better optimization of both processes. The aim of this thesis was to assess the feasibility of a two-stage PN/AMX process at mainstream conditions.

The first objective was to investigate the feasibility of applying a rather constant loading rate in an up-flow anammox sludge bed (UAnSB) reactor during a summer-to-winter temperature transition, from 20 to 10 ºC plus a three months period at 10 ºC by treating a real mainstream wastewater for a period of ca. 350 days. The UAnSB reactor was able to damp a 10 ºC drop while maintaining high and stable nitrogen removal rates throughout the operation. The successful reactor performance was attributed to the heterogenous substrate distribution along the sludge bed that allowed for an intra bed overcapacity.

The optimization of nitrogen removal efficiency and effluent quality is still a challenge of the PN/AMX process at mainstream conditions. The second objective was to design a configuration for coupling anammox to heterotrophic denitrification in a single reactor unit (i.e. the so-called CANDLE reactor, **C**oupling **A**nammox and **D**enitrification in a sing**LE** unit). The CANDLE reactor was tested with real mainstream wastewater and fed with acetate as a C-source over a temperature range from 20 to 14 ºC for ca. 200 days. The enhancement of heterotrophic denitrifying activity allowed to reduce the nitrate produced by anammox bacteria, without compromising the anammox performance and, thus, to improve effluent quality while maintaining high and constant nitrogen removal rates. The study of substrate distribution along the sludge bed and of the microbial community showed that the coupling and coexistence of both populations in a single reactor unit was possible as anammox and heterotrophic denitrifiers occupied differentiated bed compartments. Additionally, the enhancement of heterotrophic denitrifying activity allowed for mitigation of nitrous oxide (N_2O) emissions.

The potential nitrate production in the PN step is currently limiting the application of mainstream PN/AMX process. Nevertheless, successful long-term operations have been achieved in granular reactors at low temperatures. The last aim was to investigate the influence of different operational parameters in the development of an autotrophic granular sludge performing a stable nitritation using CAS as inoculum in an air-lift reactor. The enhancement of ammonium oxidation activity and the promotion of high air-flow rates showed to be effective in the development of an autotrophic granular sludge performing a stable nitritation.

Overall, this thesis demonstrated that there seems to be no obstacles for the application of the anammox process at mainstream conditions and that a successful start-up of a granular PN reactor can be attained under optimal conditions by using CAS as inoculum. Future research should be focused on gaining insight for the implementation of the PN/AMX process at full-scale conditions.

Resumen

El tratamiento de las aguas residuales es necesario para evitar riesgos sobre la salud pública, así como para evitar la contaminación sobre los distintos ecosistemas naturales. Actualmente, las estaciones depuradoras de aguas residuales (EDARs) con tratamiento de lodos activos eliminan el nitrógeno a través del proceso de nitrificación/desnitrificación. A pesar de que las instalaciones actuales son robustas y permiten obtener una buena calidad del efluente, representan un gran consumo energético y económico. Así, los últimos estudios se han enfocado en desarrollar EDARs urbanas energéticamente autosostenibles. La implementación de bacterias anammox en la línea principal de una EDAR urbana permite separar la eliminación de materia orgánica (DQO) y nitrógeno. En un primer paso, la DQO se concentra y se redirige a digestión anaeróbica para maximizar la producción de biogás. El efluente es luego tratado para la eliminación autotrófica de nitrógeno mediante dos reacciones consecutivas. En primer lugar, la nitritación parcial, donde aproximadamente la mitad del amonio se oxida a nitrógeno en condiciones aerobias a través de las bacterias amonio oxidantes. Posteriormente, el amonio no oxidado y el nitrito obtenido se convierten a nitrógeno gas (N_2) mediante las bacterias anammox en condiciones anóxicas. El proceso de nitritación parcial/anammox se ha implementado de forma satisfactoria para el tratamiento de corrientes con altas cargas de amonio en condiciones mesofílicas. Sin embargo, su implementación en la línea principal de una EDAR urbana está limitado por sus bajas temperaturas, así como por las diluidas y variables concentraciones de nitrógeno, por los estrictos límites de vertido y por la necesidad de poder ser un proceso competitivo con los tratamientos actuales.

Inicialmente, se estudió la posibilidad de implementar el proceso de nitritación parcial/anammox en la línea principal de una EDAR urbana en un sistema de una sola

etapa; sin embargo, el proceso resultó ser inestable a bajas temperaturas. En este sentido, la utilización de un sistema en dos etapas fue considerado como una alternativa para mejorar la implementación del proceso de eliminación autotrófica de nitrógeno en la línea principal de aguas. Así, el objetivo principal de la tesis fue estudiar la estabilidad del proceso de nitritación parcial/anammox en dos reactores independientes para el tratamiento de las aguas residuales urbanas.

El primer objetivo, fue investigar la estabilidad de un reactor UAnSB (del inglés *Up-flow anammox sludge bed*) de tratar un agua residual urbana real a una carga constante durante un periodo de unos 350 días simulando una transición verano-invierno en términos de temperatura, de 20 a 10 ºC, y manteniendo el reactor a 10 ºC, durante más de tres meses. El reactor no solo fue capaz de mantener una carga estable durante la operación, sino que además se consiguieron altas velocidades de eliminación de nitrógeno a bajas temperaturas. Los buenos resultados de operación se atribuyeron a la distribución heterogenia del sustrato a lo largo del reactor que permitió una sobrecapacidad del lecho, explotada a bajas temperaturas.

La optimización de la calidad del efluente y de la eficiencia de eliminación de nitrógeno aún representan un reto para la implementación del proceso de nitritación parcial/anammox en la línea principal de una EDAR urbana. El segundo objetivo de esta tesis fue acoplar el proceso anammox con la desnitrificación heterotrófica en un solo reactor llamado CANDLE (del inglés ***C**oupling **A**nammox and **D**enitrification in a sing**LE** unit*). El reactor CANDLE trató un agua residual urbana real y simultáneamente fue alimentado con acetato como fuente externa de carbono durante aproximadamente 200 días en un rango de temperaturas de 20 a 14 ºC. La actividad heterotrófica desnitrificante permitió reducir el nitrato producido por las bacterias anammox, sin comprometer la misma actividad anammox, y, por tanto, permitió mejorar la calidad del efluente mientras que se mantuvieron elevadas velocidades de eliminación de nitrógeno. El estudio de la distribución del sustrato a lo largo de lecho, así como de las comunidades microbianas indició que el acoplamiento y coexistencia de las bacterias anammox con las bacterias heterotróficas desnitrificantes fue posible ya que ambos grupos ocuparon compartimientos diferenciados a lo largo del lecho del reactor. Adicionalmente, la actividad heterotrófica desnitrificante permitió mitigar las emisiones de óxido nitroso (N_2O).

Evitar la potencial producción de nitrato durante el proceso de nitritación parcial es uno de los mayores retos para la implementación del proceso de nitritación parcial/anammox en la línea principal de una EDAR urbana. Sin embargo, varios estudios han demostrado una nitritación parcial estable a bajas temperaturas usando reactores granulares. El último objetivo de esta tesis fue investigar la influencia de distintos parámetros operacionales en el desarrollo de gránulos autotróficos para desarrollar y mantener una nitritación parcial estable usando lodos activos convencionales como inóculo en un reactor tipo *air-lift*. El hecho de favorecer la actividad amonio oxidante y promover elevados caudales de aire fomentaron el desarrollo de gránulos autotróficos que permitieron una nitritación parcial estable.

En resumen, esta tesis parece indicar que no deberían de existir obstáculos para la aplicación del proceso anammox en la línea principal de una EDAR urbana y que la puesta en marcha de un reactor granular para la nitritación estable es posible usando lodos activos como inóculo. Sin embargo, todavía se necesita una mayor labor de investigación para aplicar el proceso de nitritación parcial/anammox a escala real.

Resum

El tractament de les aigües residuals és necessari per evitar riscos sobre la salut pública, així com per evitar la contaminació sobre els diferents ecosistemes. Actualment, les estacions depuradores d'aigües residuals (EDARs) amb tractament de fangs actius eliminen el nitrogen a través del procés de nitrificació/desnitrificació. Tot i que les instal·lacions actuals són robustes i permeten obtenir una bona qualitat de l'efluent, comportem un gran consum econòmic i energètic. Així, els últims estudis s'han enfocat en desenvolupar EDARs urbanes energèticament autosostenibles. La implementació dels bacteris anammox a la línia principal d'una EDAR urbana permet separar l'eliminació de matèria orgànica (DQO) i nitrogen. En primer lloc, la DQO es concentra i es redirigeix a digestió anaeròbica per tal de maximitzar la producció de biogàs. L'efluent és posteriorment tractat per eliminar el nitrogen autotròficament en dues reaccions consecutives. El primer pas consisteix en la nitritació parcial, on, aproximadament la meitat de l'amoni s'oxida a nitrogen en condicions aeròbiques a través dels bacteris amoni oxidants. Seguidament, l'amoni no oxidat i el nitrit obtingut es converteixen a nitrogen gas (N_2) mitjançant els bacteris anammox en condicions anòxiques. El procés de nitritació parcial/anammox s'ha implementat satisfactòriament per al tractament de corrents amb elevades concentracions d'amoni en condicions mesofíliques. No obstant, la seva implementació a la línia principal d'una EDAR urbana està limitada per les baixes temperatures, així com per les diluïdes i variables concentracions de nitrogen, pels estrictes límits d'abocament i per la necessitat de ser un procés competitiu amb els tractaments actuals.

Estudis previs han demostrat que el procés de de nitritació parcial/anammox en una sola etapa a la línia principal d'una EDAR urbana resulta inestable a baixes

temperatures. Així doncs, la implementació d'un sistema en dues etapes va ser considerat com una alternativa per tal de millorar el procés d'eliminació autotròfica de nitrogen a la línia principal d'aigües. Per tant, l'objectiu principal d'aquesta tesi ha sigut estudiar l'estabilitat del procés de nitritació parcial/anammox en dos reactors independents per al tractament d'aigua residual urbana.

El primer objectiu va ser investigar l'estabilitat d'un reactor UAnSB (de l'anglès *Up-flow anammox sludge bed*) al tractar una aigua residual urbana real a una càrrega constant durant un període d'uns 350 dies simulant una transició estiu-hivern en relació a la temperatura, de 20 a 10 ºC, i mantenint el reactor a 10 ºC, durant més de tres mesos. El reactor no només va poder mantenir una càrrega estable al llarg de tota l'operació, sinó que, a més, es van aconseguir altes eficiències d'eliminació de nitrogen a baixes temperatures. Els bons resultats de l'operació s'han atribuït a la distribució heterogènia del substrat al llarg del reactor que va permetre una sobrecapacitat del llit, explotada a baixes temperatures.

L'optimització de la qualitat de l'efluent i de l'eficiència d'eliminació de nitrogen encara representen un repte per tal de poder implementar el procés de nitritació parcial/anammox a la línia principal d'una EDAR urbana. El segon objectiu d'aquesta tesi va ser desenvolupar un reactor per tal d'acoblar el procés anammox amb la desnitrificació heterotròfica en un únic reactor anomenat CANDLE (de l'anglès ***C**oupling **A**nammox and **D**enitrification in a sing**LE** unit*). El reactor CANDLE va tractar aigua residual urbana real i simultàniament va ser alimentat amb acetat com a font externa de carboni durant aproximadament 200 dies en un rang de temperatures de 20 a 14 ºC. L'activitat heterotròfica desnitrificant va permetre reduir el nitrat produït pels bacteris anammox, sense comprometre la mateixa activitat anammox, i, per tant, va millorar la qualitat de l'efluent mentre que alhora es mantenien elevades velocitats d'eliminació de nitrogen. L'estudi de la distribució del substrat al llarg del llit, així com de les comunitats microbianes van indicar que l'acoblament i la coexistència dels bacteris anammox amb els bacteris heterotròfics desnitrificants va ser possible ja que ambdós ocuparen compartiments diferenciats al llarg del llit del reactor. Alhora, l'activitat heterotròfica desnitrificant va permetre mitigar les emissions d'òxid nitrós (N_2O).

Evitar la potencial producció de nitrat durant el procés de nitritació parcial és un dels majors reptes de cara a la implementació del procés de nitritació parcial/anammox a la línia principal d'una EDAR urbana. Tanmateix, diversos estudis han demostrat que el procés de nitritació parcial pot ser estable a baixes temperatures fent servir reactors granulars. L'últim objectiu d'aquesta tesi va ser investigar la influència de diversos paràmetres operacionals en el desenvolupament de grànuls autotròfics per tal de mantenir una nitritació parcial estable fent servir fangs actius convencionals com a inòcul en un reactor tipus *air-lift*. El fet d'augmentar l'activitat amoni oxidant i promoure elevats cabals d'aire van fomentar el desenvolupament de grànuls autotròfics que van permetre una nitritació estable.

En síntesis, aquesta tesi assenyala que no haurien d'existir obstacles per l'aplicació del procés anammox a la línia principal d'una EDAR urbana i que la posada en marxa d'un reactor granular per una nitritació estable és possible fent servir fangs actius com a inòcul. Tanmateix, encara es necessita d'una investigació més extensa per tal d'aplicar el procés de nitritació parcial/anammox a escala real.

List of acronyms, abbreviations and symbols

AOB	Ammonium Oxidizing Bacteria
AOR	Ammonium Oxidizing Rate
Anammox	Anaerobic Ammonium Oxidizing bacteria
AMX	Anammox
BLAST	Basic Local Alignment Search Tool
BNR	Biological Nitrogen Removal
C	Carbon
COD	Chemical Oxygen Demand
CANON	Completely Autotrophic Nitrogen removal Over Nitrite
CAS	Conventional Activated Sludge
CANDLE	**C**oupling **A**nammox and **D**enitrification in a Sing**LE** **U**nit
A	Cross-sectional reactor area
DNA	Deoxyribonucleic Acid
DO	Dissolved oxygen
EF	Emission Factor
EPS	Extracellular Polymeric Substances
Q	Flow rate
FA	Free Ammonia
GHG	Green House Gases
Ks	Half-saturation coefficient constant
HRT	Hydraulic Retention Time
MBBR	Membrane Biological Bed Reactor
NOB	Nitrite Oxidizing Bacteria
N	Nitrogen
NLR	Nitrogen Loading Rate
NRE	Nitrogen Removal Efficency
NRR	Nitrogen Removal Rate
OLR	Organic Loading Rate
PN	Partial Nitritation
PHB	Polyhydroxybutyrate
rRNA	Ribosomal Ribonucleic Acid
RBC	Rotating Biological Contactor
SBR	Sequential Batch Reactor
SHARON	Single reactor system for High activity Ammonium Removal Over Nitrite
SRT	Sludge Retention Time
SVI	Sludge Volumetric index
ug	Superficial gas-flow velocity
T	Temperature

List of acronyms, abbreviations and symbols

TAN	Total Ammonium Nitrogen
TNN	Total Nitrite Nitrogen
TS	Total Solids
TSS	Total Suspended Solids
UASB	Up-flow Anaerobic Sludge Bed
UAnSB	Up-flow Anammox Sludge Bed
$\mathbf{V_{up}}$	Up-flow velocity
VFA	Volatile Fatty Acids
VS	Volatile Solids
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plant

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Chapter 1. Introduction

1.1. Background

Wastewater originates from a combination of domestic, industrial and commercial activities, stormwater and surface runoff. The correct treatment of wastewater plays a fundamental role in our society to reduce potential public health risks while avoiding negative effects in natural ecosystems. Wastewater pollutants, such as nutrients and organic matter, are treated in urban wastewater treatment plants (WWTPs). Conventional activated sludge (CAS) has been the most frequently applied technology in urban WWTPs since it was firstly designed in 1920's (Ardern and Lockett, 1914). CAS treatment was initially designed for the removal of particulate and soluble organic matter. Since the increase in human and industrial activities in the beginning of the 1980's, the nutrients such as nitrogen and phosphorus were included as targeted pollutants to be removed in CAS systems to avoid the associated risks of eutrophication.

Current WWTPs are robust and provide a successful effluent quality, but require for a high energy consumption and operating costs and represent a large footprint impact (Van Loosdrecht and Brdjanovic, 2014). Nowadays, wastewater systems account for approximately 3 to 4 % of energy consumption in developed countries (EPA, 2013). By 2030, global demand for energy and water is expected to grow by 40% and 50%, respectively (UN-HABITAT, 2016), whereas about half of the global population will face severe water shortage in the incoming decades (Alcamo et al., 2007). Further, WWTPs have been identified as potential sources of anthropogenic greenhouse gases (GHG) emissions produced during the biological wastewater treatment processes (CO_2, CH_4, NO and N_2O) and due to energy consumption (CO_2) (Campos et al., 2016). Besides the poor cost-effectiveness and the actual demands from a rapidly growing human population, the need for a more sustainable society bring us to rethink and redesign future WWTPs. Thus, research efforts should be directed to the development of more sustainable and energy efficient WWTPs.

1.2. Biological Nitrogen Removal

Current WWTPs with nutrient removal perform biological nitrogen removal (BNR) through the nitrification/denitrification process. The nitrification/denitrification is a stable process, but it requires high energy consumption and operational costs and needs for a high land availability. The aeration process for nitrogen and chemical oxygen demand (COD) removal accounts for ca. 60 – 80 % of the energy consumption of an urban WWTP (Zessner et al., 2010). Further, only 20 % of the organic matter is recovered in the form of biogas throughout anaerobic digestion processes (Larsen et al., 2015).

The discovery of anaerobic ammonium oxidizing (anammox) bacteria in the early 90's (Mulder et al., 1995) changed the paradigm of nitrogen removal. Anammox bacteria are chemolithotrophs organisms that belong to the Planctomycetes phylum (Kartal and Keltjens, 2016). Anammox bacteria oxidize ammonium to nitrogen gas (N_2) by using nitrite as electron acceptor (Strous et al., 1999). As anammox bacteria grow autotrophically, they are able to use CO_2 as carbon source to form new biomass. During the anammox reaction, nitrate is produced as a side product, due to nitrite oxidation. The complete stoichiometry of the anammox metabolism is presented in Eq. 1.1 as obtained by Strous et al. (1998).

$$\mathrm{NH_4^+ + 1.32NO_2^- + 0.006HCO_3^- + 0.13H^+}$$
$$\mathrm{\rightarrow 1.02N_2 + 0.26NO_3^- + 2.03H_2O + 0.066CH_2O_{0.5}N_{0.15}} \qquad \text{(Eq. 1.1)}$$

The implementation of the anammox process in the main water line of an urban WWTPs offers the possibility of uncoupling COD and nitrogen removal. A first step consists of a physical or chemical treatment in which COD is concentrated and redirected for subsequent energy recovery through anaerobic digestion. The effluent of the first stage, combined with the supernatant of the digester (i.e. sidestream), is treated autotrophically for nitrogen removal in two-consecutive reactions. Firstly, partial nitritation (PN) takes places, where roughly half of the ammonium is oxidized into nitrite by ammonium oxidizing bacteria (AOB) under aerobic conditions, and thus, the suppression of the nitrite oxidizing step performed by nitrite oxidizing bacteria (NOB) is required (Fig. 1.1). Subsequently, the remaining ammonium and the obtained nitrite are converted into N_2 by anammox bacteria under anoxic conditions (Fig. 1.1).

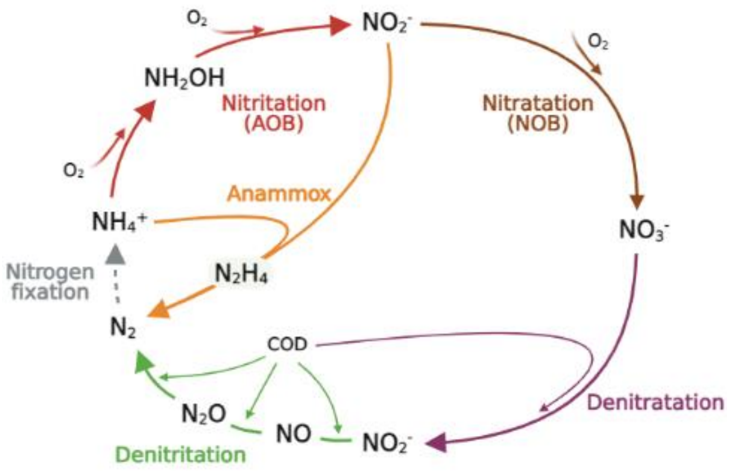

Fig. 1.1. Schematic illustration of the nitrification, denitrification, anammox and nitrogen fixation processes. AOB, Ammonium oxidizing bacteria. NOB, Nitrite oxidizing bacteria. Anammox, anaerobic ammonium oxidizing bacteria. Modified from Daims et al. (2016).

The implementation of the PN/AMX process in the main water line offers significant advantages over the conventional BNR process as (1) oxygen requirements are reduced by a 60 %, (2) no organic carbon is derived to heterotrophic denitrification and (3) the uncoupling of organic matter from nitrogen removal allows all the organic carbon to be recovered as bioenergy (methane) through anaerobic digestion (Cao et al., 2017; Kartal et al., 2010). Theoretical calculations showed that the implementation of PN/AMX in the main line would allow to recover 24 Wh person^{-1} compared to the 44 Wh person^{-1} consumed during conventional treatment (Siegrist et al., 2008). Thus, this technology offers the opportunity to achieve efficient nitrogen removal and energy neutral (or even energy generating) WWTPs, while allowing for a significant reduction in operating costs.

1.3. Challenges for autotrophic biological nitrogen removal at mainstream conditions

The PN/AMX process has been successfully implemented to treat side-stream wastewaters such as supernatant from anaerobic digestors and industrial wastewaters, characterized by high strength ammonium concentrations and mesophilic temperatures (Joss et al., 2009; van der Star et al., 2007; Wett, 2006). As of 2014, more than 100 full-scale facilities have been successfully operating worldwide (Lackner et al., 2014). The first PN/AMX process for the treatment of ammonium-rich wastewaters was developed by Paques (Rotterdam, The Netherlands) using a two-stage sequential batch reactor (SBR) configuration. PN took place in a SHARON® (Single reactor High activity Ammonium Removal Over Nitrite) reactor, while the anammox process was performed in a granular anoxic reactor (van Dongen et al., 2001). However, because of the reduced investment costs, the focus shifted to single-stage reactor configurations. In this case, AOB and anammox bacteria coexist under microaerobic conditions to avoid inhibition of anammox bacteria by oxygen and to achieve suitable conditions for PN (Strous et al., 1997). Currently, ca. 90 % of the full-scale installations are performed by using one-stage configurations and include different reactor types with the corresponding patented technologies such as SBRs (DEMON®), granular sludge processes (CANON®), moving bed biofilm bioreactor (MBBR) (ANITAMox®), rotating biological contactors (RBC) or activated sludge reactors (Lackner et al., 2014).

In the last years, great efforts have been made to implement this technology to the treatment of mainstream water. Despite PN/AMX is a well-established technology for the treatment of sidestream wastewaters, it is still a challenge at mainstream conditions and no full-scale installations have been yet reported. The main bottleneck for the implementation of PN/AMX into the main water line relies on achieving a stable PN process (i.e. NOB repression), therefore preventing aerobic nitrite oxidation. In contrast to sidestream process, the low and varying ammonium concentrations (ca. 30 – 60 mg N L^{-1}) of mainstream water result into free ammonia (FA) concentrations lower than the reported threshold for a proper NOB inhibition (Anthonisen et al., 1976; Jubany et al., 2009). The low temperatures of municipal wastewater (10 – 20 °C, in moderate climates) hinders nitrite oxidation suppression, as the maximum specific growth rate (μ_{max}) of NOB is higher than that of AOB below 20 ºC (Hellinga et al., 1998). Also, the growth rate and activity of anammox bacteria is reduced at low temperatures (Dosta et al., 2008; Lotti et al., 2015a). Further, mainstream treatments need to meet the strict effluent standards (< 10 mg N L^{-1}) according to the European Council Directive 91/271/ECC. However, the theoretical nitrogen removal efficiency of the PN/A process is limited by the produced nitrate by anammox bacteria (up to 10% of the influent nitrogen). Lastly, mainstream PN/AMX needs to be competitive with conventional treatments to achieve volumetric nitrogen removal rates above 0.03 to 0.05 g N L^{-1} d^{-1} (Metcalf & Eddy, 2003).

1.4. From one to two-stage mainstream PN/AMX

As a first approach, the performance of PN/AMX at mainstream conditions was tested in one-stage reactor configurations because of the successful results in the treatment of sidestream wastewaters. However, the main weak point of this system was that NOB proliferated at low temperatures, resulting into a reduced nitrogen removal performance through the anammox process (De Clippeleir et al., 2013; Gilbert et al., 2015). Other authors reported a good reactor performance at 15 ºC, but at the expense of maintaining low nitrogen removal rates (Laureni et al., 2019; Pedrouso et al., 2019). Promising results were reported in terms of effluent quality and of nitrogen removal performance when treating an aerobically pre-treated urban wastewater at 15 ºC (Laureni et al., 2019, 2016; Pedrouso et al., 2019), although with a marked anammox activity suppression at 11 ºC. Overall, the feasibility of the one-stage PN/AMX configuration remained to be tested at winter temperatures.

Two-stage systems have been recognized as a good alternative as it allows for a separately and better optimization of both processes. One of the strategies to achieve stable PN in a single-stage reactor at mainstream conditions is based on the assumption that NOB present a higher oxygen half-saturation coefficient than AOB (Guisasola et al., 2005; Pérez et al., 2009). Thus, operating at low dissolved oxygen (DO) has been pointed out a as a feasible strategy for NOB repression at mainstream conditions (Lotti et al., 2014a; Ma et al., 2015). However, different studies contradict the idea that AOB presents a better oxygen affinity than NOB (Manser et al., 2005; Regmi et al., 2014; Sliekers et al., 2005). As the apparent oxygen half-saturation coefficient increases with floc and colony size and NOB are found in smaller microcolonies, NOB will present a higher oxygen affinity that that of AOB (Pérez et al., 2005; Picioreanu et al., 2016).

Another strategy for hampering nitrate production in a single PN reactor is based on the use of granular biomass by maintaining a suitable DO/ammonium concentration ratio in the bulk reactor liquid (Bartrolí et al., 2010). Given a fixed DO concentration and under a certain residual ammonium concentration, AOB is able to increase its activity, resulting into a higher DO consumption and, thus, limiting nitrite oxidation because of the limiting oxygen concentrations, relegating NOB to deeper layers (Poot et al., 2016).

Experimental evidence of stratification of nitrifiers guilds, where AOB dominated in the outer shell and NOB in the inner core of the granule, was shown to be effective for a successful nitrite oxidation repression (Poot et al., 2016; Soler-Jofra et al., 2019). Indeed, different studies reported a stable PN at low temperatures by controlling the residual ammonium concentrations within a granular reactor during long-term experiments (Isanta et al., 2015b; Reino et al., 2016).

On the other hand, encouraging results were reported for the implementation of the anammox process in a single unit at mainstream conditions. Different studies reported anammox activity to be feasible at low temperatures by treating a real mainstream wastewater. However, most of the studies suffered a marked anammox activity decrease at low temperatures (12.5 ºC) (Laureni et al., 2015), use high recirculation flow-rates to achieve high up-flow velocities (Lotti et al., 2014b), worked at low nitrogen removal rates (Pedrouso et al., 2020) or needed to adapt nitrogen loading rates to the lower reactor performance caused by temperature decrease (Reino et al., 2018).

1.5. Research gaps and outline of the thesis

The overall goal of this thesis is to study the feasibility of the two-stage PN/AMX process at mainstream conditions. To date, the implementation of sewage PN/AMX is considered the most efficient scenario for the achievement of energy neutral (or even energy generating) urban WWTPs. However, different research gaps need to be further addressed as to promote the application of the PN/AMX process for mainstream wastewater treatment.

According to the state-of-the-art, one of main challenges for mainstream PN/AMX is to maintain a stable and high anammox performance under low water temperatures (< 15 ºC). An up-flow anammox sludge bed (UAnSB) reactor reported stable and high loading rates during a long-term mainstream operation at temperatures as low as 11 ºC (Reino et al., 2018). However, reactor performance was evaluated by mainly treating a synthetic influent, while the reasons behind the good reactor response over the temperature decrease were not deeply studied. In view of the successful results of Reino et al. (2018), **Chapter 3** is focused on understanding the response of an UAnSB reactor at mainstream conditions over a temperature drop from 20 to 10 ºC, plus ca. 3 months a 10 ºC. Reactor process performance is evaluated by treating a real mainstream wastewater, while maintaining stable nitrogen loading rates, higher than those of conventional treatments. Nitrogen removal performance is assessed throughout reactor operation and the effect of the heterotrophic denitrifying activity derived from COD present in the influent is also discussed. The study of substrate distribution along the sludge bed is used to study the feasibility of mainstream anammox.

The theoretical maximum nitrogen removal efficiency of mainstream PN/AMX is currently limited by the produced nitrate during the anammox process, though it can be even lower due to the instability of previous mainstream stages. Thus, one of the main bottlenecks of mainstream PN/AMX is the optimization of nitrogen removal efficiency and effluent quality. In view of the increasing stringent discharge limitations, a new reactor design is proposed to convert an UAnSB reactor (**Chapter 3**) into a single reactor unit for the combination of anammox and heterotrophic denitrification by avoiding competition between both process through the addition of an external C-source (**Chapter 4**). The nitrogen removal performance by combining the anammox and heterotrophic denitrification processes is evaluated in a temperature range from 20 to 14 ºC. The characterization of substrate distribution and of the microbial community are used to identify the regions for anammox and heterotrophic denitrification along the sludge bed. The impact on N_2O emissions derived from the enhancement of heterotrophic denitrification is also assessed.

The potential nitrate production in the PN stage is currently limiting the implementation of anammox nitrogen removal-based technologies at mainstream conditions, though successful long-term mainstream partial nitritation stability has been achieved in granular reactors at low water temperatures (10 ºC) (Isanta et al., 2015b; Reino et al., 2016). However, the specific operating conditions resulting into an effective start-up of this reactor type remains poorly understood (X. Li et al., 2020; Torà et al., 2013). The aim of **Chapter 5** is to assess the influence of different operational parameters in the development of an autotrophic granular sludge performing a stable nitritation using CAS as inoculum in an air-lift reactor. A specific start-up strategy for the rapid obtention of a nitrifying granular sludge is defined, whereas the operational conditions constraining its development are also reported.

Finally, **Chapter 6** summarizes the overall conclusions of this thesis and highlights those relevant aspects that should be considered for a potential full-scale implementation of the PN/AMX process at mainstream conditions.

Chapter 2. Thesis objectives

The goal of this dissertation is to assess the feasibility of a two-stage partial nitritation (PN/AMX) process for sewage treatment (i.e. low temperatures and low nitrogen concentrations). This thesis is focused on gaining knowledge for a potential full-scale PN/AMX implementation in the main water line of an urban wastewater treatment plant (WWTP).

The specific objectives of this thesis are here defined:

- To assess the feasibility of operating at a constant nitrogen loading rate in a up-flow anammox sludge bed (UAnSB) reactor to face a (realistic) summer-to-winter transition in terms of temperature decrease while treating a real mainstream wastewater.
- To explore the UAnSB reactor performance response over a 10 ºC drop by studying substrate distribution along the sludge bed.
- To characterise the microbial community at 20 and 10 ºC for the different sludge bed sections of the UAnSB reactor.
- To design a single reactor unit able (i) to remove the nitrogen via the anammox process while simultaneously (ii) to heterotrophically reduce the produced nitrate by anammox bacteria by feeding an external carbon source.
- To investigate the cooperation of anammox and heterotrophic denitrifying bacteria in a single stage unit by means of substrate distribution and microbial analyses of the sludge bed.
- To define the operational conditions for the start-up of an autotrophic aerobic granular sludge reactor performing stable nitritation using floccular sludge as inoculum.

Chapter 3. Effective dampening of temperature effects in an anammox reactor treating real mainstream wastewater

Chapter based on: **Juan-Díaz, X.**, Pérez, J., Carrera, J., 2021. Effective dampening of temperature effects in an anammox reactor treating real mainstream wastewater. J. Water Process Eng. doi:10.1016/j.jwpe.2020.101853

Abstract

The aim of the present study was to evaluate the capability of an up-flow anammox sludge bed reactor (13 L) of dampening a 10 °C temperature drop at mainstream conditions while operating at constant nitrogen loading rates (0.11 ± 0.01 g N L^{-1} d^{-1}). The up-flow anammox sludge bed reactor was fed with an aerobically pre-treated mainstream wastewater. The reactor temperature was controlled as to mimic a (realistic) summer-to-winter transition from 20 to 10 ºC plus a three months period at 10 ºC. During the 350 days of operation, the average nitrogen removal rate remained high (0.10 ± 0.01 g N L^{-1} d^{-1}), indicating that the 10 °C drop did not affect the overall efficiency of the reactor. This can be explained as anammox activity differed among the different sludge bed sections. At 20ºC, anammox activity was mainly located at the bottom of the sludge bed. At 10ºC, the anammox activity decrease of the bottom sludge bed was compensated by an activity increase within the upper sludge bed sections. The contribution of heterotrophic denitrifying activity to the total nitrogen removal rate was assessed to be 3-to-5 times lower than that of anammox, even at 10 °C. Microbial community of 16S rRNA gene-targeted sequencing analyses resulted in the identification of an uncharacterized Planctomycetes in high numbers. This study demonstrated that the low temperatures should not be an obstacle for the feasibility of anammox bacteria in the main water line of an urban wastewater treatment plant.

3.1. Introduction

The implementation of the anaerobic ammonium oxidation (anammox) process at mainstream conditions for nitrogen removal is an attractive alternative to achieve energy neutral (or even energy generating) urban wastewater treatment plants (WWTPs) (Kartal et al., 2010). The anammox process has been successfully used to treat side-stream wastewaters (the so-called sludge liquor) (Lackner et al., 2014), but it still faces several challenges as to be applied in the main water line of urban WWTPs. The bottlenecks at mainstream conditions are related to (1) the low temperatures, (2) the low nitrogen concentrations and (3) the presence of organic matter which hinder anammox performance (Kartal et al., 2010; Pijuan et al., 2020). Furthermore, the main challenges are focused on the need to operate at nitrogen removal rates (NRRs) similar to conventional WWTPs (Metcalf & Eddy, 2003) and, at the same time, meeting (or even improving) the strict effluent requirements (<10 mg N L^{-1}) according the European Council Directive 91/271/ECC.

Previous studies pointed out the possibility to implement the anammox process with the so-called one-stage configuration (with partial nitritation and anammox in the same reactor), as it is the most widespread approach for side-stream treatment (Lackner et al., 2014). Some one-stage configurations studies reported high NRRs in a membrane biological bioreactor (MBBR) and in rotating biological contactor (RBC) treating synthetic wastewater but nitrite accumulated and nitrate was produced by nitrite-oxidizing bacteria (NOB) when temperature decreased (De Clippeleir et al., 2013; Gilbert et al., 2015). Interestingly, other studies achieved a good effluent quality working at NRRs close to those reported for conventional WWTPs in one-stage configurations treating an aerobically pre-treated mainstream wastewater in MBBR at 16 and 15 ºC

(Laureni et al., 2019, 2015). However, they experienced an anammox activity suppression at 11 ºC (Laureni et al., 2015) or needed to daily filter and centrifuge the effluent to reintroduce the solids into the reactor to improve floc retention (Laureni et al., 2019), which could not be implemented at full-scale. Despite one-stage systems gained a lot of interest due to the successful attained results at sidestream conditions and the lower investment costs, two-stage systems have been recognized as a good alternative to avoid the associated problems in terms of nitrate production (i.e. NOB proliferation) and of anammox deterioration (Gonzalez-Martinez et al., 2016; Hendrickx et al., 2012; Pérez et al., 2015). In fact, stable partial nitration with an effective NOB repression has been reported by using granular biomass (Isanta et al., 2015b; Ma et al., 2011; Poot et al., 2016; Reino et al., 2018, 2016; Soler-Jofra et al., 2019). The advantage when splitting the nitrogen removal into two granular sludge reactors is that stratification of nitrifier guilds is feasible (Poot et al., 2016; Soler-Jofra et al., 2019), with ammonia-oxidizing bacteria (AOB) occupying the external shell of the granules whereas NOB are relegated to deeper layers where oxygen is not available. Additionally, the organic matter not totally converted in the pre-treatment could be also consumed before reaching the anaerobic ammonia oxidation reactor. In the subsequent anammox reactor, then, NOB cannot compete for nitrite with anammox because of the anoxic conditions imposed. Recently, further confirmation of the convenience of using the two-stage nitrogen removal approach has been obtained from shotgun metagenomics. Results obtained indicated that the two-stage approach led to the substantial anammox enrichment, making mainstream anammox viable (Annavajhala et al., 2018).

By using the two-stage approach, only few studies reported a long-stable anammox operation treating real wastewater at mainstream conditions. Some studies reported high

NRRs but with reactor set-up that required high recirculation flow rates (Lotti et al., 2014), which prevent scaling-up that technology. Another study reported a three-fold anammox activity decrease when temperature was decreased from 20 to 15 ºC treating a municipal wastewater (Laureni et al., 2015). In addition, high NRRs in an up-flow anammox sludge bed (UAnSB) reactor were reported at 11 ºC suggesting that the high concentrations of biomass in this type of reactor helped to face temperature decrease (Reino et al., 2018). However, NRRs decreased when changing from synthetic to an amended pre-treated mainstream wastewater which resulted into accumulation of substrates in the effluent. In all previous studies, it should be stressed that when the effect of low temperatures was evaluated, nitrogen loading rate (NLR) was decreased to avoid the accumulation of substrates (ammonium and nitrite) due to the decrease of anammox activity. According to the state-of-the-art, one of the main challenges in this technology is still to demonstrate that an anammox reactor can effectively dampen the temperature effects in the mainstream line of an urban WWTP under a roughly constant NLR. This proof of concept should be carried out by: (i) using real mainstream wastewater, (ii) employing a reactor easy to scale-up, (iii) attaining and maintaining a constant NLR higher than those usually reported for urban WWTPs and (iv) performing a realistic temperature decrease throughout a long-term experiment. Additionally, providing the reason why this technology can achieve these objectives would be a clear step forward on its development.

The UAnSB reactor was operated for almost a year as to mimic a (realistic) temperature change from 20 ºC to 10 ºC (with a period of ca. three months at 10 ºC), by working at a constant loading rate treating a real mainstream wastewater throughout the operation. An in-depth characterization of the reactor operation by evaluating substrate distribution along the sludge bed reactor was used to assess the variation of anammox activity contribution. The contribution of heterotrophic denitrification activity to the overall reactor performance was also assessed. Additionally, microbiological population analyses assisted the characterization of the microbial community in the sludge bed at 20 and 10 ºC.

3.2. Materials and Methods

3.2.1. Reactor operation and configuration

A lab-scale UAnSB reactor with an effective working volume of 13 L, excluding the gas-liquid-solid separator phase (12 L), was used for the implementation of the anammox process. The inner diameter of the column was 120 mm and the total-reactor-height to column-diameter ratio was 12:1. The UAnSB reactor of this study was different than the one used in Reino et al. (2018) since the total volume was higher (25 L vs 2 L) and the tube diameter to particle diameter ratio (D/d) was larger (166 vs 70). The change in the reactor design was planned as to minimize potential wall effects, which could lead to a flow maldistribution. The reactor had five different sampling points at heights of 0 m (S1), 0.05 m (S2), 0.16 m (S3), 0.25 m (S4) and 0.36 m (S5) (Fig. 3.1). The pH of the reactor bulk liquid was not controlled but measured off-line. Temperature was maintained by means of a cooling system connected to the reactor jacket. Reactor temperature was continuously monitored and recorded on-line using a Pt1000 sensor (Axiomatic, S.L, Spain) located in the core of the sludge bed. Different operational periods can be distinguished as a function of the reactor temperature (Table 3.1).

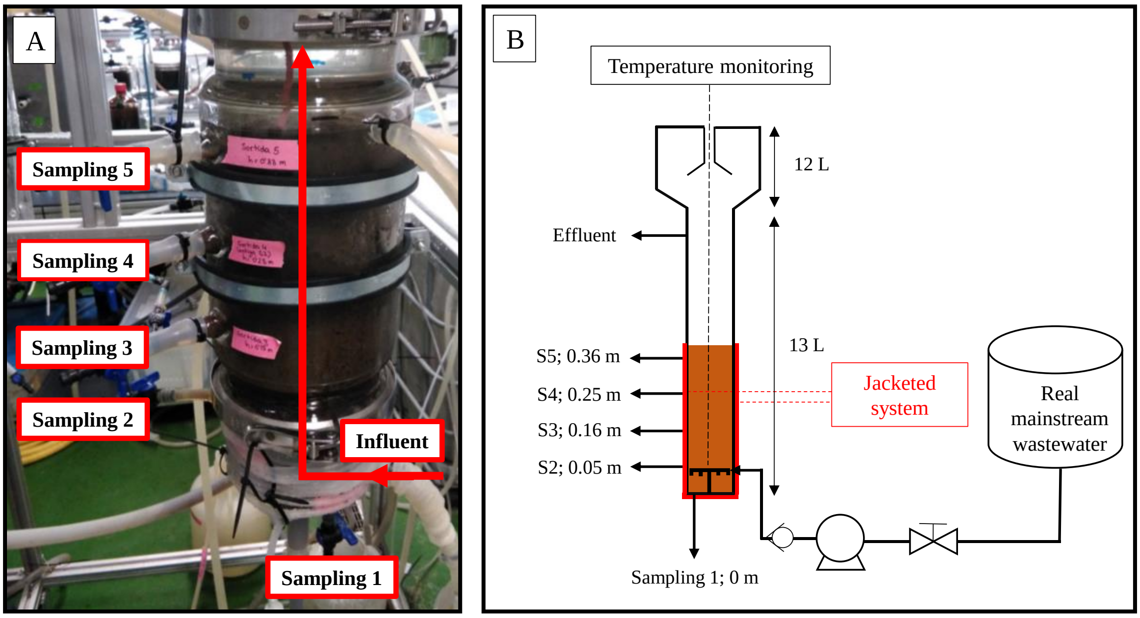

Fig. 3.1. (A) Picture of the lab-scale UAnSB reactor (sludge bed section). **(B)** Schematic diagram of the reactor set-up with the corresponding peripheral instrumentation and sampling points. The fraction of the UAnSB reactor vessel highlighted in solid orange corresponds to the sludge bed. The effective working volume is of 13 L and the gas-liquid-solid separator is of 12 L. Sampling points are denoted as S1, S2, S3, S4 and S5 and the height from the reactor base is provided for each one of them.

3.2.2. Long-term reactor operation and wastewater characteristics

The UAnSB reactor was inoculated with granular biomass from an anammox SBR operated at 33 ± 1 ºC with synthetic wastewater (Isanta et al., 2015a). The inoculum was enriched in *Candidatus* Brocadia fulgida (65 %) estimated by 16S rRNA gene-targeted sequencing analyses. The inoculum presented a maximum specific anammox activity of 0.14 ± 0.01 g N g VS^{-1} h^{-1}. The anammox granules had an average size of 868 ± 20 µm and the biomass concentration was 4.9 ± 0.3 g VS L^{-1}. After inoculation, the UAnSB reactor was operated for ca. one year at 20 ± 1 ºC (data not shown). The objective of this previous experimental period was to find the best operational strategy to obtain high and stable loading rates together with a good effluent quality without the need of using a recirculation flow-rate. At the same time, it was important to ensure the maintenance of granular sludge throughout the start-up period by controlling the up-flow velocities. The mainstream wastewater used in this study came from an urban WWTP located in an industrial area of Catalonia, NE Spain. This wastewater was a mixture of effluents coming from: (i) a high-rate activated sludge reactor (after primary sedimentation) working at sludge retention times (SRTs) of 1-2 days and (ii) a partial nitritation pilot-scale reactor treating reject water from the dewatering process of the digested sludge. Both effluents were mixed in the appropriate proportion to obtain a pre-treated mainstream wastewater suitable to be added into an anammox reactor. The wastewater was stored in a tank at 20 ± 1ºC, which was refilled every 4 to 5 weeks. The tank was flushed with N_2 every time it was refilled to keep DO concentration in the range of 0.3 to 1.3 mg O_2 L^{-1}. Further information about the characteristics of the mainstream wastewater as well as influent concentrations can be found in Table 3.1 and Fig. A3 in Appendix I, respectively.

3.2.3. Calculations

NLRs and NRRs were calculated by using the effective UAnSB reactor volume of 13 L, excluding the gas-liquid-solid separator. NLR was calculated considering the ammonium and nitrite concentrations of the real mainstream wastewater. NRR was calculated as the removal of ammonium and nitrite without considering the nitrate produced in the anammox reaction. However, nitrate production was considered for the quantification of the nitrogen removal efficiency (NRE) to clearly indicate what are the needs for a post-treatment, in case of an eventual full-scale implementation of this technology.

Table 3.1. Characterization of the real mainstream wastewater of the UAnSB reactor. This wastewater was a mixture of effluents coming from: (i) a high-rate activated sludge reactor (after primary sedimentation) working at sludge retention times (SRTs) of 1-2 days and (ii) a partial nitritation pilot-scale reactor treating reject water from the dewatering process of the digested sludge. Both effluents were mixed in the appropriate proportion to obtain a pre-treated mainstream wastewater suitable to be added into an anammox reactor. DO is the dissolved oxygen concentration. COD is the soluble chemical oxygen demand. COD/N corresponds to the ratio of soluble chemical oxygen demand to total nitrogen of the influent.

Parameter	Value	Units
[N-NH_4^+]	26 ± 8	mg N L^{-1}
[N-NO_2^-]	36 ± 9	mg N L^{-1}
[N-NO_3^-]	2 ± 2	mg N L^{-1}
pH	8.1 ± 0.3	-
DO	0.3 to 1.3	mg O_2 L^{-1}
COD	63 ± 15	mg COD L^{-1}
COD/N	1.0 ± 0.2	mg COD mg^{-1} N

3.2.4. Profile concentrations and anammox activity along the sludge bed

Ammonium, nitrite and nitrate concentrations were measured along the different sampling points of the UAnSB reactor at steady state conditions to evaluate nitrogen compounds concentrations distribution on the sludge bed. Volumetric and specific NRRs corresponding to the different sludge bed sections delimited by the sampling points were calculated using the measured ammonium and nitrite concentrations based on Eq. 3.1 and Eq. 3.2. Distribution of sampling points can be found in Fig. 3.1.

Volumetric section nitrogen removal rate (NRR, g N L^{-1} d^{-1}) and specific section nitrogen removal rate (NRR, g N g^{-1} VS d^{-1}) were calculated for each bed section (Eq. 3.1 and Eq. 3.2, respectively).

$$\text{Volumetric NRR } (h_{i+1} - h_i) = \frac{C(h_{i+1}) - C(h_i) \cdot Q}{\text{section volume } (h_{i+1} - h_i)} \quad (\text{Eq. } 3.1)$$

$$\text{Specific NRR } (h_{i+1} - h_i) = \frac{C(h_{i+1}) - C(h_i) \cdot Q}{\text{section volume } (h_{i+1} - h_i)} \cdot \frac{1}{y} \quad (\text{Eq. } 3.2)$$

where $C(h_i)$ and $C(h_{i+1})$ are the ammonium and nitrite concentrations at sampling points i and $i + 1$, respectively in g L^{-1}, Q corresponds to the influent flow rate in L d^{-1} and y corresponds to the biomass concentration being 14 ± 5 g VS L^{-1} along the whole sludge bed. Finally, section volume, in L, is calculated as follows (Eq. 3.3),

$$\text{section volume } (h_{i+1} - h_i) = \pi \cdot r^2 \cdot (h_{i+1} - h_i) \cdot 100 \ (\text{Eq. } 3.3)$$

being h_i and h_{i+1} the heights at the initial sampling i and $i + 1$ in m and r corresponds to the inner radius of the UAnSB reactor in m.

3.2.5. Heterotrophic denitrifying *ex-situ* batch activity tests

Ex-situ batch tests were used to assess heterotrophic activity within the sludge bed following the procedures detailed in van Loosdrecht et al. (2016). Tests were carried out in duplicates using septum-closed bottles of 125 mL with 2.0 ± 0.3 g VS L^{-1} from biomass of sampling S3. All bottles and substrates were flushed with N_2 to maintain anoxic conditions. Tests were conducted at 180 rpm and at 20 ºC. Three sets of experiments were performed: (1) the consumption of nitrite or nitrate without the presence of any external C-source to evaluate heterotrophic denitrification from biomass decay, (2) the consumption of nitrite and/or nitrate in the presence of acetate as an easily biodegradable carbon source under non-limiting chemical oxygen demand (COD) concentrations and (3) the consumption of nitrite and/or nitrate in the presence of acetate as an easily biodegradable carbon source under limiting COD concentrations. Sampling time depended on the consumption rate of substrates in each experiment. Substrate consumption velocities were calculated by linear regression of the nitrite or nitrate concentrations of three to six bulk liquid-phase grab samples. The employed COD/N ratios can be found in Table 3.2. The calculations of the COD/N ratios followed the methodology detailed in van Loosdrecht et al. (2016). To determine the maximum heterotrophic denitrification specific consumption rates (i.e. non-limiting COD concentrations) via nitrate or nitrite at least twice of the stoichiometric COD/N relationship of Eq. 3.4 and Eq. 3.5 was respectively considered. To determine heterotrophic denitrifying activities under limiting COD concentrations via nitrate and/or nitrite, approximately half of the COD/N ratios of Eq. 3.4 and Eq. 3.5 were applied, respectively.

$$\frac{\mathrm{COD}}{\mathrm{N}} = \frac{2.86}{1 - \mathrm{Y_{OHO}}} \qquad \text{(Eq. 3.4)}$$

$$\frac{\mathrm{COD}}{\mathrm{N}} = \frac{1.71}{1 - \mathrm{Y_{OHO}}} \qquad \text{(Eq. 3.5)}$$

where $\mathrm{Y_{OHO}}$ is the heterotrophic anoxic growth yield for acetate 0.66 g COD g^{-1} COD (Ficara and Canziani, 2007).

3.2.6. Analytical methods

Liquid samples from influent and effluent of the UAnSB reactor were analyzed 3-to-4 times per week. Samples were filtered (0.22 μm) before analysis. Nitrite and nitrate concentrations were analyzed off-line with ionic chromatography using ICS-2000 Integrated Reagent-Free IC system (DIONEX Corporation, USA). Ammonium concentrations were analyzed off-line by means of a gas selective electrode (GSE) (AMTAX sc, Hach Lange, Germany). For concentration profiles and for *ex-situ* batch activity tests, concentrations of ammonium and nitrite were determined by colorimetric Hach Lange kits (LCK303 and LCK342, respectively, Hach Lange, Germany). Soluble

COD was analyzed by using kits ranging from 0 to 1500 mg COD L^{-1} (Scharlab, Spain). Total solids (TS) and volatile solids (VS) concentration were analyzed according to Standard Methods (APHA, 2005). Average biomass particle size was measured by a laser diffraction analysis system (Malvern Mastersizer Series 2600, Malvern instruments Ltd., UK).

Table 3.2. Substrates and COD/N ratios used for the *ex-situ* heterotrophic denitrifying batch activity tests performed using acetate as C-source with sludge from sampling S3 of the UAnSB reactor. COD/N ratios were calculated following van Loosdrecht et al. (2016).

Performed test	Day of operation	Nitrite (mg N L^{-1})	Nitrate (mg N L^{-1})	COD (mg COD L^{-1})	COD/N
Only Nitrite	320	24 ± 1	-	-	-
Only Nitrate	320	-	23 ± 1	-	-
Nitrite + acetate (non-limiting COD)	343	49 ± 1	-	873 ± 37	18 ± 1
Nitrite + acetate (limiting COD)	end of operation	35 ± 1	-	158 ± 37	4.5 ± 1.0
Nitrate + acetate (non-limiting COD)	343	-	37 ± 1	879 ± 1	24 ± 1
Nitrate + acetate (limiting COD)	end of operation	-	37 ± 1	160 ± 1	4.3 ± 1.0
Nitrate + nitrite + acetate (limiting COD)	end of operation	24 ± 1	25 ± 1	172 ± 63	3.6 ± 1.3

3.2.7. Microbiological characterization and quantification

Microbiological community composition was identified by using next-generation sequencing analysis. DNA was extracted from three different sampling points (S1, S3 and S5) by using the Soil DNA Isolation Plus KitTM (Norgen Biotek Corp, Canada) following the manufacturer protocol. The quantity and quality of the extracted DNA was measured by using the NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific, USA). A 260/280 nm ratio of 1.8 ± 0.1 was used as quality cut-off and a minimum of 20 ng μL^{-1} of extracted DNA was guaranteed to perform sequencing. Paired-end sequencing of the extracted DNA was performed on an Illumina MiSeq platform by the Research and Testing Laboratory (Lubbock, Texas, USA). Bacterial 16S rRNA variable regions V2-V4 were targeted using the primer pair 515F-806R for general bacteria and the specific primer pair 368F-820R for the anammox population. Additional information of the bioinformatics protocol can be found in Appendix II.

3.3. Results and discussion

3.3.1. Performance of the UAnSB reactor during a summer-to-winter transition period by treating a real mainstream wastewater

i. UAnSB reactor operation

The UAnSB reactor was operated by maintaining a constant NLR (0.11 ± 0.01 g N L^{-1} d^{-1}) for 350 days (Table 3.3). Reactor temperature was controlled to mimic a realistic summer-to-winter transition period as reported to happen in urban WWTPs operated in temperate climates decreasing from 20 to 10 ºC in ca. 3 months (slightly more pronounced than that described in Gilbert et al. (2014)). In addition, the reactor operation was maintained at 10 ºC for c.a. 3 months to evaluate long-term effects of such low temperature in the anammox process. Overall, in the 350 days period the reactor performance was high; in average, the NRR and NRE were 0.10 ± 0.01 g N L^{-1} d^{-1}

and 83 ± 8%, respectively (see the overall performance in “all periods” row, Table 3.3 and Fig. 3.2). These results indicate that the UAnSB reactor was able to perform at rather constant loading rates by maintaining high NRRs during a realistic summer-to-winter temperature decrease (Fig. 3.2). Besides the capacity of the UAnSB reactor to withstand the temperature change as well as the cold temperatures, it is important to highlight that the average NRR achieved (0.10 ± 0.01 g N L^{-1} d^{-1}) was ca. two times higher than that reported for conventional urban WWTPs (Metcalf & Eddy, 2003).

In addition to the overall achievements already highlighted, a more detailed analysis of the experimental data is here also discussed. The reactor operation period was divided into several phases (Table 3.3). For temperatures between 20 and 12°C (periods I and II), the removal of nitrogen at high rates was achieved as reflected by the high NREs of 87 ± 4 %. During period III (10°C), an unintended increase in loading rates resulted in a slight decrease in NREs efficiencies up to 71 ± 6 %. The lack of data between days 182 and 224 corresponds to technical problems in the analysis of nitrogen species. In period IV, temperature was increased back to 20 ºC to proof the stability of the anammox process after long exposure to cold temperatures. During period IV, the UAnSB was operated at similar NLR to those of period III attaining NREs of 78 ± 8 % (Table 3.3). Changes in influent concentrations occurred throughout the operation due to variability of the real wastewater, which resulted into variations of the nitrite to ammonium feeding ratios. Interestingly, the decrease in NREs during period III and IV were related with the high nitrite to ammonium ratio fed into the reactor which led to an excess of nitrite concentrations in the influent (Table 3.3), worsening the performance of the process (see Effluent quality section for further details).

Table 3.3. Operational parameters for the different periods I, II, III, IV and V. NLR: Nitrogen Loading Rate; NRR: Nitrogen Removal Rate; NRE: Nitrogen Removal Efficiency. *Temperature is presented in accordance of the temperature range from 20 to 10 ºC for the whole operation.

Period	Temperature (ºC)	NLR (g N L^{-1} d^{-1})	NRR (g N L^{-1} d^{-1})	NRE (%)	Total effluent nitrogen (mg N L^{-1})	Ammonium effluent (mg N L^{-1})	Nitrite effluent (mg N L^{-1})	Nitrate effluent (mg N L^{-1})	Nitrite/ ammonium consumed	Nitrate produced/ ammonium consumed	Nitrite/ ammonium influent	Up-flow velocity (m h^{-1})
all periods	15 ± 5*	0.11 ± 0.01	0.10 ± 0.01	83 ± 8	11 ± 4	3 ± 3	3 ± 3	5 ± 3	1.4 ± 0.2	0.19 ± 0.05	1.4 ± 0.2	0.08 ± 0.02
I (0-81)	20	0.10 ± 0.02	0.10 ± 0.02	87 ± 3	10 ± 3	2 ± 2	2 ± 2	6 ± 2	1.4 ± 0.1	0.20 ± 0.03	1.3 ± 0.2	0.07 ± 0.01
II (81-155)	19 to 12	0.09 ± 0.01	0.09 ± 0.01	86 ± 5	9 ± 2	4 ± 2	1 ± 1	7 ± 2	1.3 ± 0.1	0.23 ± 0.04	1.3 ± 0.1	0.07 ± 0.02
III (155-260)	10	0.11 ± 0.01	0.09 ± 0.01	71 ± 6	15 ± 4	4 ± 2	7 ± 4	4 ± 2	1.5 ± 0.2	0.20 ± 0.05	1.4 ± 0.2	0.10 ± 0.01
IV (255-350)	20	0.11 ± 0.02	0.09 ± 0.02	78 ± 8	11 ± 4	3 ± 2	5 ± 4	3 ± 2	1.4 ± 0.2	0.10 ± 0.01	1.4 ± 0.2	0.10 ± 0.01

ii. Effluent quality

Ammonium, nitrite, nitrate and total nitrogen concentrations in the effluent throughout the study were plotted in Fig. 3.2. The total average effluent nitrogen concentration was slightly higher (11 ± 4 mg N L^{-1}) than legal discharge limits according to EU legislation (i.e. 10 mg N L^{-1}) (Table 3.3). The UAnSB reactor was almost able to provide a suitable effluent for an urban WWTP according to the European legislation. Nevertheless, further strategies should be implemented to guarantee a good effluent quality. One option might consist of optimizing the previous partial nitritation stage to provide a suitable nitrite to ammonium ratio for the subsequent anammox process (Guo et al., 2020; Pérez et al., 2015). In fact, the results of this study pointed out that a non-suitable nitrite to ammonium feeding ratio had a direct effect on the effluent quality of the UAnSB reactor. This was especially important on periods III and IV, where rather high total nitrogen concentrations were detected in the effluent (15 ± 4 mg N L^{-1} and 11 ± 4 mg N L^{-1}, respectively) especially in the form of nitrite (7 ± 4 mg N L^{-1} and 5 ± 4 mg N L^{-1}, respectively) (Table 3.3). Further data concerning the nitrite to ammonium feeding ratios can be found in Appendix I (see Fig. A1). Interestingly, another reason for the high nitrogen concentrations in the effluent could be related with preferential ways or to external mass transfer limitations, although with the present analysis this remains speculative. According to the overall effluent nitrate concentrations (i.e. 5 ± 3 mg N L^{-1}), a post-treatment by adding an external C-source to enhance heterotrophic denitrification within the UAnSB reactor is deeply investigated in Chapter 4.

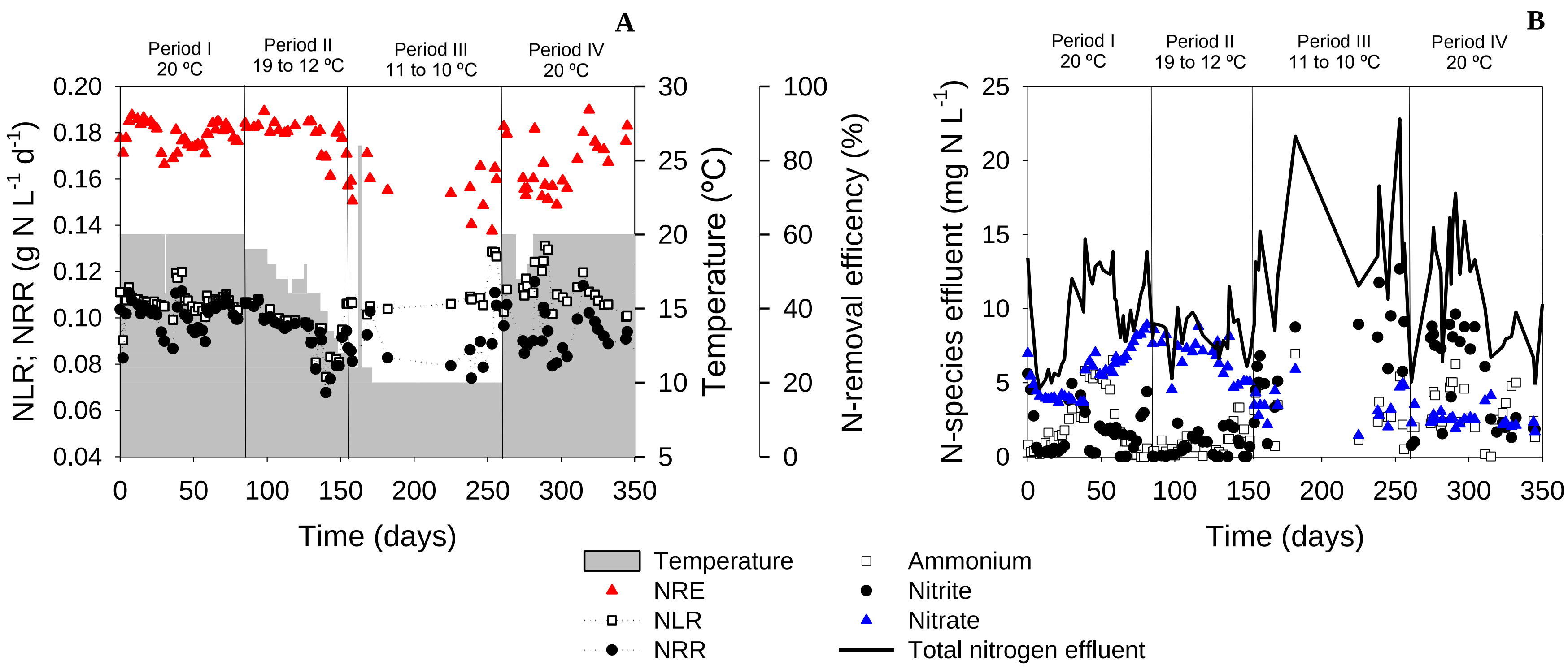

Fig. 3.2. Long-term operation of the UAnSB reactor at mainstream conditions at different temperatures. (A) Nitrogen loading and removal rates (NLRs and NRRs, respectively) and nitrogen removal efficiencies (NREs). (B) Effluent concentrations of ammonium, nitrite and nitrate. Total nitrogen concentrations in the effluent refer to the sum of ammonium, nitrite and nitrate concentrations.

iii. Solids, granule size and up-flow velocity

Throughout the reactor operation period, the average biomass concentration was 14 ± 5 g VS L^{-1} with a VS/TS ratio of 0.4 ± 0.1. Mean average size of the granules was maintained stable throughout the operation within the different sampling points (see Fig. 3.3) despite working at low up-flow velocities (0.08 ± 0.02 m h^{-1}) and at temperatures as low as 10 ºC (Table 3.3). It should be emphasized that the up-flow velocities of periods III and IV were slightly higher than those of period I and II (Table 3.3). Maintaining roughly constant loading rates throughout the operation was challenging since variations in influent concentrations had to be correspondingly compensated by manipulating the influent flow rate (see Fig. A3 for influent concentrations in Appendix I). Thus, this resulted into slight variations of the applied up-flow velocities.

The obtained results are in contrast to those reported by Reino and Carrera (2017), who suggested to operate at up-flow velocities higher than 0.4 m h^{-1} in an UAnSB reactor to maintain granulation. Interestingly, the applied up-flow velocities within the UAnSB reactor were significantly lower than those conventionally applied to up-flow anaerobic sludge bed (UASB) reactors used in anaerobic digestion (0.7 m h^{-1}) (Metcalf & Eddy, 2003). The reasons for the stability of the granule structure (in average 717 ± 43 µm, see Fig. 3.3) were probably linked to the reactor design used. The rather high H/D ratio (12:1) selected could have been favourable to preserve granule morphology and, thus, avoid biomass loss. The obtained results are in accordance with different studies that reported granule stability at low temperatures within an UAnSB reactors (He et al., 2018; Ma et al., 2013; S. Wang et al., 2020), while granule deterioration occurred in an SBR-anammox reactor (Sánchez-Guillén et al., 2016). This is a significant fact since the obtained results indicate that anammox granulation can be maintained without the need of applying high up-flow velocities, which can only be achieved by applying high recirculation flow rates, and thus, resulting into unattainable operation costs at mainstream conditions.

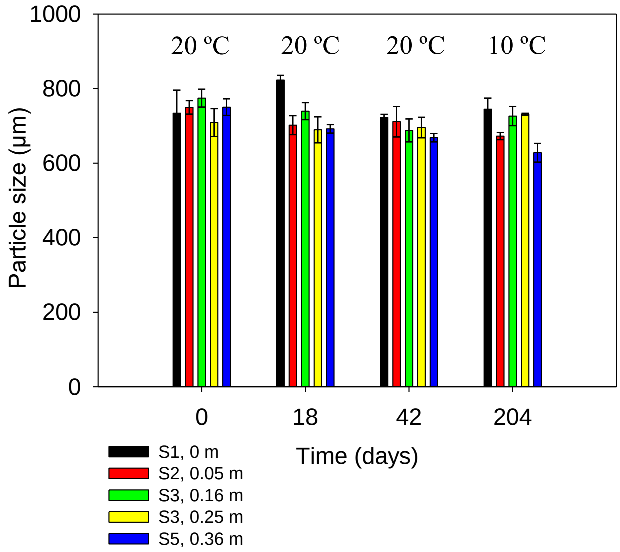

Fig. 3.3. Particle size at different reactors sampling points (S1, S2, S3, S4 and S5) of the UAnSB reactor at 20 and 10 ºC.

3.3.2. Heterogeneous distribution of the anammox activity in the sludge bed as the key point to face low temperatures at mainstream conditions

The anammox activity within the different sludge bed sections was calculated using the nitrogen compounds concentrations measured along the sampling points of the UAnSB reactor. These activities were quantified as the specific NRR achieved in each sludge bed section and they were calculated under steady state conditions at periods I (20ºC), III (10ºC) and IV (back to 20ºC) (Fig. 3.4).

At 20 ºC (period I), the specific NRR associated to the S1-S2 sludge bed section (100 ± 14 mg N g^{-1} VS L^{-1}) (see Fig. 3.1 for a detailed situation of this section in the UAnSB) was ca. 50 times higher than those specific NRRs determined for upper sludge bed sections (< 4 mg N g^{-1} VS L^{-1}) (Fig. 3.4). This fact is correlated with the almost complete absence of nitrite from section S4 onwards, resulting into substrate limitation for anammox biomass of upper sludge bed sections (Fig. 3.4). Thus, there was a significant amount of biomass not contributing to the overall anammox activity at high temperatures.

At 10 ºC (period III), the specific NRR from sludge bed section S1-S2 showed a significant decrease (63 ± 14 mg N g^{-1} VS L^{-1}) from that obtained at 20 ºC, resulting into a higher substrate availability for upper sludge bed sections (Fig. 3.4). The increase of substrate availability resulted into an activation of the starved anammox biomass of middle and upper sludge bed sections. Consequently, almost a 10-fold increase in the specific NRR was detected within sludge bed sections S2 onwards (ranging from 4 to 14 mg N g^{-1} VS L^{-1}) (Fig. 3.4). Therefore, the biomass activity decrease of the bottom sludge bed section (from 20 to 10 ºC) was counterbalanced by the activity increase of middle and upper sludge bed sections at 10 ºC. That means that the sludge bed of the UAnSB reactor presented an overcapacity that allowed to dampen a 10 ºC temperature drop while operating at high and constant nitrogen loading rates.

When temperature was increased back to 20 ºC (period IV), the specific NRR within S1-S2 sludge bed section increased up to 110 ± 9 mg N g^{-1} VS L^{-1} (Fig. 3.4), in the range of those of period I, confirming that activity within the bottom sludge bed was fully recovered after a long-period exposure to low temperatures. However, middle and upper sludge sections (from S2 to S4) did not decrease their specific NRR but maintained in the range to those values obtained at 10 ºC (Fig. 3.4). This might be related to the fact that the applied NLRs of period IV were slightly higher than the ones applied in period I (Table 3.3). This was translated into lower NREs and, thus, into higher residual ammonium and nitrite concentrations to middle and upper sections of the anammox sludge (Fig. 3.4). Further, the nitrite to ammonium stoichiometry ratios within top sludge sections of period IV were more favorable for the anammox reaction than the ones of period I (i.e. where nitrite was limiting), and this resulted into a higher specific activity within upper sludge bed sections.

The significant amount of biomass concentration (14 ± 5 g VS L^{-1}) of the sludge bed and the low up-flow velocities applied in the UAnSB reactor resulted into a heterogeneous substrate distribution along the sludge bed; that is, into a plug-flow substrate hydrodynamic pattern. Different authors suggested that this hydrodynamic pattern could limit the practical application of an UAnSB reactor because it could lead to have a pull of biomass exposed to substrate limitation, and thus, to a lower reactor performance (H. Ma et al., 2017; Strous et al., 1998). However, the overall NRR of the UAnSB did not decrease throughout the operation but maintained rather constant even after a long-term exposure to low temperatures (Fig. 3.2), in contrast of what should have been expected from the temperature dependence of the anammox reaction rate (Hendrickx et al., 2012; Lotti et al., 2015b; Sobotka et al., 2016). Therefore, the heterogenous substrate concentrations gradients within the anammox granular sludge bed granted the assistance of a sludge bed overcapacity that has been crucial to dampen a 10 ºC drop by working at high and constant loading rates.

3.3.3. Assessment of the heterotrophic denitrifying activity within the UAnSB reactor

The mainstream wastewater treated in this study contained an organic matter concentration of 60 ± 14 mg COD L^{-1} and a COD/N ratio of 1.0 ± 0.1 mg COD mg^{-1} N. Throughout the operational period, a COD removal efficiency of 60 ± 16 % was achieved in the UAnSB reactor. This result is in accordance with nitrogen mass balances of the UAnSB reactor (Fig. 3.5), which indicated a contribution of heterotrophic denitrification (5 ± 5 %) in the total nitrogen removal. Likewise, throughout the operational period, the produced nitrate to consumed ammonium ratio (Table 3.3) was lower than the proposed by Strous et al. (1998), indicating that part of the produced nitrate by anammox was being heterotrophically denitrified.

To characterize the heterotrophic activity of the sludge bed, *ex-situ* heterotrophic batch activity tests were performed using nitrite and/or nitrate at different COD/N ratios with sludge from sampling S3 (see Table 3.2).

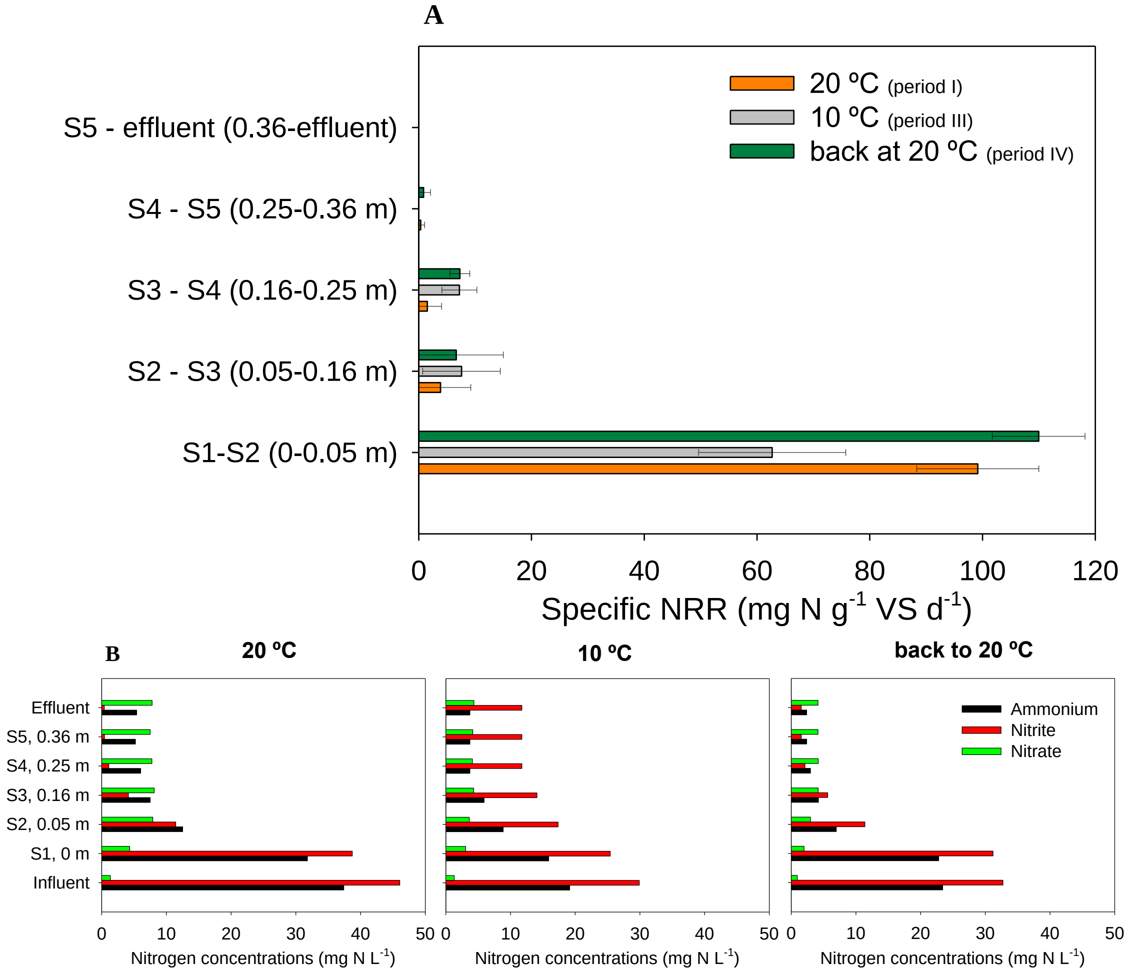

Fig. 3.4. (A) Average specific nitrogen removal rates (NRRs) and **(B)** nitrogen compounds concentrations corresponding to different sludge bed heights sections of the UAnSB reactor delimited by the sampling points (see Fig. 3.1) at different operation periods and temperatures. Nitrogen concentrations at 20 ºC, 10 ºC and back at 20 ºC correspond to days 42, 239 and 282, respectively.

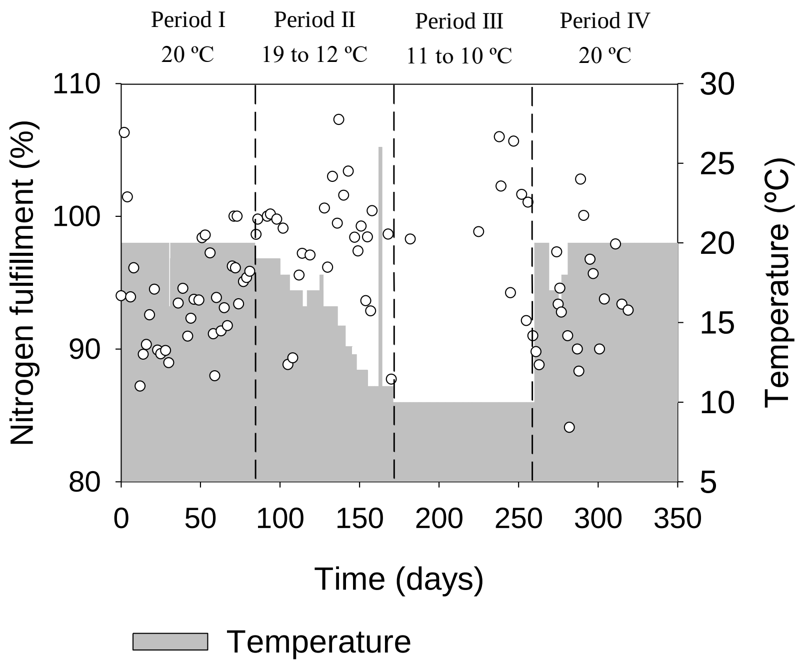

Fig. 3.5. Fulfillment of the nitrogen balance of the UAnSB reactor operation treating a real mainstream wastewater at each temperature period.

The heterotrophic denitrifying activities from decay products (i.e. without addition of an external C-source) were hardly detected while the activities achieved with the addition of acetate as external C-source ranged from 3 to 13 mg N g^{-1} VS d^{-1} depending on the electron acceptor (nitrite or nitrate) and on the employed COD/N ratio (Fig. 3.6). At low COD/N ratios, the heterotrophic activities achieved with nitrite, nitrate or a mix of both electron acceptors were almost the same (Fig. 3.6), indicating that heterotrophic bacteria did not present a preference for any of these two electron acceptors under limiting COD conditions. However, at high COD/N ratios, the heterotrophic activity achieved with nitrate was almost twice than the achieved with nitrite as electron acceptor (Fig. 3.6), indicating that heterotrophic bacteria seem to prefer nitrate under non-limiting COD conditions. This fact is interesting as it could enhance effluent quality by reducing part of the produced nitrate without competing with anammox for nitrite. Heterotrophic bacteria could outcompete anammox bacteria due to their higher growth rates (Güven et al., 2005;

Lackner et al., 2008; Leal et al., 2016; Molinuevo et al., 2009; Pijuan et al., 2020). Further, the presence of several organic compounds present in real wastewaters may hinder anammox activity (Güven et al., 2005; Molinuevo et al., 2009). However, in this study, the detected heterotrophic denitrifying activities were five to three times lower than the overall NRRs measured within the UAnSB reactor regardless the reactor temperature (i.e. at 20 or 10 ºC) (Fig. 3.6), even under non-limiting COD concentrations, which are supposed to enhance heterotrophic denitrifying activity (van Loosdrecht et al., 2016). Therefore, anammox activity dominated the overall nitrogen conversion while heterotrophic denitrifying activity slightly contributed to improve effluent quality by mainly decreasing the nitrate concentrations produced by the anammox process.

3.3.4. Microbiological characterization and quantification

The microbial community composition was studied by 16S rRNA gene-targeted sequencing analyses when UAnSB reactor reached steady state conditions. Six different libraries of reactor sampling points S1, S3 and S5 (see Fig. 3.1 for a detailed situation of these sampling points) at operational days 17 (20 ºC) and 203 (10 ºC) were constructed. The total number of sequences for each library after quality analysis and removal of low-quality sequences can be found in Appendix II (see Fig. A8). All libraries presented an average length of 290 bps per sequence. The microbial community for both temperatures and for the three sampling points was dominated by species inside the Planctomycetes phylum, ranging from 12 to 36 % (Fig. 3.7). The unclassified Planctomycetes sequence was run against BLAST and matched with an uncultured Planctomycetes (MG099764) found in a CANON bioreactor acclimated from high to low temperatures (Gonzalez-Martinez et al., 2016). The genus *Candidatus* Brocadia was also identified at both temperatures and at different reactor sampling points with an abundance from 5 to 19 %. Accordingly, the results of the specific primer used to identify the different species among the anammox population pointed out that the major abundance corresponded to the *Candidatus* Brocadia genus, while an increase of *Candidatus* Kuenenia was observed at low temperatures (Fig. 3.7). These results agree with the experimental observations indicating that the anammox process dominates the nitrogen conversions in the reactor. In addition, different abundances of heterotrophic denitrifying bacteria as *Ignavibacterium* (Chlorobi phylum) (8 to 14 %) and of Caldilineales order (4 to 5 %) were detected at 20 ºC at all the different sampling points. Also, a small abundance of other Chloroflexi (3 to 5 %) was detected at 20 ºC (Fig. 3.7). Interestingly, the decrease of temperature seemed to decrease the percentage of heterotrophic denitrifying bacteria whereas the percentage of anammox bacteria increased. This is in agreement with He et al. (2018) who found a higher abundance of Planctomycetes after decreasing temperature from 33 to 13 ºC in an UASB reactor, pointing out that anammox microbes had been adapted to cold temperatures.

The obtained sequencing analyses are in accordance with other studies where a community of heterotrophic denitrifying bacteria were detected within anammox reactors. In fact, it seems that Chlorobi bacteria may degrade and catabolize extracellular peptides bound in the EPS matrix of anammox bacteria while respiring the nitrate produced by anammox bacteria (Lawson et al., 2017). Similarly, a high abundance of *Ignavibacterium* was found after analysing different one- and two-stage partial nitritation/anammox (PN/AMX) reactor configurations at main and side-stream conditions (Annavajhala et al., 2018). Likewise, it was found that the second highest abundance in a full-scale partial-nitritation-anammox reactor corresponded to Chlorobi bacteria, indicating that these organisms could cooperate with Brocadia *sp* in anammox wastewater treatment systems (Speth et al., 2016).

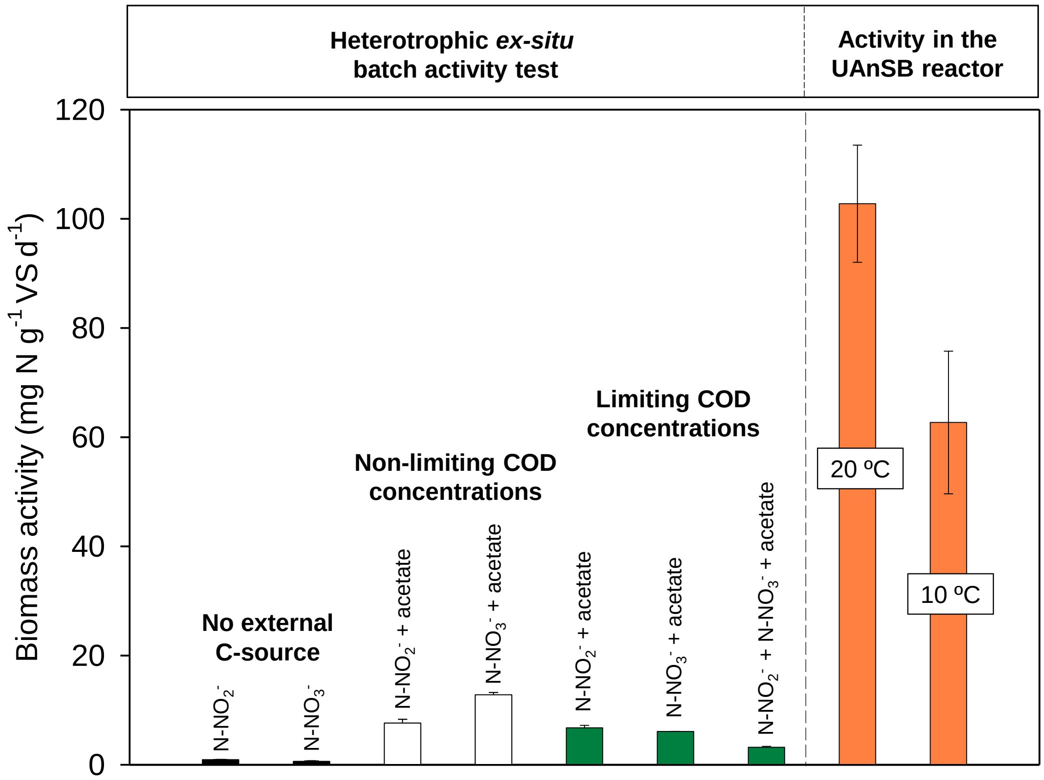

Fig. 3.6. Specific heterotrophic denitrifying activities at different COD/N ratios from *ex-situ* batch tests conducted at 20 ºC employing biomass from sampling S3. Average specific nitrogen removal rates (NRRs) within the UAnSB reactor at 20 and 10 ºC are also depicted. The applied COD/N ratios and the operational days of each batch test can be found in Table 3.2.

The coexistence of uncultured Chloroflexi bacteria was also found in anammox reactors fed with synthetic medium (i.e. no external organic matter), pointing out that these bacteria degrade and use cellular components produced by anammox bacteria while reducing nitrite and/or nitrate (Kindaichi et al., 2012). As suggested by literature, the hydrolysis of extracellular compounds bound in the extracellular polysaccharides (EPS) of anammox bacteria by Chlorobi and Chloroflexi bacteria could provide short-chain volatile fatty acids (VFAs) and alcohols which would support other microbial communities as Proteobacteria (Bhattacharjee et al., 2017). Those findings would support the obtained results found in this study since a significant abundance of Proteobacteria (7 to 10 %) and more specifically of *Denitratisoma* genus (7 to 8 %) was detected at 20 ºC, while its abundance decreased at 10 ºC. *Denitratisoma* is able to grow using different fatty acids using nitrate as electron acceptor (Fahrbach et al., 2006). As mentioned, different heterotrophic denitrifying bacteria can grow from decay products or hydrolysis of EPS which might be enhanced by the residual COD concentration from the pre-treated mainstream wastewater. As suggested by previous reports, the metabolic contribution of denitrifying bacteria in terms of the nitrogen cycle is expected to be from nitrate. These would be in accordance with the operational results of the UAnSB reactor where a COD consumption together with a lower yield of nitrate produced to ammonium consumed was observed (see Table 3.3), indicating that nitrate reduction was taking place.

Despite the UAnSB reactor presented high diversity according to biological diversity indices (see Fig. A7 in Appendix II), high NREs were attained throughout the operation. Similarly, a two times higher anammox bacteria abundance was found in a suspended than in an attached anammox reactor although both reactors presented similar NREs, suggesting that engineering reactors do not need to be highly enriched in anammox bacteria to achieve high removal efficiencies, endorsing the feasibility of mainstream anammox at full-scale conditions (Bhattacharjee et al., 2017). Additionally, biological diversity indices as well as microbial communities did not present significant differences among the different sludge bed sections. This agrees with the reported UAnSB sludge bed reactor overcapacity, which indicated that anammox activity persisted within middle and upper sludge bed sections despite a long-term exposure to starvation conditions.

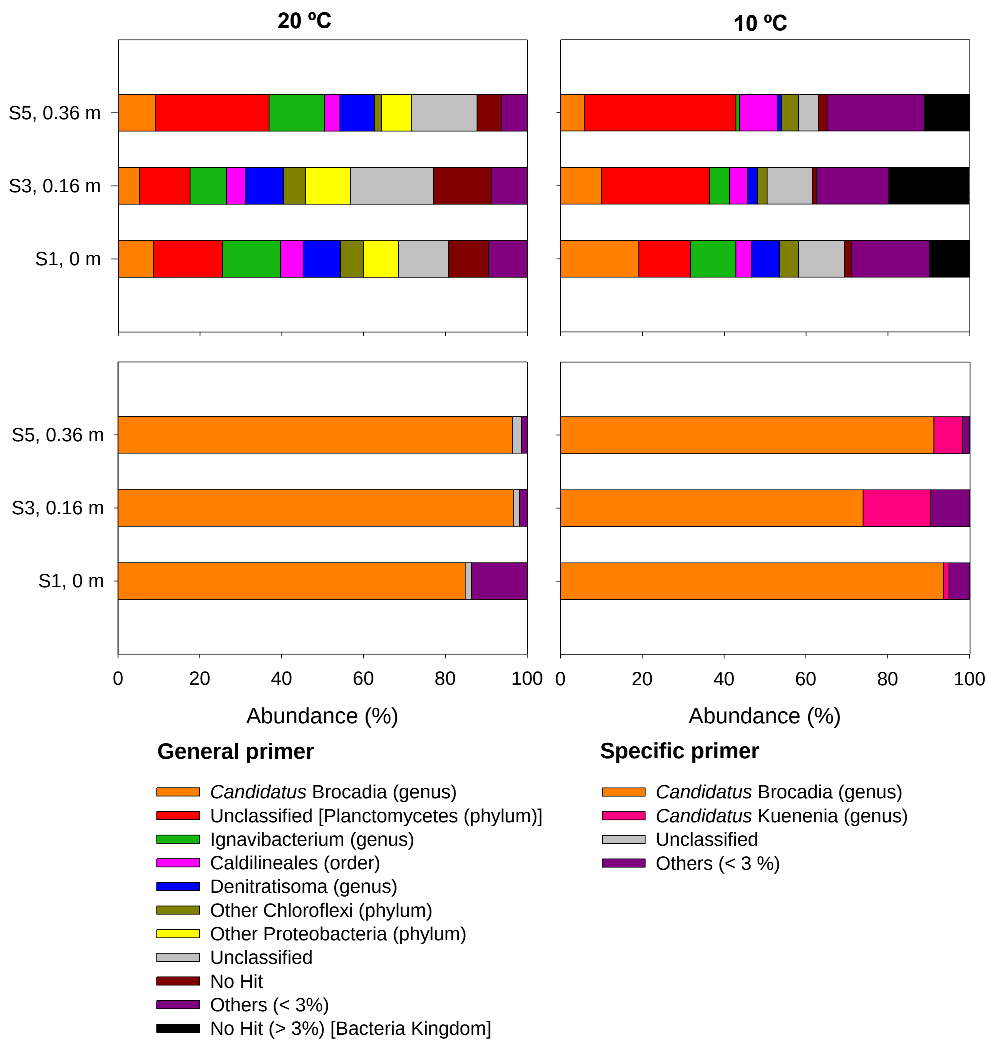

Fig. 3.7. Microbial diversity using the 515F-806R primer (top) and the specific primer 368F-826R (bottom) of libraries at 20 ºC (day 17) and at 10 ºC (day 203) at different UAnSB reactor heights: 0 m (S1), 0.16 m (S3) and 0.36 m (S5). Relative abundance was calculated only considering those microorganisms in which the number of 16S copies was higher than 3 % of the total copies.

3.4. Conclusions

- An UAnSB reactor treating mainstream wastewater fed at nearly constant loading rates (0.11 ± 0.01 g N L^{-1} d^{-1}) fully damped the effects of a 10ºC temperature drop, by roughly maintaining stable removal rates (ca. 0.10 ± 0.01 g N L^{-1} d^{-1}).

- The anammox activity differed along the UAnSB reactor, being concentrated at the bottom sludge bed section, yielding heterogeneous substrate profiles along the sludge bed.
- The biomass not contributing to the overall activity at high temperatures (i.e. the so-called reactor overcapacity) assisted to maintain high nitrogen removal rates at low temperatures (ca. 10ºC, three months).
- Due to the presence of COD in the mainstream wastewater, heterotrophic denitrification activity was detected in the sludge bed. However, anammox activity dominated the overall nitrogen conversion, even at low temperatures.

Chapter 4. Coupling anammox and heterotrophic denitrification activity at mainstream conditions in a single reactor unit

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Abstract

Mainstream partial nitritation/anammox (PN/AMX) have attracted large attention in the last decade. The two-stage PN/AMX process has been pointed out as a more advantageous technology for mainstream anammox than one-stage systems. However, the process requires of an efficiency improvement by designing a new technology to remove the nitrate produced by anammox bacteria. This study has developed a new process by coupling anammox to heterotrophic denitrification in a single reactor unit by avoiding competition between both processes. The addition of acetate as an external C-source enhanced heterotrophic denitrifying activity, allowing to reduce the nitrate produced by anammox bacteria, without hampering the anammox process. Further, a proper organic load management showed to be effective to face the drawbacks derived from previous mainstream treatment stages (i.e. undesired nitrate production), without compromising organic effluent quality. The combination of anammox and heterotrophic denitrifying in a single reactor unit was possible as anammox activity dominated within bottom sludge bed sections, while heterotrophic denitrification occurred within middle and upper sludge sections. Microbial diversity results of 16S rRNA gene-targeted sequencing analyses confirmed that anammox and heterotrophic denitrifiers communities occupied two differentiated sludge bed sections along the reactor. N_2O emissions were lower when heterotrophic denitrification occurred.

4.1. Introduction

Anammox bacteria oxidize ammonium by using nitrite as electron acceptor to produce nitrogen gas (Strous et al., 1998). The implementation of the anammox process at mainstream conditions offers the possibility to move from energy consuming to neutral (or even positive) wastewater treatment plants (WWTPs) (Kartal et al., 2010). Compared to conventional nitrification/denitrification, nitrogen removal via the anammox process allows to reduce oxygen consumption by a 60 % and does not requires any organic C-source. The treatment of municipal sewage via anammox-based processes allows the uncoupling of organic matter (i.e. chemical oxygen demand, COD) and nitrogen removal. A widely accepted configuration for the implementation of the anammox process at mainstream conditions is the adsorption/bio-oxidation (A/B) process (Böehnke, 1977; Böehnke et al., 1998; Versprille et al., 1984). The incoming organic matter is captured within the A-step via a physical-chemical or a biological treatment for its subsequent energy production via anaerobic digestion (Jetten et al., 1997; Kartal et al., 2010; Siegrist et al., 2008). The remaining nitrogen is autotrophically removed in the B-stage by the partial nitritation/anammox (PN/AMX) process. To date, two-stage PN/AMX systems have been pointed out as a feasible configuration for the implementation of the B-stage process. By using the two-stage approach, high and stable nitrogen removal rates (NRRs) has been reported at temperatures as low as 11 ºC (Laureni et al., 2015; Reino et al., 2016, 2018 and Chapter 3). The maximum nitrogen removal efficiency of the process is limited by the nitrate produced by anammox bacteria (up to 10% of the influent nitrogen). The increasing stringent discharge limitations require of a further increase in the process efficiency by designing a process that includes the removal of the nitrate produced by anammox bacteria. The conventional process upgrade would be to use an additional reactor unit for heterotrophic denitrification by adding an external C-source (e.g., acetate). An efficient design of this last treatment unit might also contribute to mitigate the negative effects of (already identified) process challenges such as the instability of the previous PN reactor during short periods of time (for instance, nitrate production at the lowest temperatures in winter (Soler-Jofra et al., 2019)); potential nitrate production in the A-stage during warm seasons (Duan et al., 2019; Zhang et al., 2021); non-ideal nitrite to ammonium feeding ratios to the anammox reactor (see section 3.3.1 in Chapter 3) or the daily nitrogen loads oscillations of the main water line (Pérez et al., 2015).

Instead of adding an additional reactor unit, an appealing alternative would be to add the external C-source in the anammox reactor. The combination of anammox and heterotrophic denitrification in a single reactor unit would allow to enhance the robustness of the PN/AMX process at mainstream conditions. Nevertheless, heterotrophic denitrifying bacteria might deteriorate the anammox process, as they present higher growth rates and could outcompete anammox bacteria utilizing nitrite as electron acceptor (J. Li et al., 2020). Also, high COD/N ratios and long-term exposure to organic substrates might result into a loss of anammox activity (Pijuan et al., 2020; Zhang et al., 2015).

An ad hoc reactor design has been developed to convert an Up-flow Anammox Sludge Bed (UAnSB) reactor into a reactor unit with minimal nitrate production (i.e. CANDLE, **C**oupling **A**nammox and **D**enitrification in a sing**LE** unit). The proposed configuration enabled the use of anammox and heterotrophic denitrifying bacteria in a single unit by preventing competition for nitrite among both groups of bacteria. The CANDLE reactor was operated for ca. 200 days at temperatures ranging from 20 to 14 ºC by treating a real mainstream wastewater. Substrate distribution along the sludge bed sections was investigated to determine the distribution of the anammox and heterotrophic denitrifying activities in the reactor. Nitrous oxide (N_2O) emissions were monitored, whereas process reactor performance was complemented by 16S rRNA gene-targeted sequencing analyses for the characterization of the microbial community.

4.2. Materials and methods

4.2.1. Reactor operation and configuration

An UAnSB reactor with an effective working volume of 13 L, excluding the gas-liquid-solid (GLS) separator phase (12 L), was converted into a CANDLE reactor for the combination of the anammox and heterotrophic denitrifying activity in a single reactor unit. The inner diameter of the column was 120 mm and the total-reactor-height to column-diameter ratio was 12:1. The CANDLE reactor was previously operated as an UAnSB reactor for 350 days at temperatures ranging from 20 to 10 ºC by treating a real mainstream wastewater (see Chapter 3). The reactor had five different sampling points at heights of 0 m (S1), 0.05 m (S2), 0.16 m (S3), 0.25 m (S4) and 0.36 m (S5) (Fig. 4.1). The main difference to the UAnSB reactor is that the CANDLE reactor presents two inlets with the corresponding diffusors located (1) in the bottom section to feed a real mainstream wastewater and (2) at 0.16 m from the reactor bottom (i.e. at the height of sampling S3) to feed an external C-source (i.e. sodium acetate) (Fig. 4.1). Reactor temperature was continuously monitored and recorded on-line using a Pt1000 sensor (Axiomatic, S.L, Spain) located in the core of the sludge bed. CANDLE reactor operation was divided in four periods.

- **Period I**. To evaluate reactor performance without the addition of an external C-source, i.e. only mainstream wastewater addition at 20 ºC. This period will be used as base line in terms of reactor performance for the assessment of the effects of adding an external C-source. During this period disturbances were applied in the inflow with regards to: (i) the nitrite to ammonium ratio compared to the theoretically desired for the anammox process; (ii) the inflow nitrate concentrations, mimicking a low performance of the previous treatment stages (e.g., nitrification during carbon removal in the A-stage and nitrate produced in the PN reactor at low temperatures).

- **Period II**. Reactor performance was evaluated by adding acetate as an external C-source at 20 ºC. Additionally, the same disturbances described in period I were applied. Further, N_2O emissions were assessed under optimal and limited COD conditions for heterotrophic denitrification.
- **Period III**. To re-evaluate reactor performance with only mainstream wastewater addition at 20 ºC. This experimental period is designed to analyse the potential negative effects of the external COD addition on the anammox process after stopping the previous acetate addition (i.e. period II). Additionally, it was also used to assess the N_2O emissions without external COD addition, for enabling comparison with periods in which the external organic source was fed to the reactor.
- **Period IV**. Reactor performance was assessed when adding acetate whereas a temperature decrease from 20 to 14 ºC was applied, to explore the influence of temperature on both process (anammox and heterotrophic denitrification) taking place in a single reactor unit. N_2O emissions were also evaluated at 17 ºC under over-dimensioned COD conditions.

4.2.2. Wastewater characteristics

The CANDLE reactor was fed with real mainstream wastewater coming from an urban WWTP located in an industrial area of Catalonia, NE Spain. As an effluent coming from a PN reactor at mainstream conditions was not available at the time of this study, nitrite or ammonium were added according to the needs of anammox stoichiometry. Ammonium and nitrite were added as $(NH_4)_2SO_4$ and $NaNO_2$, respectively to maintain a ratio between the nitrite and ammonium concentrations of 1.2 ± 0.2. Further characterization of the real mainstream wastewater after the ammonium and nitrite addition is provided (see Table 4.1). The real mainstream wastewater was stored in a tank within lab-facilities. The tank containing the influent was flushed with N_2 every time the tank was refilled (3 – 5 weeks) to keep DO concentration in the range of 0.2 to 2 mg O_2 L^{-1} and maintained at 20 ± 1°C. The stored wastewater experienced some degree of nitrification, which allowed to simulate a potential nitrate production of the previous mainstream treatment stages. Consequently, variable nitrate concentrations were measured in the inflow real mainstream wastewater throughout reactor operation, as well as variations in the feeding ammonium/nitrite concentration ratio, diverging from the theoretical one desired for the anammox process (see Fig. 4.2 and Fig. A4 in Appendix I, respectively).

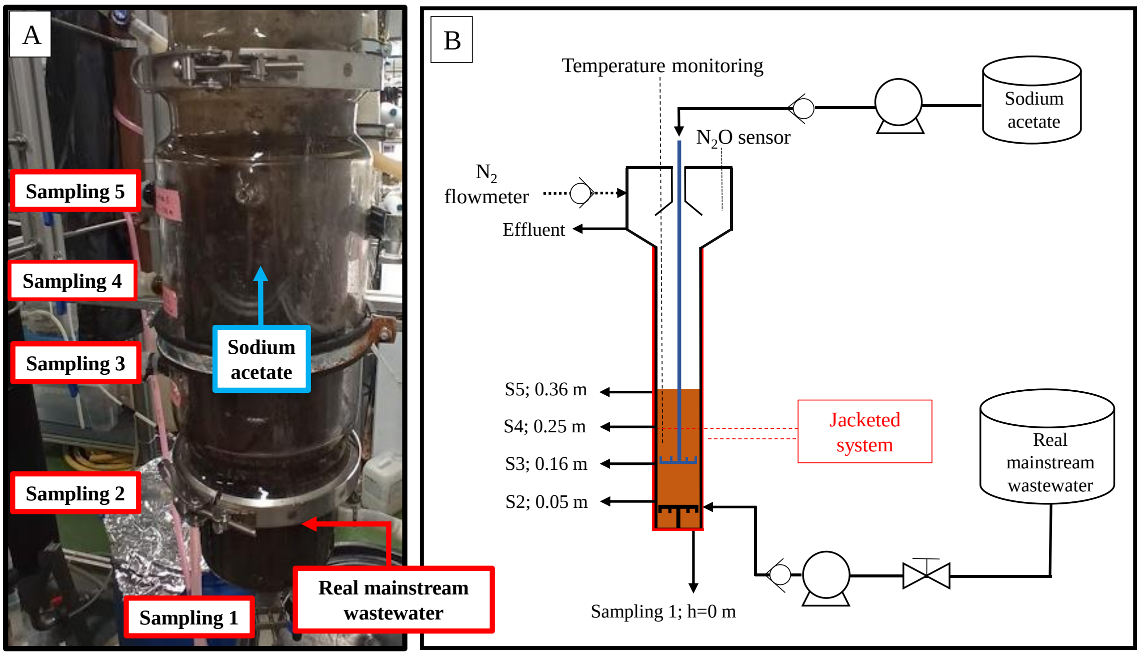

Fig 4.1. (A) Picture of the lab-scale CANDLE reactor (sludge bed section). **(B)** Schematic diagram of the reactor set-up with the corresponding peripheral instrumentation, feeding and sampling points. The fraction of the CANDLE reactor vessel highlighted in solid orange corresponds to the sludge bed. Sampling points are denoted as S1, S2, S3, S4 and S5 and the height from the reactor base is provided for each one of them.

Table 4.1. Characterization of the ammonium and nitrite amended real mainstream wastewater of the CANDLE reactor. DO is the dissolved oxygen concentration. COD_T and COD_S correspond to the total and soluble chemical oxygen demands, respectively.

Parameter	Value	Units
[N-NH_4^+]	24 ± 5	mg N L^{-1}
[N-NO_2^-]	27 ± 7	mg N L^{-1}
[N-NO_3^-]	see Fig. 4.2	-
DO	0.2 to 2	mg O_2 L^{-1}
COD_T (influent)	40 ± 5	mg COD L^{-1}
COD_S (influent)	38 ± 7	mg COD L^{-1}

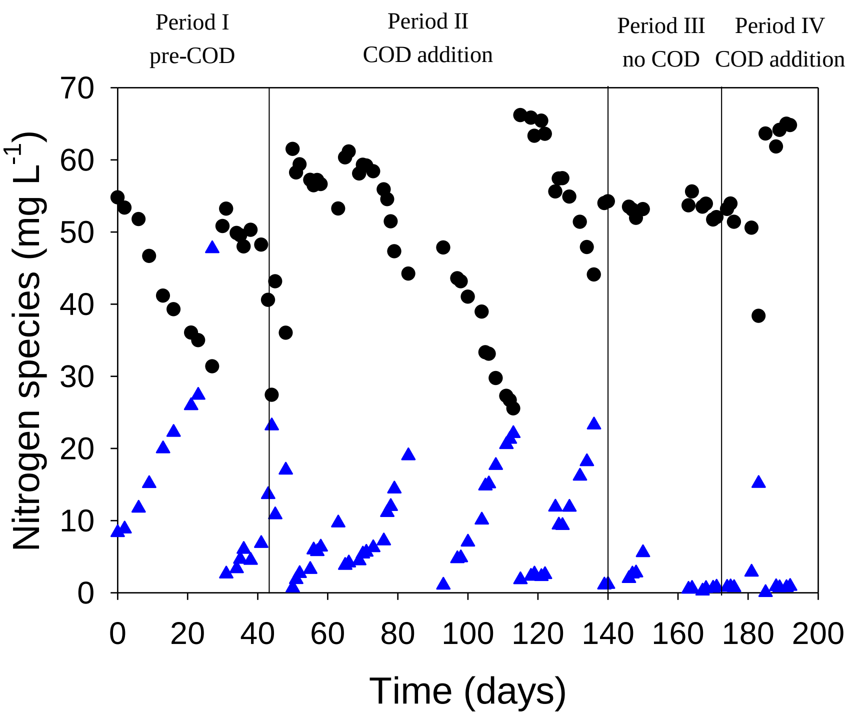

Fig. 4.2. Nitrogen concentrations in the influent as the sum of ammonium and nitrite (black dots) and nitrate (blue triangles) throughout the CANDLE reactor operation.

4.2.3. Profile concentrations along the sludge bed

Ammonium, nitrite, nitrate, COD and acetate concentrations were measured along the different sampling points of the CANDLE reactor at steady state conditions to assess the distribution and contribution of anammox and of heterotrophic denitrification activity. Further, acetate concentrations were measured along the sludge bed on day 132. Distribution of sampling points is depicted in Fig. 4.1.

4.2.4. Heterotrophic denitrifying *ex-situ* batch activity tests

Ex-situ batch tests were used to assess heterotrophic denitrifying activity before and during acetate addition on days 43 (period I) and 98 (period II), respectively. Tests were carried out in duplicates using septum-closed bottles of 125 mL with 2.5 ± 0.3 g VS L^{-1} and 0.3 ± 0.1 g VS L^{-1} for tests of day-43 and day-98, respectively. For tests of day 43, biomass from sampling S3 was employed as it corresponded to the sludge bed section where acetate was added. For tests of day 98, the employed biomass corresponded to the sludge located within the GLS separator phase. The distinct sample point explains the difference of biomass concentrations among the performed tests. The biomass was washed and re-suspended in a medium containing the corresponding nitrite and/or nitrate and C-source (i.e. acetate) concentrations together with the macro and microelements to avoid nutrient limitation (Smolders et al., 1994).

All septum bottles were flushed with N_2 to maintain anoxic conditions. Tests were conducted at 180 rpm and at 20 ºC. Two sets of experiments were performed for each sampling period (days 43 and 98): (1) the consumption of nitrite or nitrate in the presence of acetate as an easily biodegradable C-source and (2) the consumption of nitrite and nitrate in the presence of acetate to evaluate if the biomass presented a preference for any of these two electron acceptors. Sampling time depended on the consumption rate of substrates in each experiment. Substrate consumption velocities were calculated by linear regression of nitrite or nitrate concentrations of three to six bulk liquid-phase grab samples. Substrate consumption velocities were calculated by linear regression of nitrite or nitrate concentrations of three to six bulk liquid-phase grab samples. The employed COD/N ratio (4.2 ± 0.4 g COD g^{-1} N-NO_3) was slightly higher than the theoretical stoichiometric relationship for the biological heterotrophic denitrification process using acetate as a C-source (3.9 g COD g^{-1} N-NO_3) (Eq. 4.1, van den Berg et al., 2016).

$$2.3\ NO_3^- + 2.1\ CH_3COOH \rightarrow CH_{1.8}O_{0.5}N_{0.2}C_2 + 3.1\ CO_2 + 4.4\ H_2O + N_2 \quad \text{(Eq. 4.1)}$$

Further information regarding COD, nitrite and nitrate concentrations for each performed *ex-situ* batch test can be found in Table 4.2.

Table 4.2. Substrates concentrations, COD/N ratios and obtained specific activities of the *ex-situ* heterotrophic denitrifying batch activity tests performed before and during acetate addition (days 43 and 98, respectively) using sodium acetate as C-source.

Performed test	**Day of operation**	**Nitrite (mg N L^{-1})**	**Nitrate (mg N L^{-1})**	**COD (mg COD L^{-1})**	**COD/N (g COD g^{-1} N)**
Nitrite + acetate	43	37 ± 2	-	149	4.1 ± 0.3
Nitrate + acetate	43	-	38 ± 2	149	4.0 ± 0.3
Nitrate + nitrite + acetate	43	20 ± 1	20 ± 1	149	4.0 ± 0.2
Nitrite + acetate	98	36 ± 2	-	149	4.1 ± 0.1

Table S4.2 Continuation

Performed test	Day of operation	Nitrite (mg N L^{-1})	Nitrate (mg N L^{-1})	COD (mg COD L^{-1})	COD/N (g COD g^{-1} N)
Nitrate + acetate	98	-	37 ± 1	149	4.2 ± 0.1
Nitrate + nitrite + acetate	98	18 ± 2	16 ± 4	149	5 ± 1

4.2.5. Analytical methods

Liquid samples from influent and effluent of the CANDLE reactor were analysed 4 to 5 times per week. Samples were filtered (0.22 µm) before analysis. Nitrite (influent) and nitrate concentrations were analysed off-line with ionic chromatography using ICS-2000 Integrated Reagent-Free IC system (DIONEX Corporation, USA). For accurate results, effluent nitrite concentrations were measured by using colorimetric Hach Lange kits (LCK342, Hach Lange, Germany). Ammonium concentrations were analysed off-line by means of a gas selective electrode (GSE) (AMTAX sc, Hach Lange, Germany). Ammonium and nitrite samples from profile concentrations and from *ex-situ* batch activity tests were determined by colorimetric Hach Lange kits (LCK303 and LCK342, respectively, Hach Lange, Germany). Total and soluble COD concentrations were analysed by using colorimetric Hach Lange kits (LCK314 Hach Lange, Germany). Acetate concentrations were measured by gas chromatography (7820A, Agilent Technologies, USA). Total solids (TS) and volatile solids (VS) concentration were analysed according to Standard Methods (APHA, 2005). Biomass characteristics were monitored using a stereomicroscope. Average particle size was measured by a laser diffraction analysis system (Malvern Mastersizer Series 2600, Malvern instruments Ltd., UK). N_2O off-gas concentrations were analysed within the GLS phase of the CANDLE reactor with a Clark-type N_2O microsensor calibrated according to manufacturer's instructions (UNISENSE, Denmark).

4.2.6. Calculations

Nitrogen loading rates (NLRs), NRRs and organic loading rates (OLRs) were calculated by using the effective CANDLE reactor volume of 13 L, excluding the GLS separator phase. NLR was calculated considering the ammonium, nitrite and nitrate concentrations of the real mainstream wastewater. Likewise, NRR was calculated considering the removed ammonium, nitrite and nitrate concentrations. The OLR was calculated considering the necessary acetate to reduce (i) the nitrate produced by the anammox process and (ii) the inflow nitrate according to the stoichiometric relation of 3.9 g COD g^{-1} N-NO_3 (see Eq. 4.1) considering a ratio of 0.78 g COD g^{-1} sodium acetate. The variations of the acetate flow rate to meet the required OLR were manual and periodically implemented. As a result, the requirements just described were not always fully achieved due to the fluctuating inflow nitrate concentrations throughout periods II and IV. In case of a full-scale application, this could be easily managed by a proper automatic control strategy. Nitrogen removal efficiency (NREs) was calculated based on Eq. 4.2:

$$\mathrm{NRE} = \frac{(\mathrm{N-NH_4^+} + \mathrm{N-NO_2^-} + \mathrm{N-NO_3^-})_{\mathrm{inf}} - (\mathrm{N-NH_4^+} + \mathrm{N-NO_2^-} + \mathrm{N-NO_3^-})_{\mathrm{eff}}}{(\mathrm{N-NH_4^+} + \mathrm{N-NO_2^-} + \mathrm{N-NO_3^-})_{\mathrm{inf}}} \qquad \text{(Eq. 4.2)}$$

where $\mathrm{N-NH_4^+}$, $\mathrm{N-NO_2^-}$ and $\mathrm{N-NO_3^-}$ are the ammonium, nitrite and nitrate concentrations in the influent (inf) and in the effluent (eff), respectively.

The emission factor (EF) for N_2O was calculated based on the N_2O emission rates (Eq. 3) to the relative nitrogen removed by the anammox process or by both the anammox and heterotrophic denitrifying processes during a defined period.

$$r_{N_2O} = Q_{N_2} \cdot C_{N_2O} \qquad \text{(Eq. 4.3)}$$

where C_{N_2O} corresponds to the measured N_2O off-gas concentrations and Q_{N_2}corresponds to the nitrogen gas flow (9 mL min^{-1}) added within the GLS separator phase and controlled by a means of flowmeter (Bronkhorst, The Nethelands). Nitrogen produced by the anammox and heterotrophic denitrification processes were not considered in Q_{N_2} as they were 7-to-10 times lower than the external nitrogen gas supply.

4.2.7. Microbiological characterization and quantification

Microbiological community composition was identified by using next-generation sequencing analysis. Two biomass samplings were carried out: (1) before acetate addition (day 42) from samplings S1 and S3 and (2) during acetate addition (day 97) from sampling points S1, S3, S4 and from the GLS separator phase. DNA was extracted by using the Soil DNA Isolation Plus KitTM (Norgen Biotek Corp, Canada) following the manufacturer protocol. The quantity and quality of the extracted DNA was measured by using the NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific, USA). A 260/280 nm ratio of 1.9 ± 0.1 was used as quality cut-off and a minimum of 17 ng μL^{-1} of extracted DNA was guaranteed to perform sequencing. Paired-end sequencing of the extracted DNA was performed on an Illumina MiSeq platform by the Research and Testing Laboratory (Lubbock, Texas, USA). Bacterial 16S rRNA variable regions V2-V4 were targeted using the primer pair 515F-806R for general bacteria and the specific primer pair 368F-820R for the anammox population. Additional information of the bioinformatics protocol can be found in Appendix II.

4.3. Results and discussion

4.3.1. Combining anammox and heterotrophic denitrifying activity in a single reactor unit: process performance

The reactor had been previously operated as an UAnSB reactor where the main anammox activity was in the bottom sludge bed section. The UAnSB reactor treated a real mainstream wastewater at rather constant NLRs (0.11 ± 0.01 g N L^{-1} d^{-1}) during a temperature decrease from 20 to 10 ºC (see Chapter 3). The CANDLE reactor configuration was proposed to couple anammox and heterotrophic denitrification processes in a single reactor unit. To prevent the competition among anammox and heterotrophic denitrifying bacteria, an external C-source (i.e. acetate) to the middle sludge bed section (i.e. at the height of sampling S3), whereas the real mainstream wastewater was fed to the bottom sludge section (Fig. 4.3). The obtained results showed that acetate addition (period II and IV) allowed the removal of the nitrate produced by anammox bacteria, without hampering the anammox process (see Fig. 4.3 and Table 4.1). This, in turn, significantly improved effluent quality while maintaining rather high and constant NRRs (0.16 ± 0.03 g N L^{-1} d^{-1}) and high NREs (91 ± 8 %) (on average from acetate addition periods, Fig. 4.3). Reactor performance was successfully maintained when temperature decreased from 20 to 14 ºC (Fig. 4.3). In case of increasing inflow nitrate concentrations (i.e. eventual nitrate production in previous treatment stages), the OLR was adjusted to maintain a good effluent quality, without hampering the autotrophic nitrogen removal by anammox bacteria (Fig. 4.3). Even when acetate was added over the stoichiometric requirements (day 93, from days 111 – 114, day 132, from days 172 to 182 and 184 to 192), COD effluent concentrations remained in the range of COD inflow concentrations (on average for acetate addition periods 40 ± 5 and 47 ± 10 mg COD L^{-1}

Table 4.3. Operational parameters for the different periods without acetate addition (I and III) and during acetate (II and IV). NLR: Nitrogen Loading Rate; NRR: Nitrogen Removal Rate; NRE: Nitrogen Removal Efficiency.

Period	Time lapse (days)	NLR (g N L^{-1} d^{-1})	NRR (g N L^{-1} d^{-1})	Total effluent nitrogen (mg N L^{-1})	Ammonium effluent (mg N L^{-1})	Nitrite effluent (mg N L^{-1})	Nitrate effluent (mg N L^{-1})	Nitrite/ ammonium consumed	Nitrate produced/ ammonium consumed	Nitrite/ ammonium influent
Period I pre-acetate addition	43	0.15 ± 0.03	0.10 ± 0.03	83 ± 4	4 ± 4	1 ± 2	17 ± 9	1.3 ± 0.1	0.24 ± 0.04	1.2 ± 0.3
Period II Acetate addition	97	0.18 ± 0.02	0.17 ± 0.03	92 ± 7	2 ± 2	1 ± 2	3 ± 4	1.2 ± 0.2	--	1.2 ± 0.2
Period III no-acetate addition	31	0.19 ± 0.01	0.15 ± 0.02	79 ± 8	4 ± 2	3 ± 2	7 ± 2	1.3 ± 0.1	0.24 ± 0.10	1.3 ± 0.1
Period IV Acetate addition	21	0.16 ± 0.02	0.14 ± 0.01	88 ± 8	5 ± 4	2 ± 2	2 ± 2	1.4 ± 0.2	--	1.2 ± 0.2

for influent and effluent, respectively), showing that mainly the non-biodegradable COD entering the reactor was the only organic source discharged, so that the external acetate was either directly used for heterotrophic denitrifiers or stored as polymers by the cells (see Fig. 4.4 and Table 4.4). A detailed analysis of the experimental data is here discussed.

The reactor operation period was divided into four different phases (Table 4.3). The high total nitrogen concentrations in the effluent of period I (i.e. without acetate addition) was caused by the high incoming nitrate concentrations and by the oscillating nitrite to ammonium feeding ratios (1.2 ± 0.3) (see Fig 4.2 and Fig. A4 in Appendix I, respectively). As soon as heterotrophic denitrifying activity was promoted by adding acetate (period II), higher NREs (92 ± 7 %) and NRRs (0.17 ± 0.03 g N L^{-1} d^{-1}) and a better effluent quality (5 ± 4 mg N L^{-1}) were achieved by maintaining high loading rates (0.18 ± 0.02 g N L^{-1} d^{-1}) (Table 4.3). From day 140 to 171 (period III), reactor performance was reassessed to rule out the possible negative effects of the previous external C-source addition on the anammox process (i.e. only mainstream wastewater feeding) by maintaining a similar NLR than that of previous periods (0.19 ± 0.01 g N L^{-1} d^{-1}). During the first 10 days after stopping acetate addition, from days 140 to 149, the nitrate production to ammonium consumption ratio was lower than the stoichiometrically proposed for the anammox process (i.e. 0.17 ± 0.05), still detecting a small degree of heterotrophic denitrifying activity. However, reactor performance decreased compared to that of period II, resulting into lower NREs (79 ± 8 %) and NRRs (0.15 ± 0.02 g N L^{-1} d^{-1}) (Table 4.3). The lower reactor performance was attributed to the lack of an external C-source, resulting into a minimal heterotrophic denitrifying activity and, thus, into high effluent nitrate concentrations (7 ± 2 mg N L^{-1}, Table 4.3). Also, the non-adjusted nitrite to ammonium feeding ratio (see Fig. A4 in Appendix I) of this period resulted into an

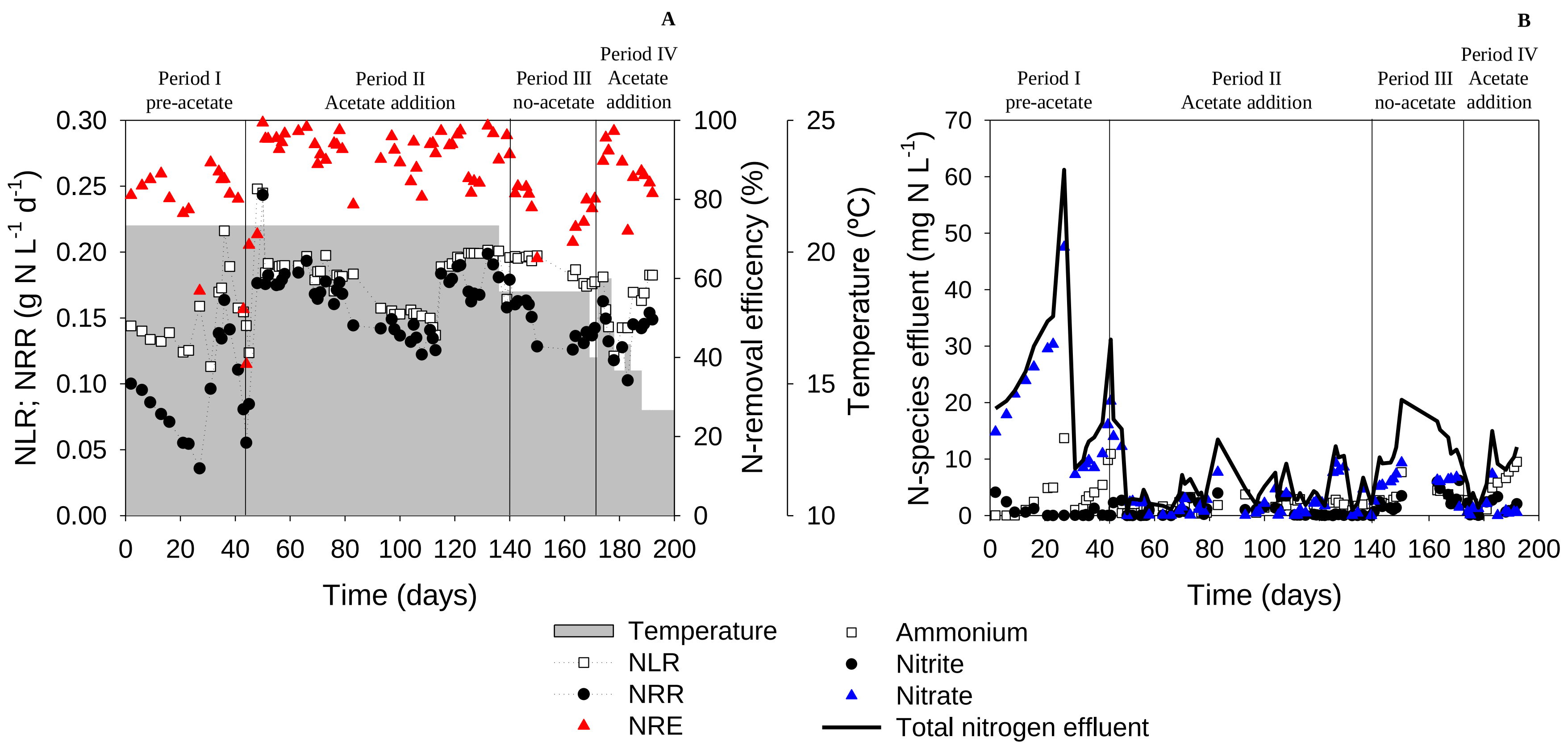

Fig. 4.3. Long-term operation of the CANDLE reactor at mainstream conditions. **(A)** Nitrogen loading and removal rates (NLRs and NRRs, respectively) and nitrogen removal efficiencies (NREs). **(B)** Effluent concentrations of ammonium, nitrite and nitrate. Total nitrogen concentrations in the effluent refer to the sum of ammonium, nitrite and nitrate.

excess of ammonium in the effluent (4 ± 2 mg N L^{-1}, Table 4.3) and thus, worsened effluent quality.

During period IV, the performance of the CANDLE reactor at low temperatures was assessed. For this purpose, reactor temperature was decreased from 20 to 14 ºC while acetate addition was reactivated by maintaining the NLR in the range of previous periods (0.16 ± 0.02 g N L^{-1} d^{-1}, Table 4.3). As the OLR was mainly over-dimensioned during period IV, nitrate concentrations remained very low in the effluent (Table 4.3). Simultaneously, high effluent ammonium concentrations were detected (5 ± 4 mg N L^{-1}, Table 4.3) caused by a misbalanced nitrite to ammonium feeding ratio to the anammox process (1.2 ± 0.1, see Fig. A4 in Appendix I). Overall, a better effluent quality and NREs were achieved compared to periods without acetate addition (Table 4.3). Despite the temperature decrease, reactor performance in period IV was only slightly lower compared to the previous acetate addition period at 20 ºC (period II), as NRR and NRE only decreased from 0.17 ± 0.03 g N L^{-1} d^{-1} and 92 ± 7 % (period II) to 0.14 ± 0.01 g N L^{-1} d^{-1} and 88 ± 8 % (period IV). The capability of an UAnSB reactor of dampening a temperature drop at mainstream conditions was previously demonstrated to be a result of the so-called reactor overcapacity. This is the capacity of the middle and upper part of the sludge bed to counterbalance the activity decrease of the bottom sludge bed section when temperature drops (Juan-Díaz et al., 2021). According to this, the CANDLE reactor also seemed to show overcapacity to damp temperature drops. Thus, the main reason of the lower NRRs and NREs of period IV can be attributed to the non-adjusted nitrite to ammonium feeding ratio, rather than to the effects of temperature decrease.

The coexistence of anammox and heterotrophic denitrifying activities in a single reactor was demonstrated to be a feasible technology to overcome the associated drawbacks of the two-stage autotrophic biological nitrogen removal process at mainstream conditions; that is, potential nitrate production or a misbalanced nitrite to ammonium ratio in the effluent of the PN reactor.

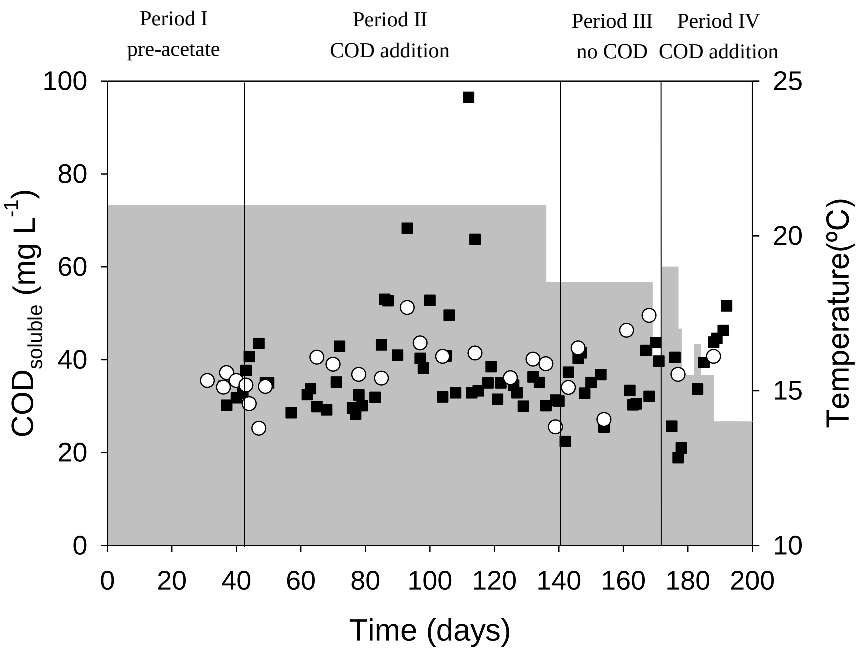

Fig. 4.4. Soluble chemical oxygen demand (COD) concentrations of the influent real mainstream wastewater (white dots) and of the effluent (black squares) during CANDLE reactor operation. The external C-source (i.e. acetate) is considered within effluent concentrations.

Table. 4.4. COD_T and COD_S are the total and soluble chemical oxygen demands corresponding to the different operational periods of the CANDLE reactor. na, not analysed.

Period		**COD_T (mg COD L^{-1})**	**COD_S (mg COD L^{-1})**
Period I pre-acetate addition	Influent	40 ± 4	36 ± 2
	Effluent	36 ± 4	33 ± 2
Period II Acetate addition	Influent	39 ± 5	37 ± 7
	Effluent	46 ± 10	39 ± 13
Period III no-acetate addition	Influent	na[a]	40 ± 10
	Effluent	na[a]	34 ± 7
Period IV Acetate addition	Influent	na[a]	39 ± 3
	Effluent	58 ± 6	37 ± 11

4.3.2. Location of anammox and heterotrophic denitrifiers along the bed prevents competition of nitrite

The anammox and heterotrophic denitrifying contributions to nitrogen removal were assessed by measuring nitrogen compounds, COD and acetate (expressed as COD) concentrations for samples withdrawn along the different sampling points of the sludge bed (see Fig. 4.1). Measurements were performed for three different operational days: during acetate supply (period II, on days 125 and 132, when acetate addition was under and over-dimensioned, respectively) and without acetate addition (day 168, period III). Additionally, biomass characteristics were examined using a stereomicroscope and particle size was measured for different withdrawn samples of the sludge bed.

Anammox activity was detected within bottom sludge sections, as observed by the reduction of ammonium and nitrite compounds from S1 to S3 sludge bed sections in both conditions, with and without acetate addition (Fig. 4.6A). The biomass located within this sludge section presented a brown-to-reddish colour, with a dense granule structure (see Fig. 4.5).

Nitrate concentrations increased along the bottom sludge bed sections, according to the nitrate produced by anammox bacteria (Fig. 4.6A). When acetate was supplied, nitrate concentrations decreased after reaching the sludge bed section corresponding to the acetate feeding point (i.e. sampling S3) (Fig. 4.6A). The highest COD concentrations corresponded to the acetate bed section addition and decreased along the upper sludge bed sections, in agreement with the reduction of nitrate concentrations by heterotrophic denitrifiers (Fig. 4.4B). The biomass located beyond the acetate feeding point presented fewer compact aggregates with a brownish colour and fluffy structure (see Fig. 4.5), although the sludge presented similar average diameter sizes than those of the bottom bed sections (see Fig. A6, Appendix I).

The obtained results indicate that heterotrophic denitrifying activity was located within middle and upper sludge sections, while anammox activity was relegated to the bottom sludge section. The reason was attributed to the suitable distribution of acetate addition beyond the middle sludge section of the CANDLE reactor. This was proved when measuring acetate concentrations along the sludge bed sections, as they were only detected within middle sludge sections, while no acetate diffusion was observed to the bottom sludge bed sections, ensuring the performance of the anammox process (Fig. 4.4B). When acetate was added above the stoichiometric requirements (day 132), the COD effluent concentration remained in the range of the COD inflow concentration, whereas only a residual nitrate concentration was detected (ca. 0.1 mg N L^{-1}) (Fig. 4.6A). Additionally, mass balances indicated that COD consumption was higher than the stoichiometrically required for nitrate reduction by using acetate as an organic source (Fig. 4.4B).

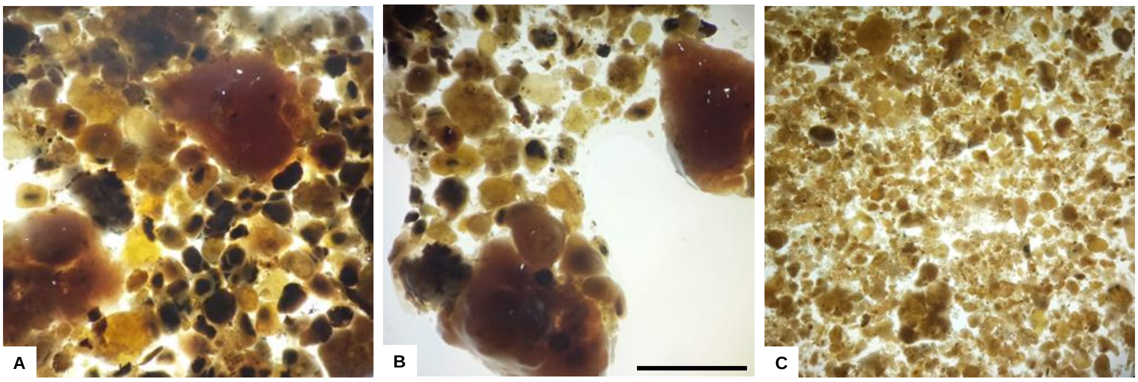

Fig. 4.5. Sludge characteristics obtained by means of a stereomicroscope. A and B, biomass corresponding to the bottom sludge section. C, biomass located beyond the acetate feeding point. Scale bar represents 1 mm.

Acetate can be intracellularly accumulated as polyhydroxybutyrate (PHB) (Liu et al., 2015; Majone et al., 2001). This is consistent with results from period III, as heterotrophic denitrification still occurred after stopping acetate addition (days 140 to 149, see section 4.3.1). This suggests that heterotrophic denitrifying bacteria stored organic compounds when acetate addition was over-dimensioned and that they were capable of further consume them under starvation conditions of an external organic feeding.

Different studies reported that anammox activity became deteriorated when influent with high COD concentrations or high COD/N ratios was fed in different reactor configurations (Chen et al., 2016; Pijuan et al., 2020). However, the results of this study not only provided evidence that anammox activity remained unaffected after the continuous external C-source supply, but also that the proposed reactor configuration allowed for the coexistence of anammox and heterotrophic denitrification processes.

4.3.3. Effect of heterotrophic denitrifying activity on N_2O emissions

N_2O off-gas concentrations were monitored within different operational periods of the CANDLE reactor with and without acetate addition at 20 ºC (period II and III, respectively) and with acetate addition at 17 ºC (period IV) (Fig. 4.7). The measured N_2O emission factor (EF) (0.002 %, on average from all available measurements) was maintained low during the different operational periods, with and without acetate addition. This is consistent for a reactor where the main biological activity is the anammox process since previous studies indicated that anammox lacks of genes producing N_2O (Kartal et al., 2007). As anammox do not produce N_2O, the measured N_2O emissions may account on other microbial pathways. They can be attributed to incomplete heterotrophic denitrification activity (Jia et al., 2018) due to the limited organic matter contained in the mainstream wastewater or because of the imposed external C-source limiting conditions. They can also be related to the ammonium oxidizing bacteria (AOB) of the incoming wastewater transported to the CANDLE reactor and located at the bottom of the sludge bed i.e. just in the entry, where they can benefit from the residual dissolved oxygen (DO) concentration of the inflow wastewater. Actually, N_2O production by AOB

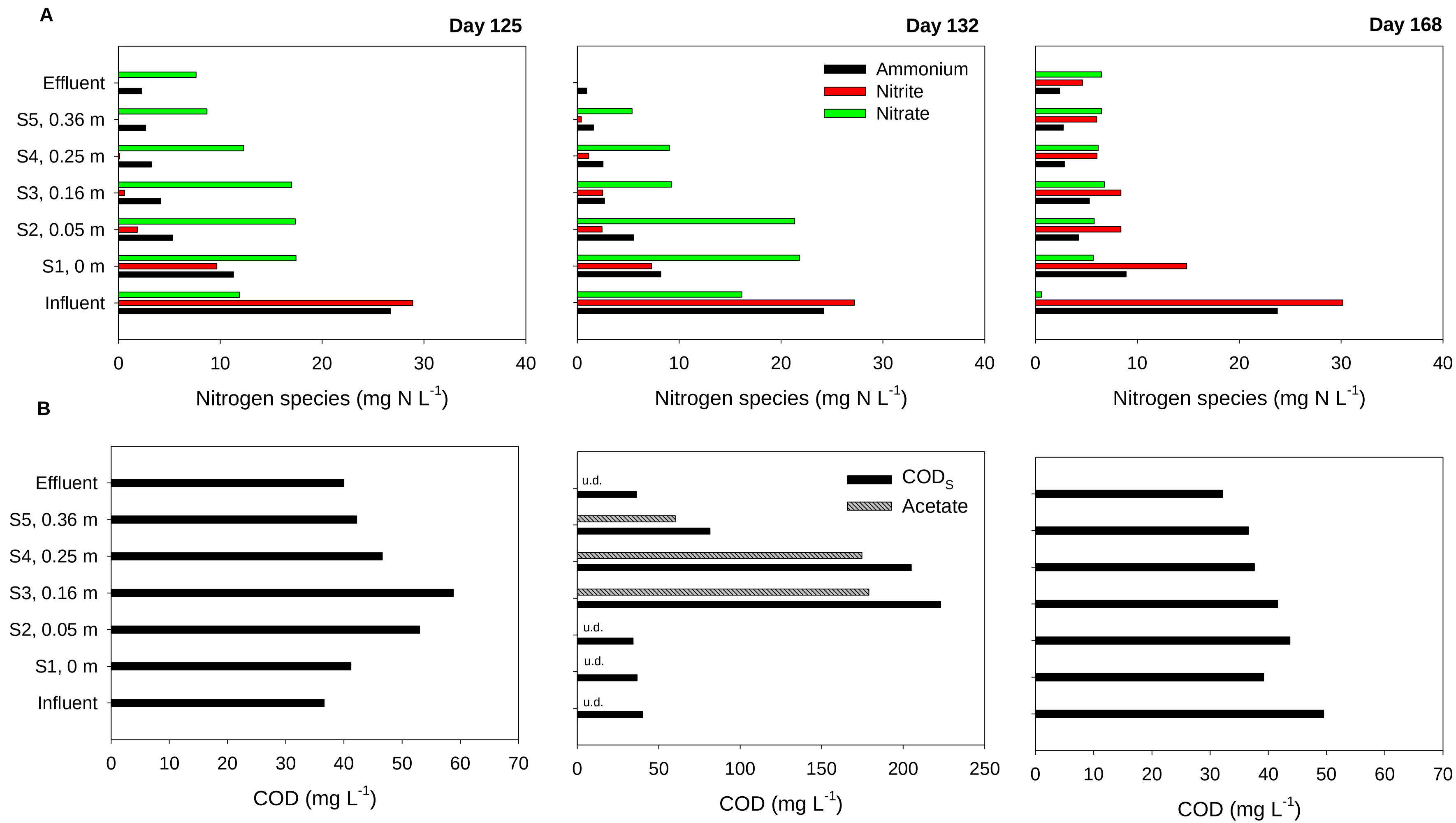

Fig. 4.6. Ammonium, nitrite, nitrate concentrations **(A)** and soluble chemical oxygen demand (COD_S) and acetate (measured as COD) concentrations **(B)** corresponding to different sludge bed heights sections 0 m (S1), 0.05 m (S2), 0.16 m (S3), 0.25 m (S4) and 0.36 (S5) of the CANDLE reactor during (period II, days 125 and 132, when acetate addition was under and over-dimensioned, respectively) and without acetate (period III, day 168) addition. *u.d, under detection limit.

is enhanced under low oxygen concentrations or anoxic environments, prevailing conditions within anammox reactors (Kampschreur et al., 2008).

The measured average N_2O off-gas concentrations of the CANDLE reactor were lower when acetate addition occurred (Fig. 4.7). This agrees with the fact that heterotrophic denitrifiers can act as an N_2O sink when COD is not limiting denitrification (Conthe et al., 2019). From days 125 to 130 (period II), acetate addition was deliberately under-dimensioned, resulting into a peak of N_2O off-gas concentrations (Fig. 4.7). This confirmed that the amount of N_2O that the denitrifiers can consume is smaller under COD limiting conditions (Jia et al., 2018). To demonstrate the positive effects of COD supply for mitigation of N_2O emissions, acetate addition was completely stopped on day 140 (period III, see Fig. 4.3). This resulted in a gradual increase of the N_2O off-gas concentrations (Fig. 4.7). However, this slow and progressive increase was not expected. During the first days after stopping acetate addition (days 140 to 149), nitrate reduction was still active (i.e. the nitrate production to ammonium consumption ratio was 0.17 ± 0.05, see Fig. A5, Appendix I). A plausible possibility of the gradual N_2O off-gas concentrations increase is related to the capability of heterotrophic denitrifying bacteria to reduce a fraction of the nitrate produced by the anammox process by using the stored organic compounds during the previous acetate addition period (i.e. when exposed to organic limiting conditions). When the nitrate produced to the ammonium consumption ratio agreed with the stoichiometric produced by the anammox process (0.29 ± 0.03; see Fig. A5, Appendix I), i.e. heterotrophic denitrifying activity was severely limited by the absence of organic matter, N_2O off-gas concentrations increased and became rather stable at a maximum value (Fig. 4.7). Finally, when acetate addition was re-established and over-dimensioned (period IV, from days 175 to 177), N_2O emissions decreased to minimal values, confirming that heterotrophic denitrifiers acted as an N_2O sink (Fig. 4.7).

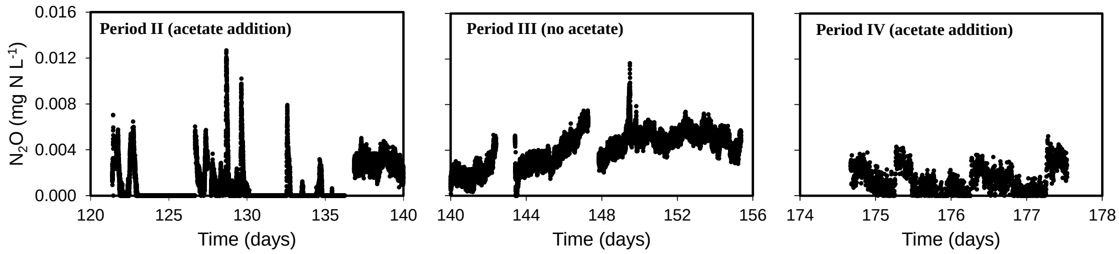

Fig. 4.7. N_2O off-gas concentrations during period II (acetate addition) and period III (no acetate) at 20 ºC and during period IV (acetate addition) at 17 ºC.

4.3.4. *Ex-situ* assessment of heterotrophic denitrifying activity

Ex-situ heterotrophic denitrifying batch tests aimed to assess heterotrophic denitrifying activity of biomass located above the external C-source feeding before (day 43, period I) and during (day 98, period II) acetate addition to the CANDLE reactor by using nitrite and/or nitrate as different electron acceptors. Before acetate addition (period I), nitrite and nitrate consumption rates were in the same range (Fig. 4.8). When both electron acceptors were added together, they were simultaneously reduced although nitrate consumption rates were higher. When assessing heterotrophic denitrifying activity after a period of acetate supply to the CANDLE reactor (i.e. day 98), nitrite and nitrate consumption rates clearly increased compared to previous tests (Fig. 4.8). Nitrate consumption rates were ca. four times higher than those of nitrite, indicating a preference of the denitrifying biomass towards nitrate as electron acceptor (Fig. 4.8). This was confirmed when both electron acceptors were added together, as nitrate was firstly reduced. Under this situation, nitrite consumption started right after nitrate depletion (ca. 22 h after starting batch activity tests). Nitrite consumption rates were ca. 5 times higher than the achieved when only nitrite was used as electron acceptor (Fig. 4.8). This might be attributed as nitrite reduction occurred during the exponential growth phase of heterotrophic denitrifying bacteria, which present doubling times of ca. 9 h at 20 ºC (Metcalf & Eddy, 2003). The *ex-situ* batch tests performed during the period of acetate addition showed that heterotrophic denitrifiers preferred to use nitrate over nitrite as electron acceptor, although in the absence of nitrate, nitrite reduction was also possible.

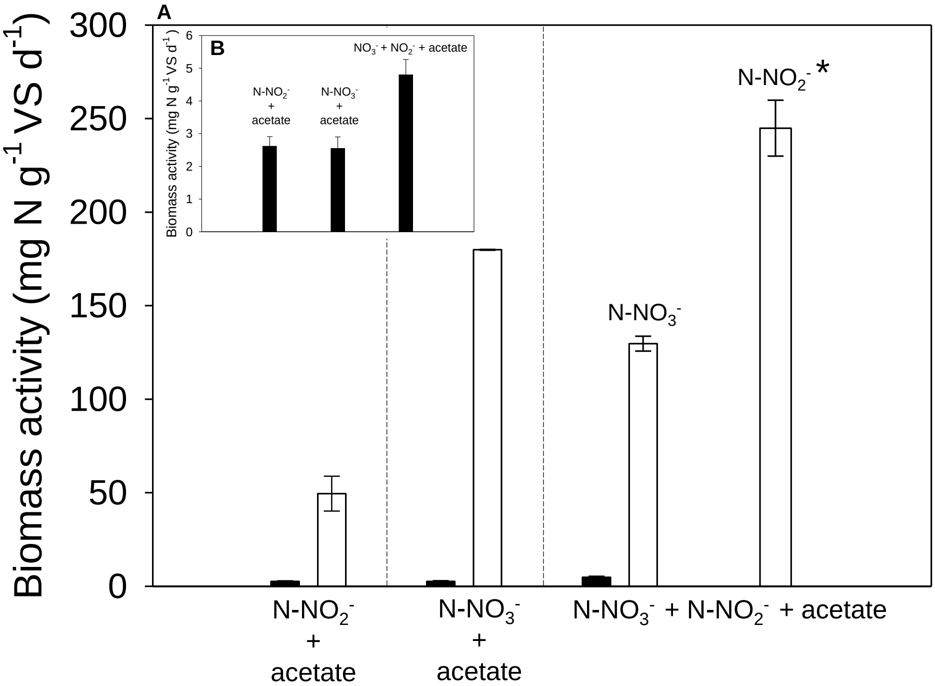

Fig. 4.8. (A) Specific heterotrophic denitrifying activities from *ex-situ* batch tests before (black) and during acetate addition (white) on days 43 and 98, respectively. **(B)** Activities before acetate addition (in black) are also depicted at a smaller Y axis. The applied COD/N ratios and nitrate and nitrite concentrations of each *ex-situ* batch test can be found in Table 4.2. *Corresponds to the nitrite consumption rate after nitrate depletion within the *ex-situ* batch tests where both electron acceptors were added.

4.3.5. Impact of acetate addition on microbial community

The microbial community composition of the CANDLE reactor was studied by 16S rRNA gene-targeted sequencing analyses before (day 42, period I) and during acetate addition (day 97, period II). Six different libraries of reactor sampling points S1 and S3 (before acetate addition) and of S1, S3, S4 and from the GLS separator phase (during acetate addition) were constructed. The total number of sequences for each library after quality analysis and removal of low-quality sequences can be found in Appendix II (see Fig. A10). All libraries presented an average length of 290 bps per sequence.

During period I (before acetate addition), the microbial community presented a high abundance of *Candidatus* Brocadia in both S1 (28 %) and S3 (12 %) (Fig. 4.9). Also, different abundances of heterotrophic denitrifying bacteria were detected. Chloroflexi, Acidobacteria and Chlorobi (specifically *Ignavibacterium* genus) phylum were observed with abundances ranging from 8 to 21 % (Fig. 4.9). These phyla represent a large fraction of the microbial diversity within anammox reactors without the addition of any external C-source (Lawson et al., 2017; Li et al., 2009; Speth et al., 2016). Under these conditions, heterotrophic denitrifying bacteria can degrade the extracellular polymeric substances (EPS) produced by anammox bacteria to use it as an organic carbon source while encoding nitrite and/or nitrate respiration (Lawson et al., 2017; Xiao et al., 2021). Overall, no significant differences were detected on the microbial community within the bottom and middle sections of the sludge bed (see similitude results from Jaccard Index in Appendix II, see Table A1). Further, anammox bacteria were present in both sludge bed sections in a significant amount.

After ca. 2 months of acetate addition, microbial composition changed within the middle sludge section S3, while it remained almost unaffected within the sludge bed section S1 (see similitude results from Jaccard Index in Appendix II, Table A1). *Candidatus* Brocadia still presented a high abundance within the bottom sludge bed section (30 %), while its abundance became significantly reduced within middle and upper sludge bed sections (< 3 %) (Fig. 4.9). However, the small anammox fraction of S3 was still dominated by the genus *Candidatus* Brocadia, according to the obtained results from the specific primer (see Fig. A11, Appendix II). Within middle and upper sludge layers, the microbial composition was strongly dominated by the genus *Thauera* (33 to 46 %), resulting into a decrease of the previously detected heterotrophic denitrifiers before acetate supply (Fig. 4.9). *Thauera* was reported to provide nitrite for anammox bacteria through reducing nitrate to nitrite in denitratation/anammox processes (Du et al., 2017; Ji et al., 2020; B. Ma et al., 2017). Also, a high abundance of *Thauera* was found in a CANON reactor (García-Ruiz et al., 2018) and when coupling anammox and denitratation in a single reactor fed with acetate as a C-source (B. Ma et al., 2017). *Thauera* prefers to use nitrate over nitrite as electron acceptor (B. Ma et al., 2017). Nevertheless, nitrite can be also reduced by *Thauera* denitrifiers after nitrate depletion if a sufficient amount of organic matter is still available (B. Ma et al., 2017). These results are in accordance with the *ex-situ* batch activity tests performed during acetate addition (i.e. day 98) when both electron acceptors were added, as nitrite reduction occurred right after nitrate consumption.

Microbial diversity results confirmed that anammox activity dominated the bottom sludge bed section of the CANDLE reactor. Results from profile concentrations showed that nitrate reduction took place beyond the acetate feeding point (i.e. sampling S3), in accordance with the developed heterotrophic denitrifying community (i.e. *Thauera*). The obtained microbial community data confirmed that the development of a significant denitrifying community in the middle and upper sections of the CANDLE reactor did not hinder the stability of the anammox community located within the bottom sludge bed section.

4.4. Conclusions

- The ad hoc reactor design for **C**oupling **A**nammox and **D**enitrification in a sing**LE** unit (i.e. the CANDLE reactor) allowed the addition of an external organic source for the removal of nitrate produced by anammox bacteria without compromising the stability of the anammox process.

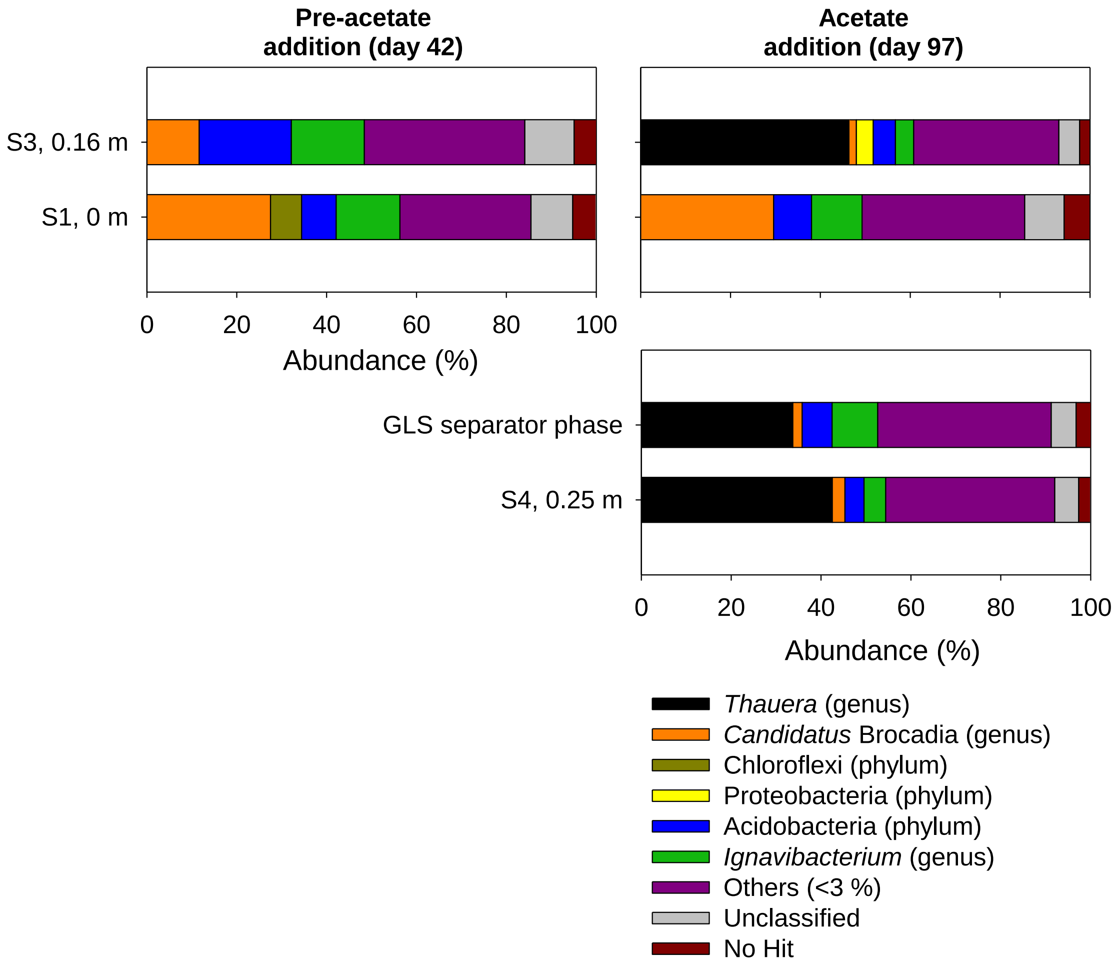

Fig. 4.9. Microbial diversity using the 515F-806R primer for libraries before and after ca. two months of acetate addition (day 42 and 97, respectively) at different CANDLE reactor heights: 0 m (S1) and 0.16 m (S3), 0.25 m (S4) and within the gas-liquid-solid (GLS). Relative abundance was calculated by only considering those microorganisms in which the number of 16S copies was higher than 3 % of the total copies.

- The coexistence of anammox and heterotrophic denitrifying bacteria in a CANDLE reactor was possible as anammox activity dominated within bottom sludge bed sections, while heterotrophic denitrification occurred within middle and upper sludge bed sections.
- N_2O emissions were lower during the addition of an external carbon source, indicating that heterotrophic denitrifiers acted as an N_2O sink.

- Microbial diversity results confirmed that both populations (anammox and heterotrophic denitrifiers) occupied differentiated niche compartments in a CANDLE reactor.
- Anammox population was dominated by the genus *Candidatus* Brocadia while the *Thauera* genus dominated among heterotrophic denitrifiers.
- The CANDLE reactor has been shown to be a feasible technology to enhance the robustness of the two-stage mainstream partial nitritation/anammox process, alleviating the negative effects from the previous treatment stages.

Chapter 5. Ammonium oxidation activity promotes stable nitritation and granulation of ammonium oxidizing bacteria

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Abstract

Two-stage partial nitritation/anammox (PN/AMX) processes have been pointed out as a feasible configuration for achieving mainstream anammox. For two-stage configurations, stable partial nitritation has been reported feasible in granular sludge reactors. This study aimed to explore the operating conditions involved in the development of an autotrophic aerobic granular sludge using floccular sludge as inoculum. The influence of different parameters such as free ammonia concentration, settling time, superficial gas flow velocity and ammonium oxidation rate was investigated. Enhancing ammonium oxidation activity since the early phase of the operation (i.e. using conventional activated sludge as inoculum enriched with a fraction of a floccular nitrifying biomass) promoted a fast development (ca. 30 days) of an autotrophic aerobic granular sludge performing a stable nitritation. When the seeded sludge presented a low nitrifying activity (i.e. not enriched), the increase of the air-flow rate triggered the formation of an autotrophic aerobic granular sludge since ammonium oxidation activity was promoted. Contrarily, imposing low settling times or strong free ammonia inhibitory conditions were shown to negatively influence the achievement of high ammonium oxidation rates, hampering the development of an autotrophic aerobic granular sludge. This study demonstrated the importance of ensuring high ammonium oxidation rates for the proper development of an autotrophic aerobic granular sludge performing stable nitritation.

5.1. Introduction

The partial nitritation/anammox (PN/AMX) process has been proposed as an efficient and cost-effective technology for nitrogen removal in urban wastewater treatment plants (WWTPs) (Gilbert et al., 2014; Hasan et al., 2021; Kartal et al., 2010; Laureni et al., 2016; Reino et al., 2018, 2016). Under this scenario, two-stage PN/AMX systems have been pointed out as a feasible configuration due to the reported successful results at temperatures as low as 11 ºC (Chen et al., 2020; Isanta et al., 2015; Juan-Díaz et al., 2021; Reino et al., 2016, 2018; Wang et al., 2020). One of the main challenges of the partial nitritation stage is the repression of nitrite oxidizing bacteria (NOB) during long-term operations, currently limiting the full-scale implementation of these systems in the mainstream of WWTPs. However, successful partial nitritation stability has been reported in granular sludge reactors at lab-scale for long-term mainstream conditions (Isanta et al., 2015b; Reino et al., 2016). Granule morphology allows the existence of oxygen and substrate gradients, which enhances stratification of ammonium oxidizing bacteria (AOB) over NOB (Poot et al., 2016). By working at certain residual ammonium concentrations, AOB increase their oxygen consumption rates, meaning that less oxygen is available for NOB, relegating them to deeper layers (Poot et al., 2016; Soler-Jofra et al., 2019). Accordingly, a modelling study revealed that AOB presented a better oxygen affinity compared to NOB simply because the position of cell clusters within a stratified granule (Picioreanu et al., 2016). Most of lab- and pilot-scale studies that attained a stable partial nitritation in granular sludge reactors at mainstream conditions used a pre-formed granular sludge (Isanta et al., 2015b; Poot et al., 2016) or supplied a substratum (i.e. activated carbon) with the inoculum (Bartrolí et al., 2010, 2011). Designing a start-up phase to form a nitrifying granular sludge in which conventional activated sludge (CAS) is used as inoculum seems to be an appealing strategy for the scale-up of this technology.

However, seeding CAS as inoculum prevents the initial suppression of nitrite oxidation activity through the same strategy designed for autotrophic aerobic granular sludge (described and applied in Bartrolí et al. (2010) and Isanta et al. (2015)). An alternative start-up strategy (i.e. in the short term) is to avoid nitrite oxidation by transient inhibition of NOB by free ammonia (FA) within floccular biomass (Jubany et al., 2009; Vadivelu et al., 2007), and simultaneously develop a granular sludge. To this end, a nitrogen concentrated wastewater, such as the one coming from the digested sludge dewatering system (i.e. reject water) could be used. Once granular sludge has been developed, FA inhibition will not be required and, thus, operation at mainstream conditions after the start-up phase could be safely imposed by maintaining suitable [DO]/[TAN] concentration ratio in the bulk reactor liquid (Isanta et al., 2015b; Reino et al., 2016).

Despite some studies focused on the achievement of an autotrophic aerobic granular sludge performing partial nitritation (X. Li et al., 2020; Torà et al., 2013), the particular set of operating conditions leading to an effective start-up of this reactor type remains to date not well defined. In contrast, the development of heterotrophic aerobic granular sludge for the simultaneously removal of COD, nitrogen and phosphorus has been widely studied (e.g., NEREDA®) (Pronk et al., 2015). Heterotrophic aerobic granular sludge has been mostly cultivated from CAS in sequential batch reactor (SBR) operations (Liu and Tay, 2004). The heterotrophic aerobic granulation process is known to be enhanced under short settling times, high shear stress and feast-famine conditions, whereas the exposure to insoluble and particulate COD can comprise the granulation process as filamentous bacteria tend to proliferate (Liu and Liu, 2006; Liu and Tay, 2004). Nevertheless, it is unknown whether these conditions could also favour autotrophic aerobic granulation.

The aim of this study is to investigate the operating conditions that can contribute into the development of an autotrophic aerobic granular sludge performing stable nitritation. For this purpose, an air-lift reactor seeded with CAS was used by working under a SBR mode. The effect of different parameters such as: (i) FA concentrations, (ii) settling time, (iii) shear stress forces -in terms of superficial gas flow velocity- and (iv) ammonium oxidation activity - in terms of ammonium oxidation rates, AORs- were evaluated in four different operational start-up strategies.

5.2. Materials and methods

5.2.1. Reactor configuration and operation

An air-lift reactor with a working volume of 16.3 L operated in a SBR mode was used (Fig. 5.1). The reactor presented a downcomer-to-separator diameter ratio of 0.36, a total length-to-downcomer diameter ratio of 11 and a riser height-to-diameter (H_r/D_r) ratio of 19.4. The riser diameter was of 0.05 m and it was located at ca. 10 cm above the reactor bottom. The SBR operation consisted of four phases: a static feeding phase (16 min), an aeration phase which lasted until total ammonia nitrogen (TAN=N-NH_4^+ + N-NH_3) decreased to the concentration fixed set-point and a settling phase with variable time length and a discharge phase (16 min). Experiments were classified in numbered strategies, as detailed in section 5.2.5 (see Table 5.1). The volumetric exchange ratio was set at 40 %, except for strategy-III, where it was correspondingly adjusted according to the operational needs (Table 5.1). The feeding was directly supplied from the bottom of the reactor. During the aeration phase, air (and nitrogen gas, if applicable) were supplied through a porous membrane diffusor located at 30 mm above of the reactor bottom. Air and nitrogen flow rates were controlled by two different rotameters. DO was measured on-line by a means of a DO electrode (DO 60-50, Crison Instruments, Spain). The pH was measured on-line with a pH-electrode (pH 53-33, Crison Instruments, Spain) with a variable set-point by adding dissolved $NaHCO_3$. Ammonium concentrations and temperature in the bulk liquid were measured by using an on-line probe (AISE sc with a CARTRICAL cartridge, Hach Lange, Germany). Temperature was maintained at 20 ± 1 ºC. All sensors and actuators were monitored and connected to the PLC system.

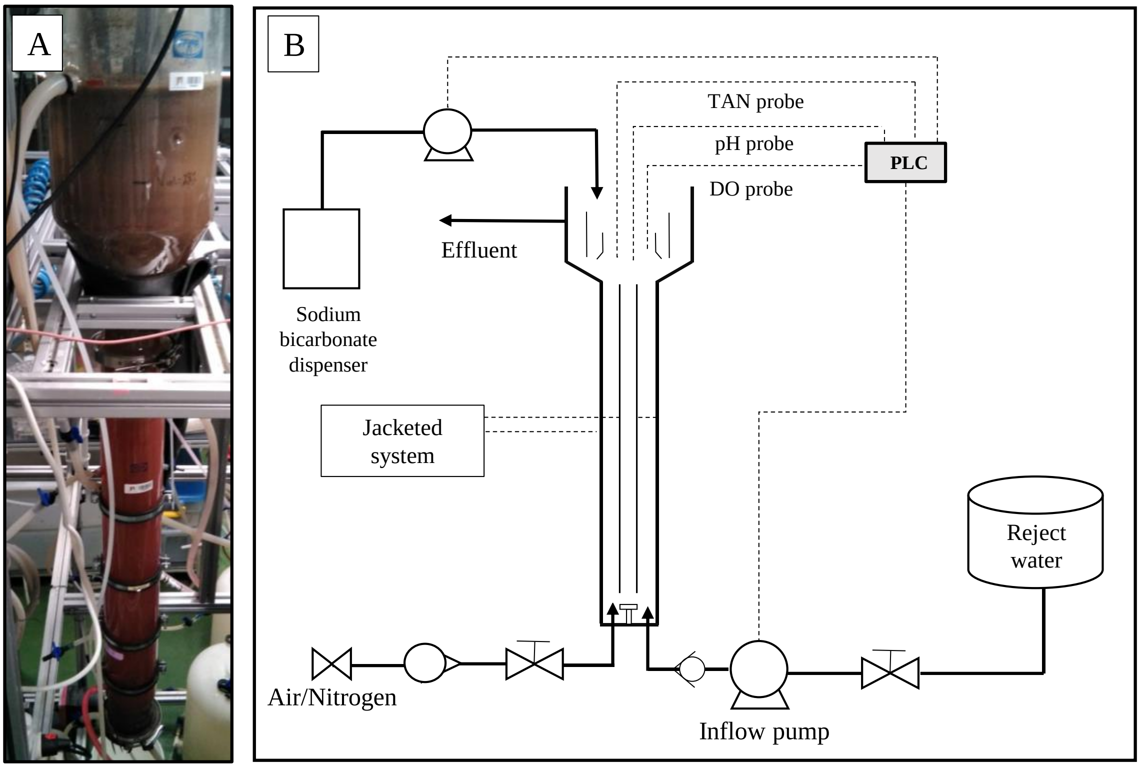

Fig. 5.1. (A) Picture of the air-lift reactor. **(B)** Schematic diagram of the reactor set-up with the corresponding peripheral instrumentation.

5.2.2. Inoculum and wastewater characteristics

The air-lift reactor was inoculated with CAS from a municipal WWTP in each start-up strategy. For the last experiment (strategy-IV), the air-lift reactor was also inoculated with flocculent sludge coming from a pilot-scale reactor treating a sidestream water successfully performing partial nitritation.

The air-lift reactor was fed in all operational strategies with a sidestream water coming from the dewatering of the digested sludge of a municipal WWTP (i.e. reject water). The used reject water was stored in a tank at 13 ± 1 ºC. During strategy-I, the reactor was fed with a sidestream water coming from the Manresa WWTP (Catalonia, Spain). The composition of the sidestream presented the following characteristics: TAN: 238 - 309 mg N L^{-1}; total nitrite nitrogen (TNN = $N\text{-}NO_2^-$ + HNO_2): 1 to 28 mg N L^{-1}; nitrate: 0.5 - 17 mg N L^{-1}; pH: 7.9 ± 0.1. To work at higher FA concentrations, a sidestream water coming from a different municipal WWTP (Rubí – Valldoreix WWTP, Catalonia, Spain) was used in strategies II, III and IV. The characteristics of this sidestream wastewater were: TAN: 512 - 963 mg N L^{-1}; TNN: 0 - 97 mg N L^{-1}; nitrate: 0 – 21 mg N L^{-1}; pH: 8.1 ± 0.1. The wide range of TAN concentrations depended on the anaerobic digester performance. The variability of TNN and nitrate concentrations were because nitrification occurred within the stored wastewater tank.

5.2.3. Analytical methods

Influent and effluent samples of the air-lift reactor were regularly analysed to determine TAN, TNN and nitrate concentrations. Samples were previously filtered (0.22 μm) before analysis. TAN concentrations were analysed off-line by means of a gas selective electrode (GSE) (AMTAX sc, Hach Lange, Germany) or by using colorimetric Hach Lange kits (LCK303 or LCK302, Hach Lange, Germany). TNN and nitrate concentrations were analysed off-line with ionic chromatography using ICS-2000 Integrated Reagent-Free IC system (DIONEX Corporation, USA). Total suspended solids (TSS), volatile suspended solids (VSS) concentrations and sludge volume index (SVI) were analysed according to Standard Methods (APHA, 2005). Average biomass particle size was measured by a laser diffraction analysis system (Malvern Mastersizer Series 2600, Malvern instruments Ltd., UK). The percentage of granular sludge was determined as the volumetric fraction of particles with a diameter higher than 200 μm (de Kreuk et al., 2007).

5.2.4. Calculations

The nitrogen loading rate (NLR) and AOR were calculated based on Eq. 5.1 and Eq. 5.2, respectively,

$$\mathrm{NLR} = \frac{\mathrm{TAN_{INF}}}{\mathrm{HRT}} \qquad \text{(Eq. 5.1)}$$

$$\mathrm{AOR} = \frac{\mathrm{TAN_{INF}} - \mathrm{TAN_{EFF}}}{\mathrm{HRT}} \qquad \text{(Eq. 5.2)}$$

where

$$\mathrm{HRT} = \frac{\mathrm{V_R}}{\mathrm{V_R} \cdot \mathrm{VER} \cdot (\text{number of cycles/day})} \qquad \text{(Eq. 5.3)}$$

and $\mathrm{TAN_{INF}}$ corresponds to influent TAN concentrations and $\mathrm{TAN_{EFF}}$ to effluent TAN concentrations (g N L^{-1}). $\mathrm{V_R}$ and VER correspond to the effective reactor volume (L) and to the volume exchange ratio, respectively.

By using the corresponding TAN concentrations, FA concentrations at the beginning of the cycle ($\mathrm{FA_{BC}}$) and at the end of the cycle ($\mathrm{FA_{EFF}}$) were calculated based on the acid-base equilibrium (Anthonisen et al., 1976),

$$\mathrm{FA} = \frac{\mathrm{TAN} \cdot 10^{\mathrm{pH}}}{e^{\frac{6344}{T+273}} + 10^{\mathrm{pH}}} \qquad \text{(Eq. 5.4)}$$

where FA corresponds to the FA concentration (mg N L^{-1}), TAN corresponds to ammonium concentrations at the end of the cycle ($\mathrm{TAN_{EFF}}$) (i.e. effluent) or to the ammonium concentrations at the beginning of the cycle ($\mathrm{TAN_{BC}}$) (mg N L^{-1}). $\mathrm{TAN_{BC}}$ concentrations were calculated considering the volumetric exchange ratio and the effective reactor volume. pH and T (ºC) correspond to the values of pH and temperature in the air-lift reactor.

The superficial gas flow velocity (u_g, cm s^{-1}) was calculated using the following equation,

$$u_g = \frac{q_{air} + q_{N_2}}{A} \qquad \text{(Eq. 5.5)}$$

where q_{air} and q_{N_2} correspond to the air and nitrogen gas flow rates (cm^3 s^{-1}), respectively and A corresponds to the area of the riser (cm^2). The superficial gas flow velocity was used as an indirect form of quantifying the shear stress conditions applied to the biomass of the airlift reactor.

5.2.5. Operational strategies

The following parameters: (i) FA concentrations in the bulk liquid, (ii) settling time, (iii) shear stress forces (in terms of superficial gas flow velocity), and (iv) ammonium oxidation activity (in terms of AOR) were combined in four different operational start-up strategies to achieve an autotrophic granular sludge performing partial nitritation (Table 5.1). Detailed information of each strategy can be found below.

- **Strategy-I.** The aim of strategy-I was to operate at moderate FA concentrations with a fast decrease of settling time and a low initial superficial gas flow velocity. The applied FA concentrations were 7 ± 2 mg N L^{-1} (Table 5.1), in the range reported for an effectively NOB repression within floccular sludge (Abeling and Seyfried, 1992; Chung et al., 2006). Settling time was decreased from 30 to 10 min (Table 5.1). Superficial gas flow velocity was maintained constant in the first part of the experiment by exclusively supplying air. Then, superficial gas velocity was stepwise increased (Table 5.1) when the granulation process was still not detected after decreasing settling time below 20 min. The reactor was seeded with CAS with an initial biomass concentration of 1 g VSS L^{-1}.

- **Strategy-II.** During strategy-II, high initial FA concentrations were imposed with a progressive decrease of settling time and a low initial superficial gas flow velocity. During the first 8 days of operation, FA concentrations were maintained at 57 mg N L^{-1} (Table 5.1). From day-8 onwards, FA concentrations were maintained at 17 ± 4 mg N L^{-1} until the end of the operation. Settling time was decreased from 30 to 10 min at slower pace than in strategy-I (Table 5.1). Superficial gas flow velocity was controlled by exclusively supplying air as in strategy-I (Table 5.1). The reactor was seeded with CAS with an initial biomass concentration of 2 g VSS L^{-1}.
- **Strategy-III.** The objective of strategy-III was to start the operation at low FA concentrations followed by a sharp increase during a short period to try to knock down NOB, when granule development was still incipient (Table 5.1). After this initial marked increase, FA concentrations were decreased and maintained at 21 ± 7 mg N L^{-1} until the end of the operation (Table 5.1). Settling time was decreased as in strategy-II (Table 5.1). Additionally, nitrogen gas was supplied together with air to operate at higher superficial gas flow velocity (compared to strategies I and II), and thus, to explore the effects of shear stress on granulation (see Fig. 5.5, for details). The reactor was seeded with CAS with an initial biomass concentration of 1 g VSS L^{-1}.
- **Strategy-IV.** The aim of strategy-IV was to enhance ammonium oxidation activity since the early phase of the operation. The airlift reactor was seeded with CAS (26.1 g VSS) but also with a fraction of floccular biomass performing partial nitritation (12.1 g VSS) (ratio ca. 2:1, respectively). To reduce the influence of other parameters, FA_{BC} concentrations were maintained similar as in strategy-I. Settling time was not decreased but maintained high and roughly constant throughout the operation

(Table 5.1). The superficial gas flow velocity was kept at the same initial value than strategy-III, though by exclusively supplying air.

5.3. Results

The time course concentrations of TAN, TNN and nitrate in the effluent as well as TAN influent concentrations for each operational strategy can be found in Fig. 5.2. Particle size together with settling time and superficial gas flow velocity, as well as AORs have been plotted in Fig. 5.4. FA concentrations, nitrate influent and effluent concentrations and solids concentrations can be found in Fig. 5.3.

5.3.1. Strategy-I: operating at moderate FA concentrations with a fast decrease of settling time and low initial superficial gas flow velocity

Initial inoculum size was 93 ± 5 µm and slightly increased to 139 ± 15 µm after 17 days of operation, with a settling time decrease from 30 to 15 min (Fig. 5.4). Imposing a FA concentration (7 ± 2 mg N L^{-1}, Table 5.1) in the reactor bulk liquid resulted in a fast achievement of partial nitritation as observed by the gradually nitrate depletion from 28 to 1 ± 1 mg N L^{-1} in 13 days of operation (Fig. 5.2). Around day-20, particle size increased up to 200 µm (Fig. 5.4), a threshold claiming that biomass moved from flocculent to granular sludge. Some days before reaching this milestone, both superficial gas flow velocity and AOR increased. Thereafter, particle size gradually increased, reaching a diameter of 270 ± 21 µm after 38 days of operation (Fig. 5.4). At the end of the operation (i.e. day 38), 58 % of particles were greater than 200 µm with good settleability properties (SVI_5/SVI_{30}=1), indicating a shift towards granular sludge (Fig. 5.7). However, from day 22-onwards, partial nitritation was progressively deteriorated, achieving up to a maximum nitrate concentration of 121 mg N L^{-1} in only 11 days (Fig. 5.2), despite FA concentrations remained stable throughout the operation (Table 5.1).

In the next experiment (strategy-II), higher FA inhibitory concentrations were applied (compared to strategy-I, see Fig. 5.3), whereas settling time decrease and superficial gas flow velocity followed the same pattern than in strategy-I.

5.3.2. Strategy-II: operating at initial inhibitory FA concentrations with a progressive decrease of settling time and low initial superficial gas flow velocity

Initial particle size was 98 ± 4 µm and only increased to 149 ± 25 µm in the first 51 days of operation (Fig. 5.4), despite decreasing settling time from 30 to 20 min. To avoid nitrate production throughout the operation, as occurred in strategy-I, high inhibitory FA conditions were applied (57 mg N L^{-1}, Table 5.1). However, during the first 8 days of operation, neither nitrite nor nitrate concentrations were detected, indicating an inhibition of both AOB and NOB (Fig. 3). To reduce the FA inhibitory conditions, a cycle was manually performed at day 8 by filling the 40 % of the reactor volume with tap water. This operation resulted into a decrease of FA concentrations from 57 to 9 mg N L^{-1}. After alleviating the imposed conditions, FA concentrations were maintained roughly constant throughout the operation (17 ± 4 mg N L^{-1}, Table 5.1). Consequently, nitrifying activity was detected as observed by the gradual production of TNN and nitrate (up to 12 mg N L^{-1}). Nevertheless, due to the low initial nitrifying activity, the system completed its first cycle after 13 days of operation. Despite the initial nitrate production, partial nitritation was successfully achieved and maintained for 44 days as nitrate concentrations were maintained at 1.9 ± 1.1 mg N L^{-1} (Fig. 5.2). On day 56, particle size increased up to ca. 300 µm. Just before reaching this goal, the superficial gas flow velocity incremented while the AOR experienced a threefold increase (Fig. 5.4).

Table 5.1. Experimental conditions for each operational strategy.

	FA (mg N L^{-1}) and pH	Settling time (min)	Superficial gas flow velocity (cm s^{-1})
Strategy-I	7 ± 2 (pH=8.0 ± 0.2)	30 to 10 in 18 days	0.06 (day 1 to day 18) 0.37 (day 20 to day 40)
Strategy-II	57 (day 0 to 8, pH=8.5) 17 ± 4 (day 8 to end of operation, pH=8.0 ± 0.2)	30 to 10 in 44 days	0.06 (day 1 to day 56) 0.23 (day 57 to day 80)
Strategy-III	15 ± 4 (day 0 to 7, pH=8.1 ± 0.1)(1) 88 (day 8, pH=8.6)(1) 113 (day 14, pH= 8.6)(2) 47 (day 18, pH=8.3)(1) 21 ± 7 (day 19 to end of operation, pH=8.1 ± 0.2)(1)	30 to 10 in 50 days	0.26 ± 0.07(3)
Strategy-IV	14 ± 4 (pH=8.0 ± 0.1)	30	0.28 (day 1 to day 20) 0.13 ± 0.04 (day 21 to day 60)

[1]Volumetric exchange ratio was maintained at (1)17 and (2)50 %, respectively. (3)Air and nitrogen gas were supplied in strategy-III. The corresponding air and nitrogen gas contributions into the superficial gas flow velocity are shown in **Fig. 5.5**.

A size of 535 ± 25 µm was reached at the end of the operation (Fig. 5.4). Further, 60 % of sludge particles surpassed the 200 µm threshold µm with a SVI_5/SVI_{30} ratio of 1 (Fig. 5.7). The difference in size distribution at the initial and at the end of the operation confirmed the achievement of an autotrophic aerobic granular sludge, more developed than in strategy-I (Fig. 5.7). However, after attaining a granular sludge, nitrite built-up became compromised after 60 days of operation, as observed for the unceasing nitrate production, resulting into a complete failure of the partial nitritation process.

Based on the obtained results in previous strategies, strategy-III was designed to evaluate the effect of shear stress forces on the development of an autotrophic aerobic granular sludge. On this sense, nitrogen gas was supplied together with air to work at higher initial shear stress conditions than those of previous strategies. Higher air flow rates were required than in previous strategies to maintain adequate bulk oxygen concentrations (i.e. as oxygen mass transfer rate was reduced because nitrogen gas addition) (see Fig. 5.5 and Fig. 5.6, respectively). To reduce the influence of other parameters, settling time was decreased as in strategy-II, while initial FA concentrations were maintained to guarantee an initial ammonium oxidation activity and they were subsequently increased to ensure a proper NOB inhibition.

5.3.3. Strategy-III: progressive increase of FA concentration from moderate to high inhibitory concentrations with a progressive decrease of settling time and higher initial superficial gas flow velocity

The main difference of strategy-III compared to previous strategies was the higher superficial gas flow velocity applied from the beginning of the experiment, as settling time was decreased at the same pace than in strategy-II (Fig. 5.4). Initial particle size of CAS inoculum was 129 ± 3 µm and progressively increased, reaching an average particle size of 257 ± 15 µm after 50 days of operation (Fig. 5.4). Nevertheless, it decreased to 155 ± 47 µm from day 50 to 79 (Fig. 5.4) with a simultaneous biomass loss (Fig. 5.3). FA concentrations were initially maintained at 15 ± 4 mg N L^{-1} (Table 5.1). Full nitrification was detected during the first 4 days of the operation, as observed by the increasing nitrate production. To reverse nitrate production, FA concentrations were gradually increased to avoid AOB inhibition until reaching a maximum of 113 mg N L^{-1} (Table 5.1). This resulted into a progressive nitrate rate depletion. To avoid negative effects within AOB activity, FA concentrations were progressively decreased and maintained at 21 ± 7 mg N L^{-1} until the end of the operation (Table 5.1). After this, nitrite built-up took place, while nitrate concentration maintained low (3 ± 2 mg N L^{-1}), resulting into a stable partial nitritation (Fig. 5.2). From day 78-onwards, nitrogen gas supply was almost zero (Fig. 5.5). This change affected the oxygen transfer in the bulk liquid, increasing the DO concentration (see Fig. 5.6) and causing a momentary increase of the AOR (Fig. 5.4). From then on, granule sludge size increased up to 409 ± 76 µm presenting good settleability properties (SVI_5/SVI_{30}=1) (Fig. 5.4). The concentration of granular sludge increased in a 30 % within the last 30 days of operation, confirming the achievement of a significant amount of granular sludge at the end of the operation (ca. 60 % of particles with size above 200 µm) (Fig. 5.7). In contrast to previous strategies, stable partial nitritation was successfully maintained until the end of the operation (Fig. 5.2).

According to the obtained results, a last experiment (i.e. strategy-IV) was planned to work at higher AORs compared to previous experiments and to assess its effects on the development of an autotrophic aerobic granular sludge. The reactor was inoculated with CAS but also with a fraction of an enriched AOB floccular biomass. FA concentrations and settling time were maintained roughly constant throughout the operation. The superficial gas flow velocity was set to maintain the same shear stress forces as in strategy-III but supplying only air.

5.3.4. Strategy-IV: enhancing ammonium oxidation activity by working at moderate FA concentrations and constant settling time and superficial gas flow velocity

Initial particle size was 107 ± 1 µm and rapidly increased up to 465 ± 11 µm in 27 days of operation, crossing the threshold of 200 µm in less than 20 days (Fig. 5.4). During this period, settling time was maintained high (30 min) while the initial superficial gas flow velocity was in the range of strategy-III (Table 5.1). Superficial gas flow velocity unintentionally decreased during the operation as reactor diffusor became progressively clogged. The main difference compared to other strategies was that the achieved AOR was, at least, one order of magnitude higher since the beginning of the operation (Fig. 5.4). Nevertheless, AOR decreased during reactor operation, along with a progressive biomass loss, yet it remained higher than in previous strategies (Fig. 5.4 and 5.3, respectively). Particle size was maintained at 470 ± 61 µm (SVI_5/SVI_{30}=1), with essentially 50 % of aggregates surpassing the 200 µm threshold (Fig. 5.4 and Fig. 5.7, respectively). Partial nitritation was attained since the early stage of the operation as nitrate concentrations remained low (2 ± 1 mg N L^{-1}) (Fig. 5.2). It should be stressed that nitrate gradually increased to 7 mg N L^{-1} (from day 27 to 42), though it was properly reversed within next days (Fig. 5.2).

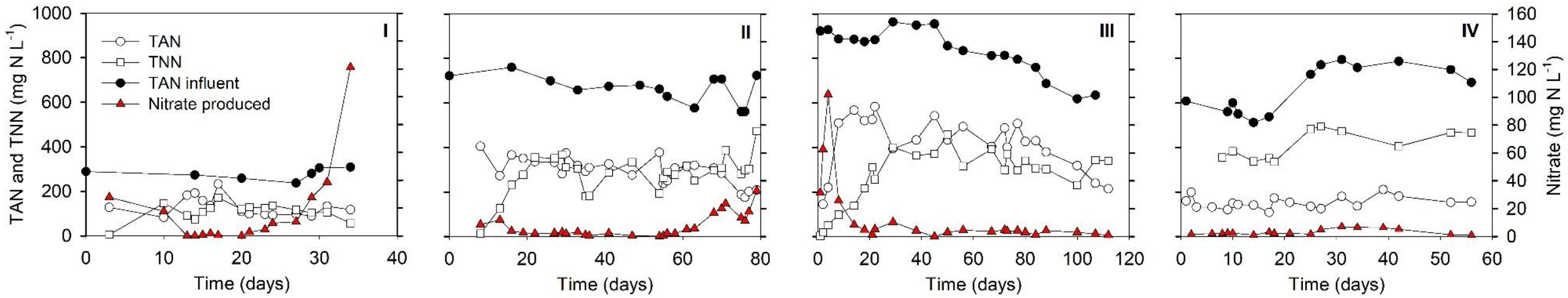

Fig. 5.2. Operational parameters and reactor performance for the four (I, II, III and IV) start-up strategies.

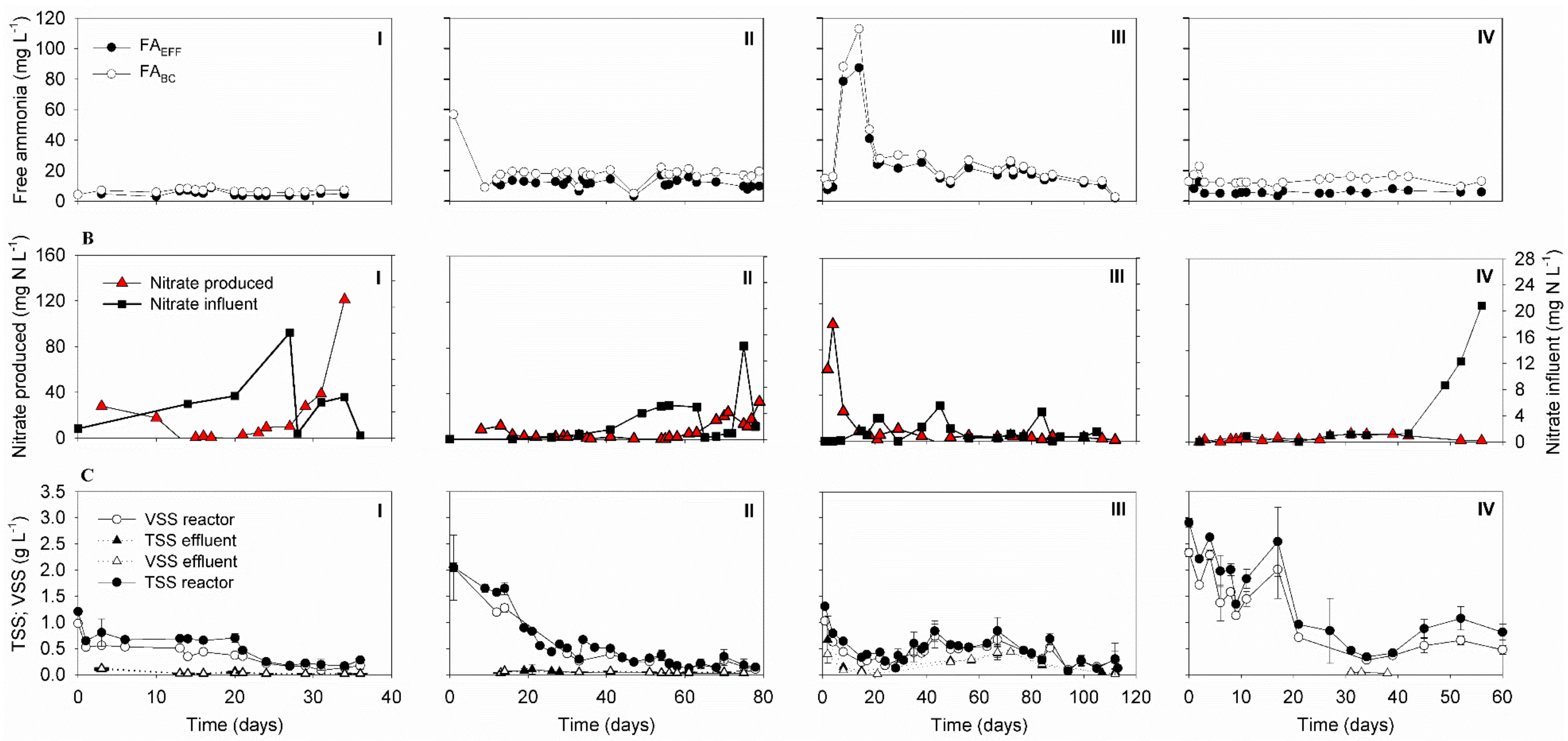

Fig. 5.3. Operational parameters and reactor performance for the four (I, II, III and IV) start-up strategies. **(A)** Time course free ammonia concentrations at the end of the cycle (FA_{EFF}) (i.e. effluent) and at the beginning of the cycle (FA_{BC}); **(B)** Time course of produced nitrate and influent nitrate concentrations and **(C)** Time course solids concentrations.

5.4. Discussion

5.4.1. Effect of free ammonia and settling time decrease in the development of an autotrophic aerobic granular sludge performing partial nitritation

During the first 13 days of strategy-I, a significant amount of nitrate was produced (Fig. 5.2), despite working at the reported inhibitory FA concentrations for NOB inhibition within CAS (7 ± 2 mg N L^{-1}) (Abeling and Seyfried, 1992; Chung et al., 2006). The initial NOB persistence was reduced in strategy-II by applying a higher FA inhibitory concentration (i.e. 57 mg N L^{-1}) (Fig. 5.3). However, this FA concentration severely reduced ammonium oxidation activity (i.e. null AORs) (Fig. 5.4). Indeed, ammonium oxidation activity was only detected some days after alleviating the applied FA inhibitory conditions (Fig. 5.4). During the initial phase of strategy-III, FA concentrations were maintained at levels to ensure an initial ammonium oxidation activity, regardless of nitrate production (Fig. 5.3). Then, FA was progressively increased up to high inhibitory conditions (i.e. 113 mg N L^{-1}), which resulted into a consecutively nitrite built-up (Fig. 5.2). However, as CAS was not adapted to tolerate high FA concentrations, these were relaxed to avoid negative effects on AOB metabolism (Liu et al., 2019; Vadivelu et al., 2007) (Fig. 5.3). Along with the suppression of NOB within the seeding sludge, settling time was simultaneously decreased in strategies I, II and III. The reduction of settling time have been described as a mechanism contributing to the achievement of heterotrophic aerobic granulation, mainly due to the selection of slow-growing heterotrophic bacterial species (Liu and Tay, 2004). However, no granule development was observed in this study when settling time was progressively decreased from 30 to 15 min (Fig. 5.4). In fact, the reduction of settling time led to a progressively biomass loss (ca. 0.5 g VSS L^{-1}, Fig. 5.3) and, thus, limited the achievement of high AORs (Fig. 5.4).

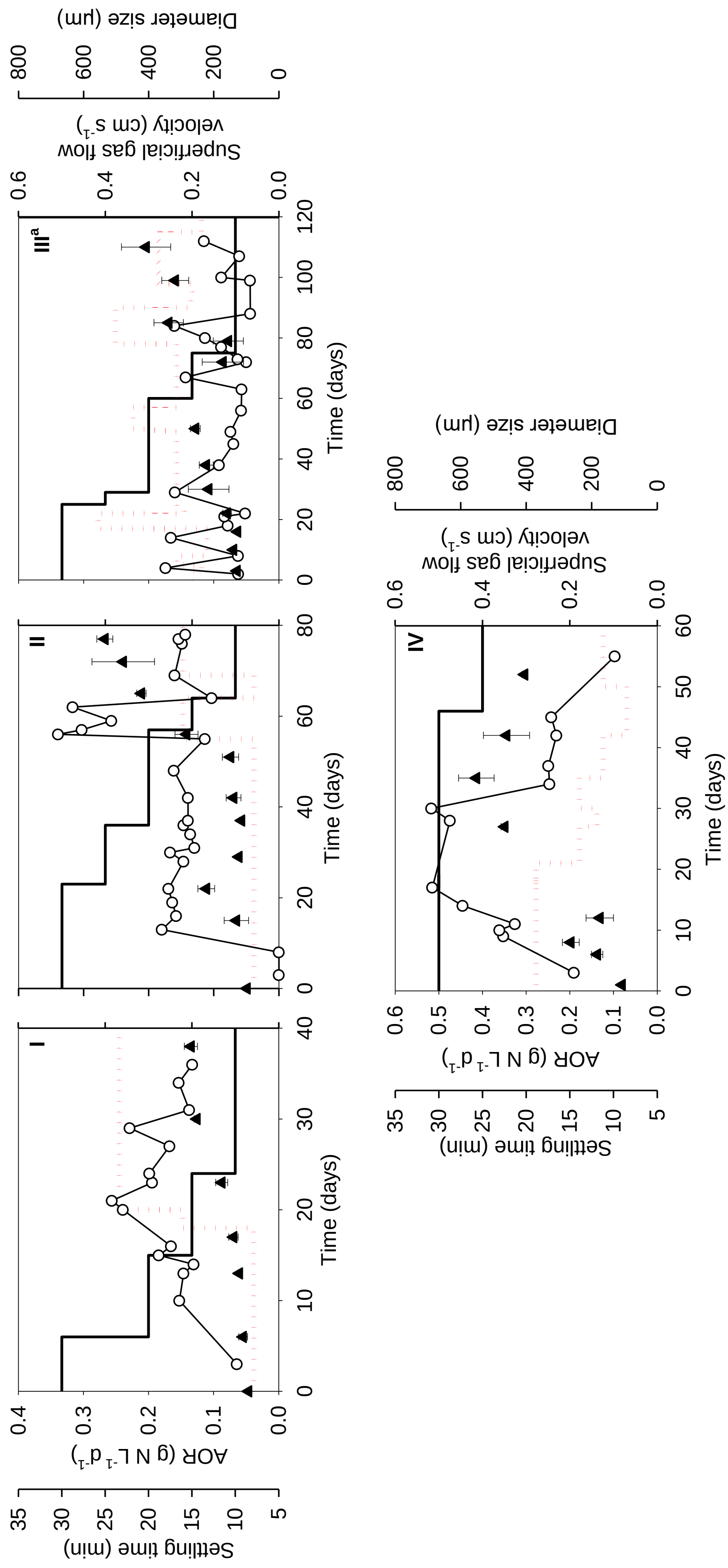

Fig. 5.4. Time course superficial gas-flow velocity (u_g) (red dashed line), ammonium oxidation rates (AORs) (white dots), diameter size (black triangles), settling time (black line) for the four (I, II, III and IV) start-up strategies. [(a)]The contribution of air and nitrogen flow rates of strategy-III can be found in Fig. 5.5.

A fast decrease in settling time negatively influenced the ability of retaining a sufficient concentration of nitrifying bacteria. Also, applying high FA shocks to a non-acclimated sludge showed to present disadvantages in terms of attaining a high ammonium oxidation activity. Consequently, the inhibitory FA concentrations with the concomitant reduction of settling time resulted into low AORs, which were not beneficial for the development of an autotrophic aerobic granular sludge.

5.4.2. Is the enhancement of superficial gas flow velocity promoting autotrophic aerobic granulation?

Operating at high superficial gas flow velocities allows for higher shear stress conditions, which can promote EPS secretion, contributing to the attachment and self-immobilization process of bacteria (Di Iaconi et al., 2005; Tay et al., 2001). This mechanism is well-established for heterotrophic aerobic granular sludge, but it is unclear for autotrophic aerobic biomass. Also, working at high air flow rates grant for greater bulk oxygen concentrations, allowing AOB to increase their activity. The obtained results showed that granule development was achieved in first strategies (I and II) just after increasing the air flow rate, in turn resulting in an increase of the AOR (Fig. 5.4). To evaluate which mechanisms derived from incrementing the air flow rate triggered the development of an autotrophic aerobic granular sludge (i.e. shear stress conditions vs. ammonium oxidation activity), a third experiment (strategy-III) was performed. In strategy-III, a higher superficial gas flow velocity was applied since the early stage of the operation by supplying both air and nitrogen gas. During this strategy, a faster increase of particle size was detected, from 128 ± 3 μm to 257 ± 15 μm in 50 days, in accordance with the higher superficial gas flow velocity (Fig. 5.4). However, particle size subsequently decreased from day 50 to 79 (Fig. 5.4) due to detachment, as a significant increase in the effluent VSS concentration was measured (Fig. 5.3). The high detachment rates declined after day

80, consistent with the interruption of nitrogen gas supply. When only air was supplied (day 78, Fig. 5.5), an increase in granule size was measured (from 155 ± 47 µm on day 79 to 339 ± 46 µm on day 85, see Fig. 5.4). This change also resulted into a marked increase in the ammonium oxidation activity (i.e. AOR) (Fig. 5.4) and higher bulk DO concentrations (see Fig. 5.6). Likewise, granular sludge development of previous strategies (I and II) occurred just after incrementing the superficial gas flow velocity and, consequently, observing an increase in the AORs (see Fig. 5.4 and Fig. 5.5, respectively). Experiments indicate that the development of an autotrophic aerobic granular sludge was promoted by the higher ammonium oxidation activity derived from boosting air flow rate (i.e. higher DO), whereas the effects of shear stress forces alone were not enough, because they could also trigger significant detachment events. Indeed, high shear stress conditions caused AOB detachment and derived into a failure of the nitritation process in a stratified nitrifying granule structure; the high detachment rates resulted into a thinner AOB layer and into a smaller granular sludge size, incrementing oxygen availability to the inner layers and, thus, allowing nitrite oxidation (Liu et al., 2020).

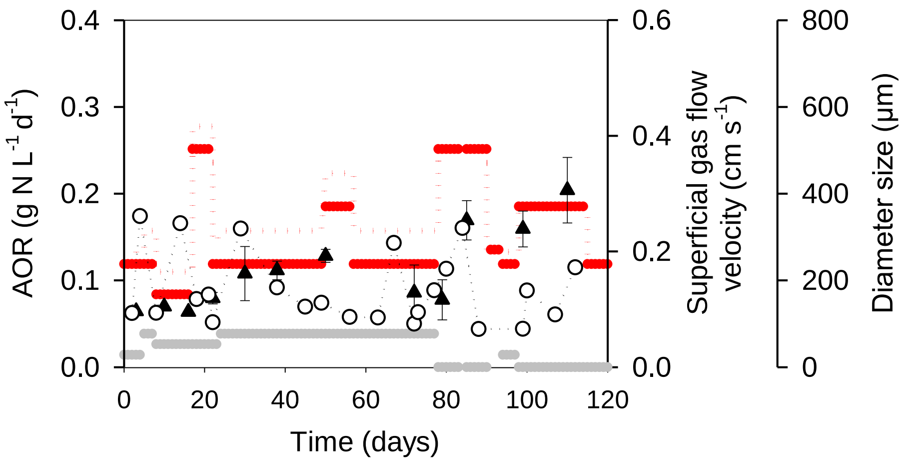

Fig. 5.5. Time course superficial gas flow velocity (u_g) (red dashed line), air (red dots) and nitrogen (grey dots) flow velocity, ammonium oxidation rates (AORs) (white dots), and diameter size (black triangles) corresponding to start-up strategy-III.

5.4.3. Linking the effects of ammonium oxidation activity to the development of an autotrophic aerobic granular sludge performing partial nitritation

The role of ammonium oxidation activity on the development of an autotrophic aerobic granular sludge from CAS was further investigated in strategy-IV. Compared to previous strategies, higher AORs were obtained since the beginning of strategy-IV, as the inoculum was enriched with a fraction of nitrifying biomass (Fig. 5.4).

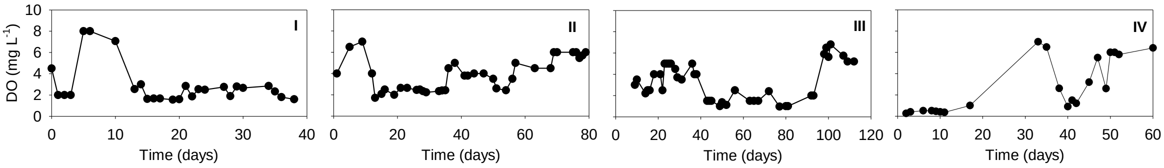

Fig. 5.6. Time course oxygen concentrations for the four (I, II, III and IV) start-up strategies.

The high ammonium oxidation activity derived into a high oxygen consumption (i.e. low bulk DO concentrations, see Fig. 5.6). Under higher AORs (compared to previous strategies), granular sludge was rapidly developed while partial nitritation was successfully achieved and maintained stable throughout the operation (see Fig. 5.4 and 5.2, respectively). This is consistent with a study that allowed for a fast start-up of the nitritation process within an aerobic granular sludge by enhancing AOB at high DO concentrations (3 – 5 mg O_2 L^{-1}), resulting into even higher and more compact granules (Wang et al., 2021). As the applied shear stress conditions were maintained similar than in strategy-III and settling time remained high, the development of an autotrophic aerobic granular sludge was shown to be promoted by the enhancement of ammonium oxidation activity in the early phase of the operation.

A period of nitrate production (up to 7 mg N L^{-1}) was detected from day 27 to 42 (Fig. 5.2) related to an increase in bulk DO concentrations (up to 8 mg O_2 L^{-1}), which could not be monitored due to a failure of the bulk DO monitoring system that lasted for 15 days (see Fig. 5.6). The higher oxygen availability derived into a transient nitrite oxidation, as nitrate production successfully decreased (≤ 2 mg N L^{-1}, Fig. 5.2) once bulk

DO concentration was lowered to 1.5 mg O_2 L^{-1}, and further maintained at ca. 6 mg O_2 L^{-1} (day 40-onwards, see Fig. 5.6). The bulk DO concentration affects the oxygen penetration depth since when bulk DO concentration increases, oxygen can reach deeper layers of the granule. This fast shift from nitrification to partial nitritation when DO is manipulated has been identified as a strong indication of the stratification of nitrifier guilds in granular sludge (Picioreanu et al., 2016; Poot et al., 2016; Soler-Jofra et al., 2019).

Previous strategies demonstrated that the reduction of settling time was not promoting the development of an autotrophic granular sludge. Also, the effects of shear stress forces derived from incrementing the superficial gas flow velocity (by adding nitrogen gas) showed to be not fully determinant if a high ammonium oxidation activity was not simultaneously promoted. The obtained results in strategy-IV showed that the high initial ammonium oxidation activity, measured as AOR, accounted for the development of an autotrophic aerobic granular sludge successfully performing partial nitritation. The representative fraction corresponding to nitrifiers in CAS is rather low (i.e. 3 %) (Harms et al., 2003; Torà et al., 2013). Thus, promoting high AORs in the early stage of the operation can be challenging. Under these conditions, the positive effects derived from increasing air flow rate were demonstrated to be effective in promoting autotrophic aerobic granular sludge formation as ammonium oxidation activity was enhanced.

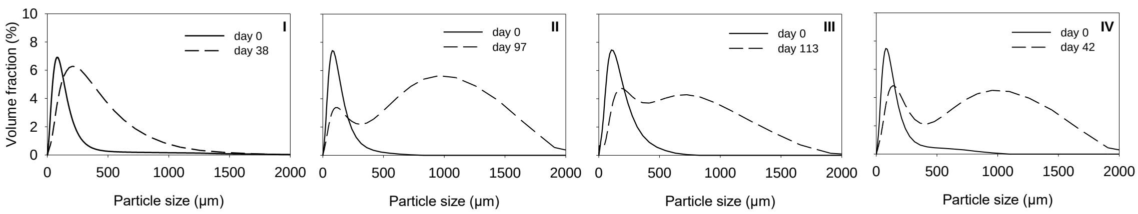

Fig. 5.7. Size distribution of the sludge for the four (I, II, III and IV) start-up strategies at the initial (solid line) and at the end-phase (dashed line) of the operation.

5.4.4. NOB influent inoculation deteriorates partial nitritation stability

Partial nitritation was not successfully maintained in strategies I and II (see Fig. 5.2), even after attaining a significant fraction of granular sludge at the end of reactor operation (ca. 50 - 60 %). In contrast, nitrite oxidation was almost completely suppressed in strategy-III (Fig. 5.2). The difference among these strategies were not the FA concentrations maintained throughout the operation (Fig. 5.2), neither the [DO]/[TAN] ratio (0.014 ± 0.006). Indeed, this last parameter was lower than the reported to maintain a successfully stable nitritation in a granular sludge reactor (Bartrolí et al., 2010). The reason that could explain the recovery of nitrite oxidation activity in strategies I and II is the continuous NOB reinoculation with the inflow. The existence of this reinoculation can be inferred from the presence or absence of nitrate influent concentrations (Fig. 5.3). The used reject water was stored in a tank several weeks and part of the TAN concentration was progressively oxidized to nitrite, and, further to nitrate (Fig. 5.3). Hence, the continuous TAN oxidation clearly indicated the existence of AOB and NOB populations within the storage wastewater and, consequently, both were continuously seeded to the reactor. In contrast, the presence of nitrate in the influent of strategy-III was almost not detected (Fig. 5.3). The NOB population developed in the storing tank probably possessed a high tolerance to FA, which resulted in failure of the partial nitritation process (Duan et al., 2019). Interestingly, in strategy-IV there was a significant amount of nitrate in the influent from day 40-onwards (Fig. 5.3), yet this did not result into nitrate production within the reactor. Compared to previous strategies, the achieved AORs were, at least, one order of magnitude higher (Fig. 5.4). When NOB inoculation took place in strategy-IV, a significant fraction of granular sludge was already obtained (ca. 50 % of particles being greater than 200 μm), which decreased the impact of the NOB seeding, because the number of cycles per day was higher at higher AORs (i.e. shorter hydraulic retention time (HRT) and higher washout of the reinoculated NOB). The wastewater storage during the development of a granular sludge performing partial nitritation should be done in conditions to avoid NOB proliferation. For eventual full-scale applications of the partial nitritation process, wastewater storage is not required, therefore this issue would not require particular attention.

5.5. Conclusions

- A specific operational start-up strategy based on enhancing ammonium oxidation activity promoted the development of an autotrophic aerobic granular sludge performing a stable partial nitritation by using conventional activated sludge as inoculum in a short period of time (ca. 30 days).
- When the inoculum has a low activity, the ammonium oxidation activity of the seeding sludge can be enhanced by increasing the air flow rate to allow for a higher bulk oxygen concentration.
- Against expectation –based on the reported conditions for granulation of heterotrophic aerobic sludge– settling time decrease appeared to be not linked to autotrophic aerobic granulation. In contrast, high settling times allowed to retain a suitable biomass concentration, beneficial for attaining higher ammonium oxidation activities.
- Strong free ammonia inhibitory conditions in the early start-up phase (i.e. seeding sludge) resulted into low ammonium oxidation rates, hindering the development of an autotrophic aerobic granular sludge.

Chapter 6. Conclusions

The goal of this thesis was to study the feasibility of a two-stage partial nitritation/anammox (PN/AMX) process at mainstream conditions. The PN/AMX process brings the possibility to achieve energy neutral (or even energy generating) wastewater treatment plants (WWTPs). The main challenges associated to mainstream PN/AMX are related to the low temperatures and nitrogen concentrations which hinder nitrite oxidizing bacteria (NOB) repression and decrease anammox growth rate, the need to meet effluent requirements and the need to be competitive with conventional treatments by achieving high and stable loading rates.

The first objective was to investigate the feasibility of anammox bacteria to treat a real mainstream wastewater at the typical conditions of a full-scale WWTP. For this purpose, an up-flow anammox sludge bed (UAnSB) reactor was operated for ca. 350 days by maintaining high and stable nitrogen removal rates (0.11 ± 0.01 g N L^{-1} d^{-1}) during a (realistic) summer-to-winter temperature transition, from 20 to 10 ºC plus a three months period at 10 ºC. Substrate distribution profile showed that the anammox activity was mainly concentrated in the bottom sludge bed section at 20 ºC, though it experienced a significant decrease at 10 ºC. The activity decrease of the bottom sludge bed section was counterbalanced by the biomass activity in middle and upper sludge bed sections, which experienced an almost 10-fold activity increase at 10 ºC. Thus, the successful UAnSB reactor performance of dampening a 10 ºC drop while maintaining a stable removal performance was attributed to the intra bed overcapacity resulting from the heterogenous substrate distribution measured along the sludge bed. Besides the capacity of the UAnSB reactor of facing cold temperatures, the average NRR achieved were high enough to be competitive with those of conventional treatments. Anammox activity dominated the overall nitrogen conversion, although the detected small degree of heterotrophic denitrifying activity helped to improve effluent quality by decreasing the nitrate concentrations produced by the anammox process. Finally, results from 16S rRNA sequencing showed that the microbial community was dominated by species of the Planctomycetes phylum, along the different sludge bed sections of the UAnSB reactor at both 20 and 10 ºC. In conclusion, there seems to be no obstacle for the application of the anammox process in the main water line of an urban WWTP.

One of the main bottlenecks of mainstream PN/AMX is still the optimization of nitrogen removal efficiency and effluent quality. Consequently, an ad hoc reactor was designed for coupling anammox to heterotrophic denitrification in a single reactor unit (i.e. the so-called CANDLE reactor, **C**oupling **A**nammox and **D**enitrification in a sing**LE** unit). The CANDLE reactor was tested with real mainstream wastewater and fed with acetate as an external C-source over a wide temperature range (from 20 to 14 ºC) for ca. 200 days. The obtained results showed that the enhancement of heterotrophic denitrifying activity allowed to reduce the nitrate produced by anammox bacteria, without compromising the anammox performance. This, in turn, allowed to significantly improve effluent quality while maintaining high and constant NRRs (0.16 ± 0.03 g N L^{-1} d^{-1}) throughout reactor operation. Further, a proper organic load management showed to be effective to face the drawbacks derived from previous mainstream treatment stages (i.e. undesired nitrate production), without compromising organic effluent quality. The study of substrate profile concentrations along the CANDLE reactor indicated that the successful coupling and coexistence of anammox and heterotrophic denitrifying was possible as anammox activity dominated within bottom sludge bed sections, whereas heterotrophic denitrification occurred within middle and upper sludge bed sections. Microbial community results obtained by 16S rRNA sequencing confirmed that both populations (anammox and heterotrophic denitrifiers) occupied differentiated sludge bed compartments dominated by the *Candidatus* Brocadia and *Thauera* genus, respectively.

Additionally, the enhancement of heterotrophic denitrifying activity allowed to mitigate nitrous oxides (N_2O) emissions. Overall, the CANDLE reactor is here presented as an appropriate technology to enhance the robustness of the two-stage mainstream PN/AMX process and represent a step-forward towards the efficiency improvement of this technology.

The main challenge for the application of PN/AMX for sewage treatment is related to the potential nitrate production in the PN step, although a successful long-term operation has been achieved in granular reactors at low water temperatures (10 ºC). The last aim of this dissertation was to investigate the influence of different operational parameters in the development of an autotrophic granular sludge performing a stable nitritation using conventional activated sludge (CAS) as inoculum in an air-lift reactor in four different operational start-up strategies. Enhancing ammonium oxidation activity in the early phase of the operation by enriching the inoculum with a fraction of a floccular nitrifying biomass was shown to be the reason of the fast development (ca. 30 days) of an autotrophic aerobic granular sludge performing a stable nitritation. When the seeded sludge presented a low nitrifying activity (i.e. not enriched), increasing the air flow rate was demonstrated to be effective in promoting autotrophic aerobic granular sludge formation as ammonium oxidation activity was enhanced. In contrast, the applied low settling and the imposed inhibitory free ammonia (FA) concentrations tested within the first start-up operational strategies hampered the development of an autotrophic aerobic granular sludge. This study demonstrated the importance of ensuring high ammonium oxidation rates for the proper development of an autotrophic aerobic granular sludge performing stable nitritation. Nevertheless, further research efforts need to be addressed on better understanding the aerobic autotrophic granulation mechanisms by deeply studying the effects of other operational parameters such as dissolved oxygen (DO) and the effect of different reactor configurations, optimizing the enhancement of the inoculum activity and improving the long-term granulation and PN stability.

Chapter 7. References

Abeling, U., Seyfried, C.F., 1992. Anaerobic-aerobic treatment of high-strength ammonium wastewater - Nitrogen removal via nitrite, in: Water Science and Technology. https://doi.org/10.2166/wst.1992.0542

Alcamo, J., Flörke, M., Märker, M., 2007. Future long-term changes in global water resources driven by socio-economic and climatic changes. Hydrol. Sci. J. 52, 247–275. https://doi.org/10.1623/hysj.52.2.247

Annavajhala, M.K., Kapoor, V., Santo-Domingo, J., Chandran, K., 2018. Structural and functional interrogation of selected biological nitrogen removal systems in the United States, Denmark, and Singapore using shotgun metagenomics. Front. Microbiol. 9, 2544. https://doi.org/10.3389/fmicb.2018.02544

Anthonisen, A.C., Loehr, R.C., Prakasam, T.B.S., Srinath, E.G., 1976. Inhibition of Nitrification by Ammonia and Nitrous Acid. Water Pollut. Control Fed. 48, 835–852.

APHA, 2005. APHA, Standard methods for the examination of water and wastewater, 21st ed., American Public Health Association, Washington, DC, USA.

Ardern, E., Lockett, W.T., 1914. Experiments on the oxidation of sewage without the aid of filters. J. Soc. Chem. Ind. 33, 523–539. https://doi.org/10.1002/jctb.5000331005

Bartrolí, A., Carrera, J., Pérez, J., 2011. Bioaugmentation as a tool for improving the start-up and stability of a pilot-scale partial nitrification biofilm airlift reactor. Bioresour. Technol. 102, 4370–4375. https://doi.org/10.1016/j.biortech.2010.12.084

Bartrolí, A., Pérez, J., Carrera, J., 2010. Applying ratio control in a continuous granular reactor to achieve full nitritation under stable operating conditions. Environ. Sci. Technol. 44, 8930–8935. https://doi.org/10.1021/es1019405

Bhattacharjee, A.S., Wu, S., Lawson, C.E., Jetten, M.S.M., Kapoor, V., Domingo, J.W.S., McMahon, K.D., Noguera, D.R., Goel, R., 2017. Whole-Community Metagenomics in Two Different Anammox Configurations: Process Performance and Community Structure. Environ. Sci. Technol. 51, 4317–4327. https://doi.org/10.1021/acs.est.6b05855

Böehnke, B., 1977. Das Adsorptions-Belebungsverfahren. Korrespondenz Abwasser 24, 121–127.

Böehnke, B., Schulze-Rettmer, R., Zuckut, S., 1998. Cost-effective reduction of high-strength wastewater by adsorption-based activated sludge technology. J. Wat. Eng. Manag. 145, 1–34.

Campos, J.L., Valenzuela-Heredia, D., Pedrouso, A., Val Del Río, A., Belmonte, M., Mosquera-Corral, A., 2016. Greenhouse Gases Emissions from Wastewater Treatment Plants: Minimization, Treatment, and Prevention. J. Chem. https://doi.org/10.1155/2016/3796352

Cao, Y., van Loosdrecht, M.C.M., Daigger, G.T., 2017. Mainstream partial nitritation–anammox in municipal wastewater treatment: status, bottlenecks, and further studies. Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-016-8058-7

Chen, C., Sun, F., Zhang, H., Wang, J., Shen, Y., Liang, X., 2016. Evaluation of COD effect on anammox process and microbial communities in the anaerobic baffled reactor (ABR). Bioresour. Technol. 216, 571–578. https://doi.org/10.1016/j.biortech.2016.05.115

Chen, Y., Zhao, Z., Liu, H., Ma, Y., An, F., Huang, J., 2020. Chemosphere Achieving stable two-stage mainstream partial-nitrification/anammox (PN/A) operation via intermittent aeration. Chemosphere 245, 125650. https://doi.org/10.1016/j.chemosphere.2019.125650

Chung, J., Shim, H., Park, S.J., Kim, S.J., Bae, W., 2006. Optimization of free ammonia concentration for nitrite accumulation in shortcut biological nitrogen removal process. Bioprocess Biosyst. Eng. 28, 275–282. https://doi.org/10.1007/s00449-005-0035-y

Conthe, M., Lycus, P., Arntzen, M., Ramos da Silva, A., Frostegård, Å., Bakken, L.R., Kleerebezem, R., van Loosdrecht, M.C.M., 2019. Denitrification as an N2O sink. Water Res. 151, 381–387. https://doi.org/10.1016/j.watres.2018.11.087

Daims, H., Lücker, S., Wagner, M., 2016. A New Perspective on Microbes Formerly Known as Nitrite-Oxidizing Bacteria. Trends Microbiol. 24, 699–712. https://doi.org/10.1016/j.tim.2016.05.004

De Clippeleir, H., Vlaeminck, S.E., De Wilde, F., Daeninck, K., Mosquera, M., Boeckx, P., Verstraete, W., Boon, N., 2013. One-stage partial nitritation/anammox at 15 C on pretreated sewage: Feasibility demonstration at lab-scale. Appl. Microbiol.

Biotechnol. 97, 10199–10210. https://doi.org/10.1007/s00253-013-4744-x

de Kreuk, M.K., Kishida, N., van Loosdrecht, M.C.M., 2007. Aerobic granular sludge - State of the art. Water Sci. Technol. 55, 75–81. https://doi.org/10.2166/wst.2007.244

Di Iaconi, C., Ramadori, R., Lopez, A., Passino, R., 2005. Hydraulic shear stress calculation in a sequencing batch biofilm reactor with granular biomass. Environ. Sci. Technol. 39, 889–894. https://doi.org/10.1021/es0400483

Dosta, J., Fernández, I., Vázquez-Padín, J.R., Mosquera-Corral, A., Campos, J.L., Mata-Álvarez, J., Méndez, R., 2008. Short- and long-term effects of temperature on the Anammox process. J. Hazard. Mater. 154, 688–693. https://doi.org/10.1016/j.jhazmat.2007.10.082

Du, R., Cao, S., Li, B., Niu, M., Wang, S., Peng, Y., 2017. Performance and microbial community analysis of a novel DEAMOX based on partial-denitrification and anammox treating ammonia and nitrate wastewaters. Water Res. https://doi.org/10.1016/j.watres.2016.10.051

Duan, H., Ye, L., Wang, Q., Zheng, M., Lu, X., Wang, Z., Yuan, Z., 2019. Nitrite oxidizing bacteria (NOB) contained in influent deteriorate mainstream NOB suppression by sidestream inactivation. Water Res. 162, 331–338. https://doi.org/10.1016/j.watres.2019.07.002

Edgar, R.C., 2013. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10, 996–998. https://doi.org/10.1038/nmeth.2604

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461. https://doi.org/10.1093/bioinformatics/btq461

EPA, 2013. Energy Efficiency in Water and Wastewater Facilities: A Guide To Developing and Implementing Greenhouse Gas Reduction Programs, Local Government Climate and Energy Strategy Guides.

Fahrbach, M., Kuever, J., Meinke, R., Kämpfer, P., Hollender, J., 2006. Denitratisoma oestradiolicum gen. nov., sp. nov., a 17 β-oestradiol-degrading, denitrifying betaproteobacterium. Int. J. Syst. Evol. Microbiol. 56, 1547–1552. https://doi.org/10.1099/ijs.0.63672-0

García-Ruiz, M.J., Maza-Márquez, P., González-López, J., Osorio, F., 2018. Nitrogen removal capacity and bacterial community dynamics of a Canon biofilter system at different organic matter concentrations. Chemosphere 193, 591–601. https://doi.org/10.1016/j.chemosphere.2017.11.066

Gilbert, E.M., Agrawal, S., Karst, S.M., Horn, H., Nielsen, P.H., Lackner, S., 2014. Low Temperature Partial Nitritation/Anammox in a Moving Bed Biofilm Reactor Treating Low Strength Wastewater. Environ. Sci. Technol. 48, 8784–8792. https://doi.org/10.1021/es501649m

Gilbert, E.M., Agrawal, S., Schwartz, T., Horn, H., Lackner, S., 2015. Comparing different reactor configurations for Partial Nitritation / Anammox at low temperatures. Water Res. 81, 92–100. https://doi.org/10.1016/j.watres.2015.05.022

Gonzalez-Martinez, A., Rodriguez-Sanchez, A., Garcia-Ruiz, M.J., Muñoz-Palazon, B., Cortes-Lorenzo, C., Osorio, F., Vahala, R., 2016. Performance and bacterial community dynamics of a CANON bioreactor acclimated from high to low operational temperatures. Chem. Eng. J. 287, 557–567. https://doi.org/10.1016/j.cej.2015.11.081

Guisasola, A., Jubany, I., Baeza, J.A., Carrera, J., Lafuente, J., 2005. Respirometric estimation of the oxygen affinity constants for biological ammonium and nitrite oxidation. J. Chem. Technol. Biotechnol. 80, 388–396. https://doi.org/10.1002/jctb.1202

Guo, Y., Chen, Y., Webeck, E., Li, Y.Y., 2020. Towards more efficient nitrogen removal and phosphorus recovery from digestion effluent: Latest developments in the anammox-based process from the application perspective. Bioresour. Technol. 299, 122560. https://doi.org/10.1016/j.biortech.2019.122560

Güven, D., Dapena, A., Kartal, B., Schmid, M.C., Maas, B., van de Pas-Schoonen, K., Sozen, S., Mendez, R., Op den Camp, H.J.M., Jetten, M.S.M., Strous, M., Schmidt, I., 2005. Propionate oxidation by and methanol inhibition of anaerobic ammonium-oxidizing bacteria. Appl. Environ. Microbiol. 71, 1066–71. https://doi.org/10.1128/AEM.71.2.1066-1071.2005

Harms, G., Layton, A.C., Dionisi, H.M., Gregory, I.R., Garrett, V.M., Hawkins, S.A., Robinson, K.G., Sayler, G.S., 2003. Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. Environ. Sci. Technol. 37, 343–351. https://doi.org/10.1021/es0257164

Hasan, M.N., Altaf, M.M., Khan, N.A., Khan, A.H., Khan, A.A., Ahmed, S., Kumar, P.S., Naushad, M., Rajapaksha, A.U., Iqbal, J., Tirth, V., Islam, S., 2021. Recent technologies for nutrient removal and recovery from wastewaters: A review. Chemosphere 277, 130328. https://doi.org/10.1016/j.chemosphere.2021.130328

He, S., Chen, Y., Qin, M., Mao, Z., Yuan, L., Niu, Q., Tan, X., 2018. Effects of temperature on anammox performance and community structure. Bioresour. Technol. 260, 186–195. https://doi.org/10.1016/j.biortech.2018.03.090

Hellinga, C., Schellen, A.A.J.C., Mulder, J.W., Van Loosdrecht, M.C.M., Heijnen, J.J., 1998. The SHARON process: An innovative method for nitrogen removal from ammonium-rich waste water, in: Water Science and Technology. No longer published by Elsevier, pp. 135–142. https://doi.org/10.1016/S0273-1223(98)00281-9

Hendrickx, T.L.G., Wang, Y., Kampman, C., Zeeman, G., Temmink, H., Buisman, C.J.N., 2012. Autotrophic nitrogen removal from low strength waste water at low temperature. Water Res. 46, 2187–2193. https://doi.org/10.1016/j.watres.2012.01.037

Isanta, E., Bezerra, T., Fernández, I., Suárez-Ojeda, M.E., Pérez, J., Carrera, J., 2015a. Microbial community shifts on an anammox reactor after a temperature shock using 454-pyrosequencing analysis. Bioresour. Technol. 181, 207–213. https://doi.org/10.1016/j.biortech.2015.01.064

Isanta, E., Reino, C., Carrera, J., Pérez, J., 2015b. Stable partial nitritation for low-strength wastewater at low temperature in an aerobic granular reactor. Water Res. 80, 149–158. https://doi.org/10.1016/j.watres.2015.04.028

Jetten, M.S.M., Horn, S.J., Van Loosdrecht, M.C.M., 1997. Towards a more sustainable municipal wastewater treatment system. Water Sci. Technol. 35, 171–180. https://doi.org/10.1016/S0273-1223(97)00195-9

Ji, J., Peng, Y., Li, X., Zhang, Q., Liu, X., 2020. A novel partial nitrification-synchronous anammox and endogenous partial denitrification (PN-SAEPD) process for advanced nitrogen removal from municipal wastewater at ambient temperatures. Water Res. 115690. https://doi.org/10.1016/j.watres.2020.115690

Jia, M., Castro-Barros, C.M., Winkler, M.K.H., Volcke, E.I.P., 2018. Effect of organic matter on the performance and N2O emission of a granular sludge anammox reactor. Environ. Sci. Water Res. Technol. 4, 1035–1046. https://doi.org/10.1039/c8ew00125a

Joss, A., Salzgeber, D., Eugster, J., König, R., Rottermann, K., Burger, S., Fabijan, P., Leumann, S., Mohn, J., Siegrist, H.R., 2009. Full-scale nitrogen removal from digester liquid with partial nitritation and anammox in one SBR. Environ. Sci. Technol. 43, 5301–5306. https://doi.org/10.1021/es900107w

Juan-Díaz, X., Pérez, J., Carrera, J., 2021. Effective dampening of temperature effects in an anammox reactor treating real mainstream wastewater. J. Water Process Eng. 101853. https://doi.org/10.1016/j.jwpe.2020.101853

Jubany, I., Lafuente, J., Baeza, J.A., Carrera, J., 2009. Total and stable washout of nitrite oxidizing bacteria from a nitrifying continuous activated sludge system using automatic control based on Oxygen Uptake Rate measurements. Water Res. 43, 2761–2772. https://doi.org/10.1016/j.watres.2009.03.022

Kampschreur, M.J., Tan, N.C.G., Kleerebezem, R., Picioreanu, C., Jetten, M.S.M., Van Loosdrecht, M.C.M., 2008. Effect of dynamic process conditions on nitrogen oxides emission from a nitrifying culture. Environ. Sci. Technol. 42, 429–435. https://doi.org/10.1021/es071667p

Kartal, B., Keltjens, J.T., 2016. Anammox Biochemistry: a Tale of Heme c Proteins. Trends Biochem. Sci. https://doi.org/10.1016/j.tibs.2016.08.015

Kartal, B., Kuenen, J.G., Van Loosdrecht, M.C.M., 2010. Sewage treatment with anammox. Science (80-.). 328, 702–703. https://doi.org/10.1126/science.1185941

Kartal, B., Kuypers, M.M.M., Lavik, G., Schalk, J., Op Den Camp, H.J.M., Jetten, M.S.M., Strous, M., 2007. Anammox bacteria disguised as denitrifiers: Nitrate reduction to dinitrogen gas via nitrite and ammonium. Environ. Microbiol. 9, 635–642. https://doi.org/10.1111/j.1462-2920.2006.01183.x

Kindaichi, T., Yuri, S., Ozaki, N., Ohashi, A., 2012. Ecophysiological role and function of uncultured Chloroflexi in an anammox reactor. Water Sci. Technol. 66, 2556–2561. https://doi.org/10.2166/wst.2012.479

Lackner, S., Gilbert, E.M., Vlaeminck, S.E., Joss, A., Horn, H., van Loosdrecht, M.C.M., 2014. Full-scale partial nitritation/anammox experiences - An application survey.

Water Res. 55, 292–303. https://doi.org/10.1016/j.watres.2014.02.032

Lackner, S., Terada, A., Smets, B.F., 2008. Heterotrophic activity compromises autotrophic nitrogen removal in membrane-aerated biofilms: Results of a modeling study. Water Res. 42, 1102–1112. https://doi.org/10.1016/j.watres.2007.08.025

Larsen, T.A., Udert, K.M., Lienert, J., 2015. Source Separation and Decentralization for Wastewater Management, Source Separation and Decentralization for Wastewater Management. https://doi.org/10.2166/9781780401072

Laureni, M., Falås, P., Robin, O., Wick, A., Weissbrodt, D.G., Nielsen, J.L., Ternes, T.A., Morgenroth, E., Joss, A., 2016. Mainstream partial nitritation and anammox: Long-term process stability and effluent quality at low temperatures. Water Res. 101, 628–639. https://doi.org/10.1016/j.watres.2016.05.005

Laureni, M., Weissbrodt, D.G., Szivák, I., Robin, O., Nielsen, J.L., Morgenroth, E., Joss, A., 2015. Activity and growth of anammox biomass on aerobically pre-treated municipal wastewater. Water Res. 80, 325–336. https://doi.org/10.1016/j.watres.2015.04.026

Laureni, M., Weissbrodt, D.G., Villez, K., Robin, O., de Jonge, N., Rosenthal, A., Wells, G., Nielsen, J.L., Morgenroth, E., Joss, A., 2019. Biomass segregation between biofilm and flocs improves the control of nitrite-oxidizing bacteria in mainstream partial nitritation and anammox processes. Water Res. 154, 104–116. https://doi.org/10.1016/j.watres.2018.12.051

Lawson, C.E., Wu, S., Bhattacharjee, A.S., Hamilton, J.J., McMahon, K.D., Goel, R., Noguera, D.R., 2017. Metabolic network analysis reveals microbial community interactions in anammox granules. Nat. Commun. 8, 15416. https://doi.org/10.1038/ncomms15416

Leal, C.D., Pereira, A.D., Nunes, F.T., Ferreira, L.O., Coelho, A.C.C., Bicalho, S.K., Mac Conell, E.F.A., Ribeiro, T.B., de Lemos Chernicharo, C.A., de Araújo, J.C., 2016. Anammox for nitrogen removal from anaerobically pre-treated municipal wastewater: Effect of COD/N ratios on process performance and bacterial community structure. Bioresour. Technol. 211, 257–266. https://doi.org/10.1016/j.biortech.2016.03.107

Li, J., Li, Jianwei, Peng, Y., Wang, S., Zhang, L., Yang, S., Li, S., 2020. Insight into the impacts of organics on anammox and their potential linking to system performance of sewage partial nitrification-anammox (PN/A): A critical review. Bioresour. Technol. 300, 122655. https://doi.org/10.1016/j.biortech.2019.122655

Li, X., Du, R., Peng, Y., Zhang, Q., Wang, J., 2020. Characteristics of sludge granulation and EPS production in development of stable partial nitrification. Bioresour. Technol. 303, 122937. https://doi.org/10.1016/j.biortech.2020.122937

Li, X.R., Du, B., Fu, H.X., Wang, R.F., Shi, J.H., Wang, Y., Jetten, M.S.M., Quan, Z.X., 2009. The bacterial diversity in an anaerobic ammonium-oxidizing (anammox) reactor community. Syst. Appl. Microbiol. 32, 278–289. https://doi.org/10.1016/j.syapm.2009.03.002

Liu, W., Yin, F., Yang, D., 2020. Granules abrasion cause deterioration of nitritation in a mainstream granular sludge reactor with high loading rate. Chemosphere 243, 125433. https://doi.org/10.1016/j.chemosphere.2019.125433

Liu, Y., Liu, Q.S., 2006. Causes and control of filamentous growth in aerobic granular sludge sequencing batch reactors. Biotechnol. Adv. https://doi.org/10.1016/j.biotechadv.2005.08.001

Liu, Y., Ngo, H.H., Guo, W., Peng, L., Wang, D., Ni, B., 2019. The roles of free ammonia (FA) in biological wastewater treatment processes: A review. Environ. Int. https://doi.org/10.1016/j.envint.2018.11.039

Liu, Y., Peng, L., Guo, J., Chen, X., Yuan, Z., Ni, B.J., 2015. Evaluating the Role of Microbial Internal Storage Turnover on Nitrous Oxide Accumulation during Denitrification. Sci. Rep. 5, 1–10. https://doi.org/10.1038/srep15138

Liu, Y., Tay, J.H., 2004. State of the art of biogranulation technology for wastewater treatment. Biotechnol. Adv. 22, 533–563. https://doi.org/10.1016/j.biotechadv.2004.05.001

Lotti, T., Kleerebezem, R., Abelleira-Pereira, J.M., Abbas, B., van Loosdrecht, M.C.M., 2015a. Faster through training: The anammox case. Water Res. 81, 261–268. https://doi.org/10.1016/j.watres.2015.06.001

Lotti, T., Kleerebezem, R., Hu, Z., Kartal, B., Jetten, M.S.M., van Loosdrecht, M.C.M., 2014a. Simultaneous partial nitritation and anammox at low temperature with granular sludge. Water Res. 66, 111–121.

https://doi.org/10.1016/j.watres.2014.07.047

Lotti, T., Kleerebezem, R., Van Erp Taalman Kip, C., Hendrickx, T.L.G., Kruit, J., Hoekstra, M., Van Loosdrecht, M.C.M., 2014b. Anammox growth on pretreated municipal wastewater. Environ. Sci. Technol. 48, 7874–7880. https://doi.org/10.1021/es500632k

Lotti, T., Kleerebezem, R., van Loosdrecht, M.C.M., 2015b. Effect of temperature change on anammox activity. Biotechnol. Bioeng. 112, 98–103. https://doi.org/10.1002/bit.25333

Ma, B., Bao, P., Wei, Y., Zhu, G., Yuan, Z., Peng, Y., 2015. Suppressing nitrite-oxidizing bacteria growth to achieve nitrogen removal from domestic wastewater via anammox using intermittent aeration with low dissolved oxygen. Sci. Rep. 5, 1–9. https://doi.org/10.1038/srep13048

Ma, B., Peng, Y., Zhang, S., Wang, J., Gan, Y., Chang, J., Wang, Shuying, Wang, Shanyun, Zhu, G., 2013. Performance of anammox UASB reactor treating low strength wastewater under moderate and low temperatures. Bioresour. Technol. 129, 606–611. https://doi.org/10.1016/j.biortech.2012.11.025

Ma, B., Qian, W., Yuan, C., Yuan, Z., Peng, Y., 2017. Achieving Mainstream Nitrogen Removal through Coupling Anammox with Denitratation. Environ. Sci. Technol. 51, 8405–8413. https://doi.org/10.1021/acs.est.7b01866

Ma, H., Niu, Q., Zhang, Y., He, S., Li, Y.Y., 2017. Substrate inhibition and concentration control in an UASB-Anammox process. Bioresour. Technol. 238, 263–272. https://doi.org/10.1016/j.biortech.2017.04.017

Majone, M., Beccari, M., Dionisi, D., Levantesi, C., Renzi, V., 2001. Role of storage phenomena on removal of different substrates during pre-denitrification, in: Water Science and Technology. https://doi.org/10.2166/wst.2001.0131

Manser, R., Gujer, W., Siegrist, H., 2005. Consequences of mass transfer effects on the kinetics of nitrifiers. Water Res. 39, 4633–4642. https://doi.org/10.1016/j.watres.2005.09.020

Metcalf & Eddy, 2003. Wastewater Engineering Teatment and Reuse, 5th edition. New York. https://doi.org/10.1016/0309-1708(80)90067-6

Molinuevo, B., García, M.C., Karakashev, D., Angelidaki, I., 2009. Anammox for ammonia removal from pig manure effluents: Effect of organic matter content on process performance. Bioresour. Technol. 100, 2171–2175. https://doi.org/10.1016/j.biortech.2008.10.038

Mulder, A., van de Graaf, A.A., Robertson, L.A., Kuenen, J.G., 1995. Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiol. Ecol. 16, 177–183. https://doi.org/10.1016/0168-6496(94)00081-7

Pedrouso, A., Trela, J., Val del Rio, A., Mosquera-Corral, A., Plaza, E., 2019. Performance of partial nitritation-anammox processes at mainstream conditions in an IFAS system. J. Environ. Manage. 250, 109538. https://doi.org/10.1016/j.jenvman.2019.109538

Pedrouso, A., Val del Rio, A., Morales, N., Vázquez-Padín, J.R., Campos, J.L., Mosquera-Corral, A., 2020. Mainstream anammox reactor performance treating municipal wastewater and batch study of temperature, pH and organic matter concentration cross-effects. Process Saf. Environ. Prot. 145, 195–202. https://doi.org/10.1016/j.psep.2020.07.052

Pérez, J., Costa, E., Kreft, J.U., 2009. Conditions for partial nitrification in biofilm reactors and a kinetic explanation. Biotechnol. Bioeng. 103, 282–295. https://doi.org/10.1002/bit.22249

Pérez, J., Isanta, E., Carrera, J., 2015. Would a two-stage N-removal be a suitable technology to implement at full scale the use of anammox for sewage treatment? Water Sci. Technol. 72, 858–864. https://doi.org/10.2166/wst.2015.281

Pérez, J., Picioreanu, C., Van Loosdrecht, M., 2005. Modeling biofilm and floc diffusion processes based on analytical solution of reaction-diffusion equations. Water Res. 39, 1311–1323. https://doi.org/10.1016/j.watres.2004.12.020

Picioreanu, C., Pérez, J., van Loosdrecht, M.C.M., 2016. Impact of cell cluster size on apparent half-saturation coefficients for oxygen in nitrifying sludge and biofilms. Water Res. 106, 371–382. https://doi.org/10.1016/j.watres.2016.10.017

Pijuan, M., Ribera-Guardia, A., Balcázar, J.L., Micó, M.M., de la Torre, T., 2020. Effect of COD on mainstream anammox: Evaluation of process performance, granule morphology and nitrous oxide production. Sci. Total Environ. 712, 136372. https://doi.org/10.1016/j.scitotenv.2019.136372

Poot, V., Hoekstra, M., Geleijnse, M.A.A., van Loosdrecht, M.C.M., Pérez, J., 2016. Effects of the residual ammonium concentration on NOB repression during partial nitritation with granular sludge. Water Res. 106, 518–530. https://doi.org/10.1016/j.watres.2016.10.028

Pronk, M., de Kreuk, M.K., de Bruin, B., Kamminga, P., Kleerebezem, R., van Loosdrecht, M.C.M., 2015. Full scale performance of the aerobic granular sludge process for sewage treatment. Water Res. 84, 207–217. https://doi.org/10.1016/j.watres.2015.07.011

Regmi, P., Miller, M.W., Holgate, B., Bunce, R., Park, H., Chandran, K., Wett, B., Murthy, S., Bott, C.B., 2014. Control of aeration, aerobic SRT and COD input for mainstream nitritation/denitritation. Water Res. 57, 162–171. https://doi.org/10.1016/j.watres.2014.03.035

Reino, C., Carrera, J., 2017. Low-strength wastewater treatment in an anammox UASB reactor: Effect of the liquid upflow velocity. Chem. Eng. J. 313, 217–225. https://doi.org/10.1016/j.cej.2016.12.051

Reino, C., Suárez-Ojeda, M.E., Pérez, J., Carrera, J., 2018. Stable long-term operation of an upflow anammox sludge bed reactor at mainstream conditions. Water Res. 128, 331–340. https://doi.org/10.1016/j.watres.2017.10.058

Reino, C., Suárez-Ojeda, M.E., Pérez, J., Carrera, J., 2016. Kinetic and microbiological characterization of aerobic granules performing partial nitritation of a low-strength wastewater at 10 °C. Water Res. 101, 147–156. https://doi.org/10.1016/j.watres.2016.05.059

Sánchez-Guillén, J.A., Lopez-Vazquez, C.M., de Oliveira Cruz, L.M., Brdjanovic, D., van Lier, J.B., 2016. Long-term performance of the Anammox process under low nitrogen sludge loading rate and moderate to low temperature. Biochem. Eng. J. 110, 95–106. https://doi.org/10.1016/j.bej.2016.02.004

Siegrist, H., Salzgeber, D., Eugster, J., Joss, A., 2008. Anammox brings WWTP closer to energy autarky due to increased biogas production and reduced aeration energy for N-removal. Water Sci. Technol. 57, 383–388. https://doi.org/10.2166/wst.2008.048

Sliekers, A.O., Haaijer, S.C.M., Stafsnes, M.H., Kuenen, J.G., Jetten, M.S.M., 2005. Competition and coexistence of aerobic ammonium- and nitrite-oxidizing bacteria at low oxygen concentrations. Appl. Microbiol. Biotechnol. 68, 808–817. https://doi.org/10.1007/s00253-005-1974-6

Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M., Heijnen, J.J., 1994. Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence. Biotechnol. Bioeng. https://doi.org/10.1002/bit.260430605

Sobotka, D., Czerwionka, K., Makinia, J., 2016. Influence of temperature on the activity of anammox granular biomass. Water Sci. Technol. 73, 2518–2525. https://doi.org/10.2166/wst.2016.103

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

Speth, D.R., In'T Zandt, M.H., Guerrero-Cruz, S., Dutilh, B.E., Jetten, M.S.M., 2016. Genome-based microbial ecology of anammox granules in a full-scale wastewater treatment system. Nat. Commun. 7, 11172. https://doi.org/10.1038/ncomms11172

Strous, M., Fuerst, J.A., Kramer, E.H.M., Logemann, S., Muyzer, G., Van De Pas-Schoonen, K.T., Webb, R., Kuenen, J.G., Jetten, M.S.M., 1999. Missing lithotroph identified as new planctomycete. Nature 400, 446–449. https://doi.org/10.1038/22749

Strous, M., Heijnen, J.J., Kuenen, J.G., Jetten, M.S.M., 1998. The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. Appl. Microbiol. Biotechnol. 50, 589–596. https://doi.org/10.1007/s002530051340

Strous, M., Van Gerven, E., Kuenen, J.G., Jetten, M., 1997. Effects of aerobic and microaerobic conditions on anaerobic ammonium- oxidizing (anammox) sludge. Appl. Environ. Microbiol. 63, 2446–2448. https://doi.org/10.1128/aem.63.6.2446-2448.1997

Tay, J., Liu, Q.S., Liu, Y., 2001. Microscopic observation of aerobic granulation in sequential aerobic sludge blanket reactor. J. Appl. Microbiol. 91, 168–175. https://doi.org/10.1046/j.1365-2672.2001.01374.x

Torà, J.A., Moliné, E., Carrera, J., Pérez, J., 2013. Efficient and automated start-up of a pilot reactor for nitritation of reject water: From batch granulation to high rate continuous operation. Chem. Eng. J. 226, 319–325. https://doi.org/10.1016/j.cej.2013.04.013

UN-HABITAT, 2016. Urbanization and Development: Emerging Futures World Cities Report 2016, International Journal.

Vadivelu, V.M., Keller, J., Yuan, Z., 2007. Free ammonia and free nitrous acid inhibition on the anabolic and catabolic processes of Nitrosomonas and Nitrobacter. Water Sci. Technol. 56, 89–97. https://doi.org/10.2166/wst.2007.612

van den Berg, E.M., Boleij, M., Kuenen, J.G., Kleerebezem, R., van Loosdrecht, M.C.M., 2016. DNRA and denitrification coexist over a broad range of acetate/N-NO3- ratios, in a chemostat enrichment culture. Front. Microbiol. 7, 1–12. https://doi.org/10.3389/fmicb.2016.01842

van der Star, W.R.L., Abma, W.R., Blommers, D., Mulder, J.W., Tokutomi, T., Strous, M., Picioreanu, C., van Loosdrecht, M.C.M., 2007. Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam. Water Res. 41, 4149–4163. https://doi.org/10.1016/j.watres.2007.03.044

van Dongen, U., Jetten, M.S.M., van Loosdrecht, M.C.M., 2001. The SHARON®-Anammox® process for treatment of ammonium rich wastewater, in: Water Science and Technology. IWA Publishing, pp. 153–160. https://doi.org/10.2166/wst.2001.0037

Van Loosdrecht, M.C.M., Brdjanovic, D., 2014. Anticipating the next century of wastewater treatment. Science (80-.). https://doi.org/10.1126/science.1255183

van Loosdrecht, M.C.M., Nielsen, P.H., Lopez-Vazquez, C.M., Brdjanovic, D., 2016. Experimental Methods in Wastewater Treatment. Water Intell. Online 15. https://doi.org/10.2166/9781780404752

Versprille, A.I., Zuurveen, B., Stein, T., 1984. The A-B process: a novel two-stage wastewater treatment system. Water Sci. Technol. 17, 235–246.

Wang, J., Lei, Z., Wang, L., Yang, S., Zhao, Y., Li, Y.Y., Chen, R., 2020. Insight into using up-flow anaerobic sludge blanket-anammox to remove nitrogen from an anaerobic membrane reactor during mainstream wastewater treatment. Bioresour.

Technol. 314, 123710. https://doi.org/10.1016/j.biortech.2020.123710

Wang, J., Song, J., Yin, F., Shen, Y., Yang, D., Liu, W., 2021. Insight into how high dissolved oxygen favors the startup of nitritation with aerobic granules. Chemosphere 270, 128643. https://doi.org/10.1016/j.chemosphere.2020.128643

Wang, S., Ishii, K., Yu, H., Shi, X., Smets, B.F., Palomo, A., Zuo, J., 2020. Stable nitrogen removal by anammox process after rapid temperature drops: insights from metagenomics and metaproteomics. Bioresour. Technol. 320, 124231. https://doi.org/10.1016/j.biortech.2020.124231

Wett, B., 2006. Solved upscaling problems for implementing deammonification of rejection water, in: Water Science and Technology. IWA Publishing, pp. 121–128. https://doi.org/10.2166/wst.2006.413

Xiao, R., Ni, B.-J., Liu, S., Lu, H., 2021. Impacts of organics on the microbial ecology of wastewater anammox processes: Recent advances and meta-analysis. Water Res. 191, 116817. https://doi.org/10.1016/j.watres.2021.116817

Zessner, M., Lampert, C., Kroiss, H., Lindtner, S., 2010. Cost comparison of wastewater treatment in Danubian countries. Water Sci. Technol. 62, 223–230. https://doi.org/10.2166/wst.2010.271

Zhang, C., Guisasola, A., Baeza, J.A., 2021. Achieving simultaneous biological COD and phosphorus removal in a continuous anaerobic/aerobic A-stage system. Water Res. 190, 116703. https://doi.org/10.1016/j.watres.2020.116703

Zhang, J., Kobert, K., Flouri, T., Stamatakis, A., 2014. PEAR: A fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30, 614–620. https://doi.org/10.1093/bioinformatics/btt593

Zhang, X., Zhang, H., Ye, C., Wei, M., Du, J., 2015. Effect of COD/N ratio on nitrogen removal and microbial communities of CANON process in membrane bioreactors. Bioresour. Technol. 189, 302–308. https://doi.org/10.1016/j.biortech.2015.04.006

Appendix I

Chapter 3. Reactor operation

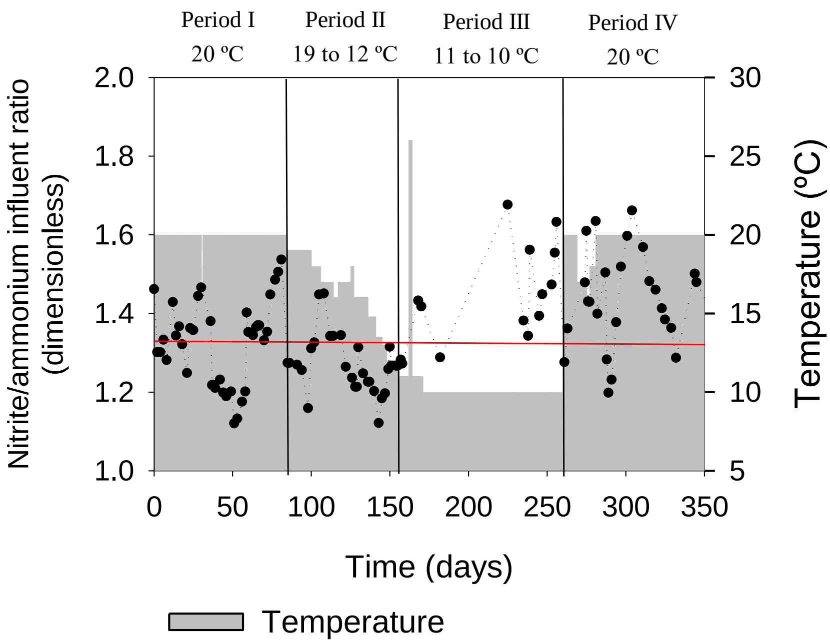

Fig. A1. Nitrite to ammonium feeding ratio throughout the UAnSB reactor operation treating a real mainstream wastewater at each temperature period. Red line corresponds to the stoichiometrically ratio of anammox bacteria according to Strous et al. (1998).

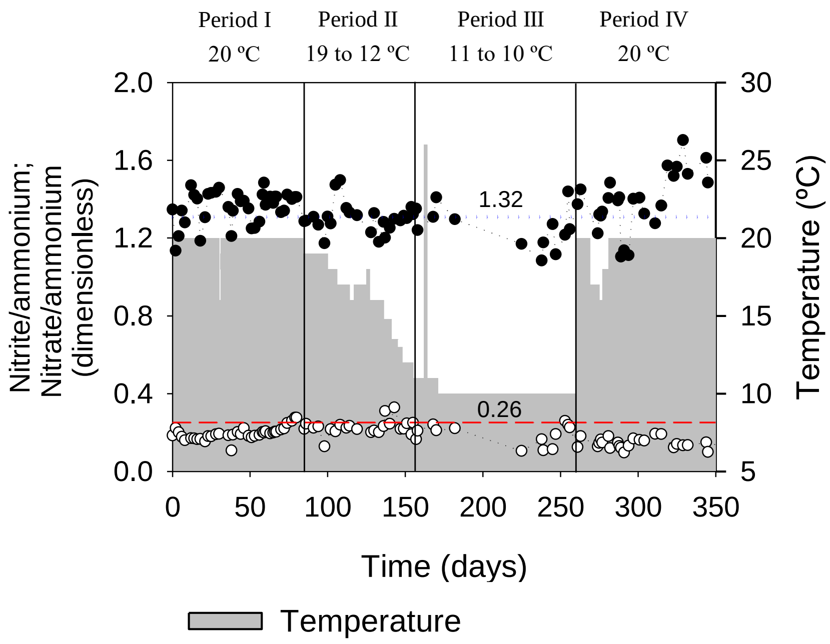

Fig. A2. Nitrite to ammonium consumed (black dots) and nitrate produced to ammonium consumed (white dots) yields throughout the UAnSB reactor operation treating a real mainstream wastewater at each temperature period. Dotted red and dashed blue lines correspond to the stoichiometric ratio of nitrite to ammonium consumed (1.32) and to the produced nitrate to ammonium consumed ratio (0.26), respectively according to Strous et al. (1998).

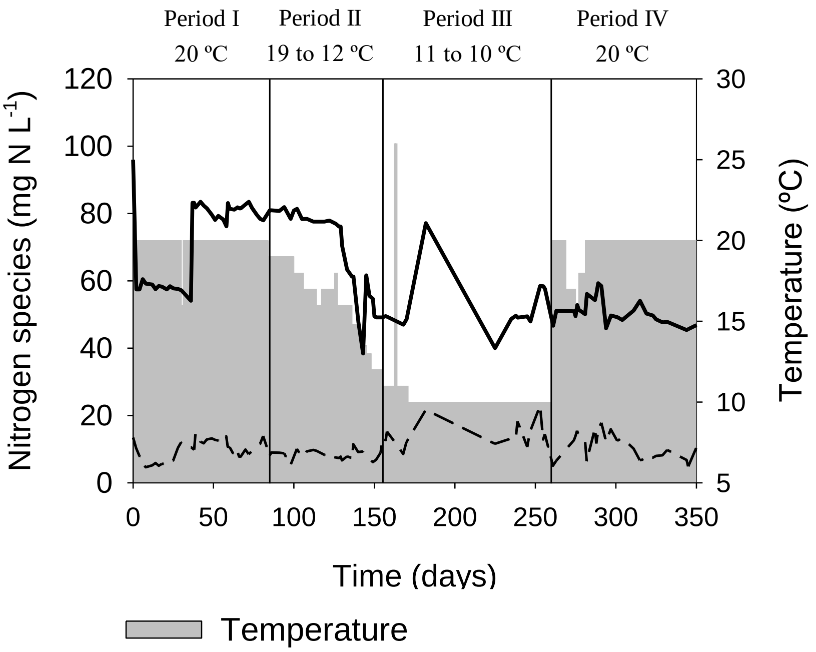

Fig. A3. Total nitrogen concentrations in the influent as the sum of ammonium and nitrite (solid line) and in the effluent (dashed line) as the sum of ammonium, nitrite and nitrate throughout the UAnSB reactor operation treating a real mainstream wastewater at each temperature period.

Chapter 4. Rector Operation

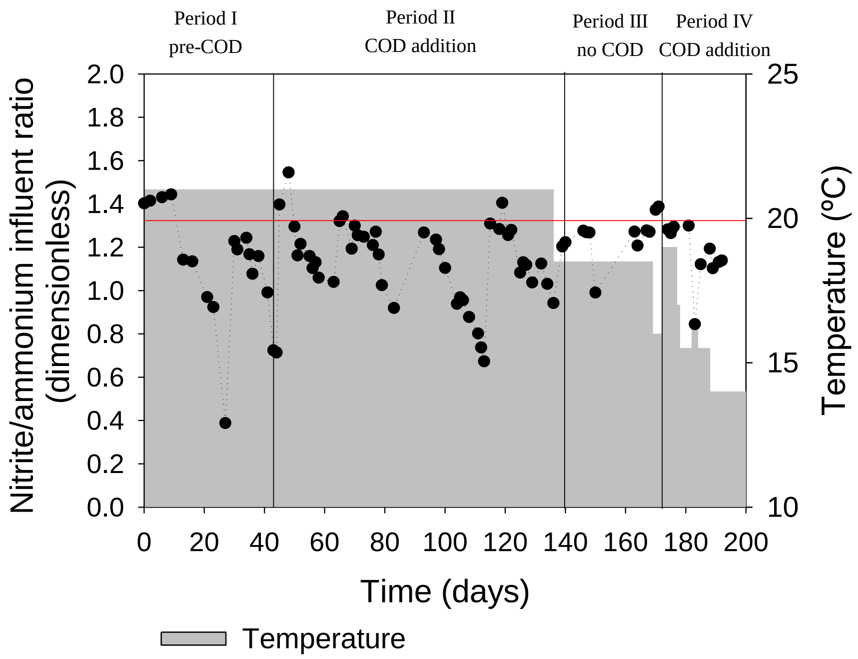

Fig. A4. Nitrite to ammonium feeding ratio throughout the CANDLE reactor operation treating a real mainstream wastewater at each temperature period. Red line corresponds to the stoichiometrically ratio of anammox bacteria according to Strous et al. (1998).

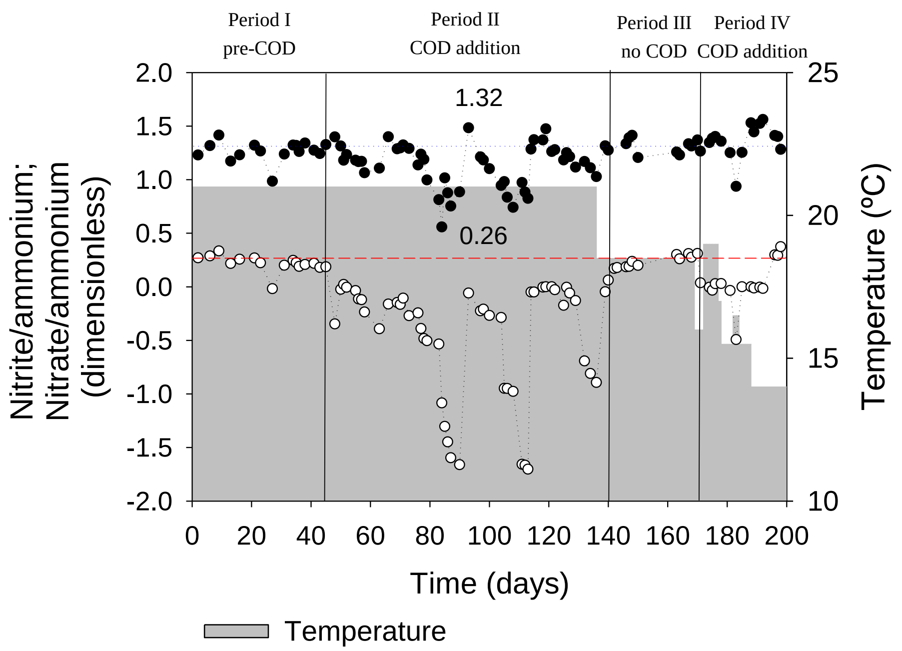

Fig. A5. Nitrite to ammonium consumed (black dots) and nitrate produced to ammonium consumed (white dots) yields throughout the CANDLE reactor operation. Dotted and dashed lines correspond to the stoichiometric ratio of nitrite to ammonium consumed (1.32) and to the produced nitrate to ammonium consumed (0.26) ratio, respectively according to Strous et al. (1998).

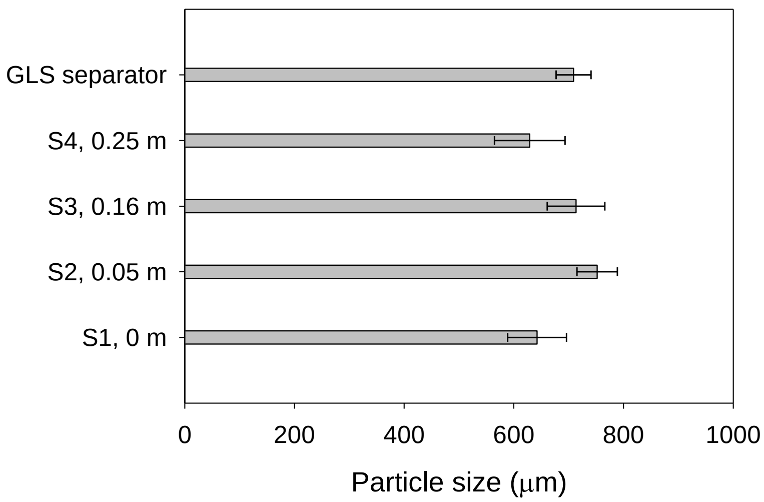

Fig. A6. Particle size at different sludge bed reactor heights 0 m (S1), 0.05 m (S2), 0.16 m (S3), 0.25 m (S4) and from the gas-liquid-solid (GLS) separator phase of the CANDLE reactor on day 97 (period II).

Appendix II

Bioinformatics protocol

The 16S rRNA gene-targeted sequencing analyses were used to characterize the diversity and relative abundance of the different microorganisms present in the sludge of the UAnSB and CANDLE reactors (Chapter 3 and Chapter 4, respectively). Appendix II compiles information of the bioinformatics protocol of the paired-end sequencing DNA data performed on an Illumina MiSeq platform by the Research and Testing Laboratory (Lubbock, Texas, USA).

For denoising performance, sequence reads were merged together using the PEAR Illumina paired-end merger (Zhang et al., 2014) and sorted by length from longest to shortest. Then, prefixed dereplication and clustering at a 4 % divergence was performed using the USEARCH (Edgar, 2010) clustering algorithm. Chimera checking was performed on the selected OTUs using the UCHIME chimera detection software executed in de novo move. The different clusters were classified into Operational Taxonomic Units (OTUs) using the UPARSE OUT selection algorithm (Edgar, 2013). Each one of the OTUs was then identified using the USEARCH global alignment algorithm against a data base of high-quality sequences derived from the NCBI database. For each OTU, the top six matches from the high-quality database were kept and confidence values were assigned to each taxonomic level by taking the number of taxonomic matches that agreed with the best match at that level and dividing that by the number of high-quality sequences matches that were found. Each OTU was then assigned to taxonomic information using the lowest common taxonomic level whose confidence value was above 51%. OTUs that received no matches against the high-quality sequences were identified as “No Hit”. After resolving the number of sequences per OTU, the percentage of each organism was individually calculated for each sample. Relative abundances of reads were calculated by taxonomic level for each library. Values represent the percentage of reads of sequences obtained at each taxonomic identity (according to the degree that of similarity described above) within the total set of readings from the library. When the taxonomy of an OTU was not assigned by using the protocol mentioned above (resulted as either in “Unclassified”, ”Unknown” or “No Hit”), an attempt to identify it was made by using the Basic Local Alignment Search Tool (BLAST) from the U.S. National Library of Medicine freely available. To assign a taxonomic classification a quality control cut-off higher than 97 % for the identity and the query cover values together with an E-value equal to zero were set.

Biological diversity indices (Shannon, Chao1, Evenness) were obtained for all libraries at 97 % of similitude to confirm enough expression of bacterial diversity by high-throughput sequencing methods. Rarefaction curves were performed at 95, 97 and 99 % of similitude to test proper expression of bacterial assembly. The Jaccard Index was also calculated for libraries of Chapter 4 at 97 % of similitude. Calculation of the biological diversity index and rarefaction curves were performed by using the on-line free available RDPipeline software.

Microbiological quantification results

Chapter 3

Results of the Shannon, the Chao1 and the Evenness indices of Chapter 3 showed an equally species richness and diversity among the different libraries (Fig. A.7). No significant differences among the different sludge bed sections at the different reactor operation temperatures (20 and 10 ºC) were detected. Rarefaction curves indicated a good coverage of diversity (Fig. A.8).

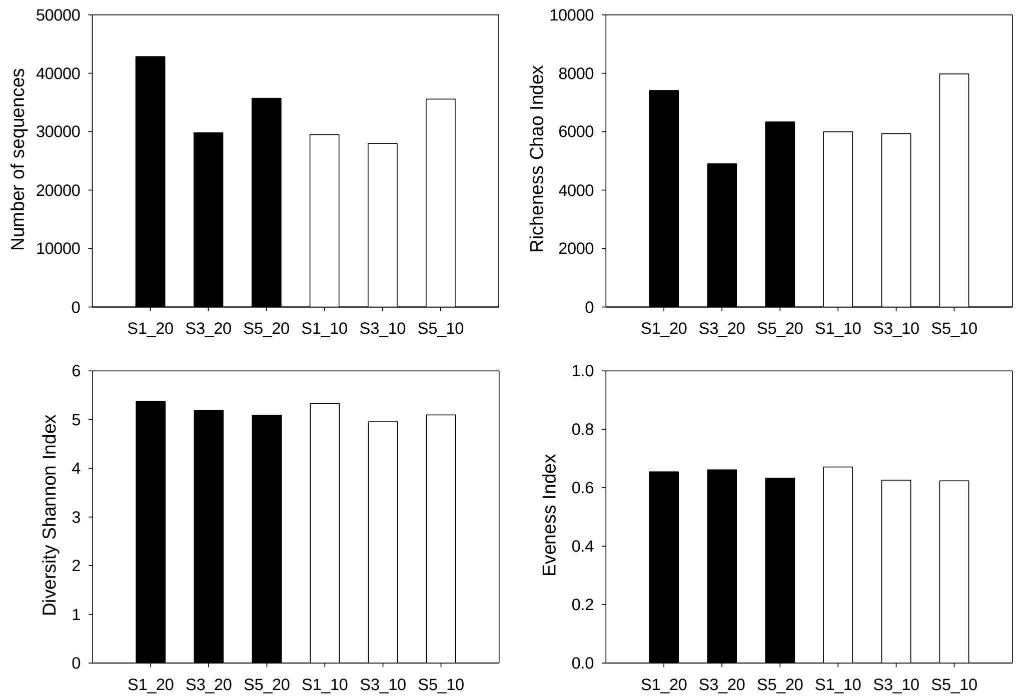

Fig. A7. Number of sequences and biological diversity indices (Chao, Shannon and Evenness) for the libraries of day 17 (20 ºC) (black columns) and of day 203 (10 ºC) (white columns) for sampling points S1, S3 and S5.

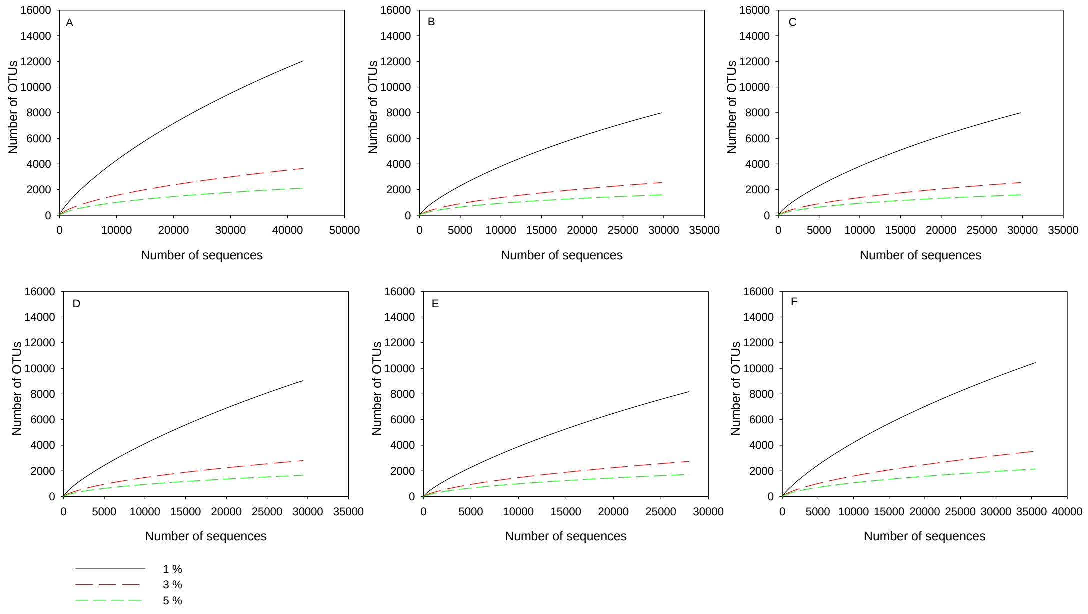

Fig. A8. Rarefaction curves for the libraries of day 17 at 20 ºC for sampling points S1 (A), S3 (B) and S5 (C) and of day 203 at 10 ºC for sampling points S1 (D), S3 (E) and S5 (F). OTUs were defined at 1 %, 3 % and 5 % distances at 1 %, 3 % and 5 % distances, respectively.

Chapter 4

Results from Chapter 4 indicated that libraries were comparable in terms of abundance percentages and that a good coverage of diversity was reached (Fig. A10), whereas biological indices showed an equally species richness and diversity among the different libraries (Fig. A9). The Jaccard Index presented a low degree of similitude of the microbial sludge community within bottom and middle and upper sludge bed sections before and after ca. 2 months of an external C-source addition, respectively (Table A1).

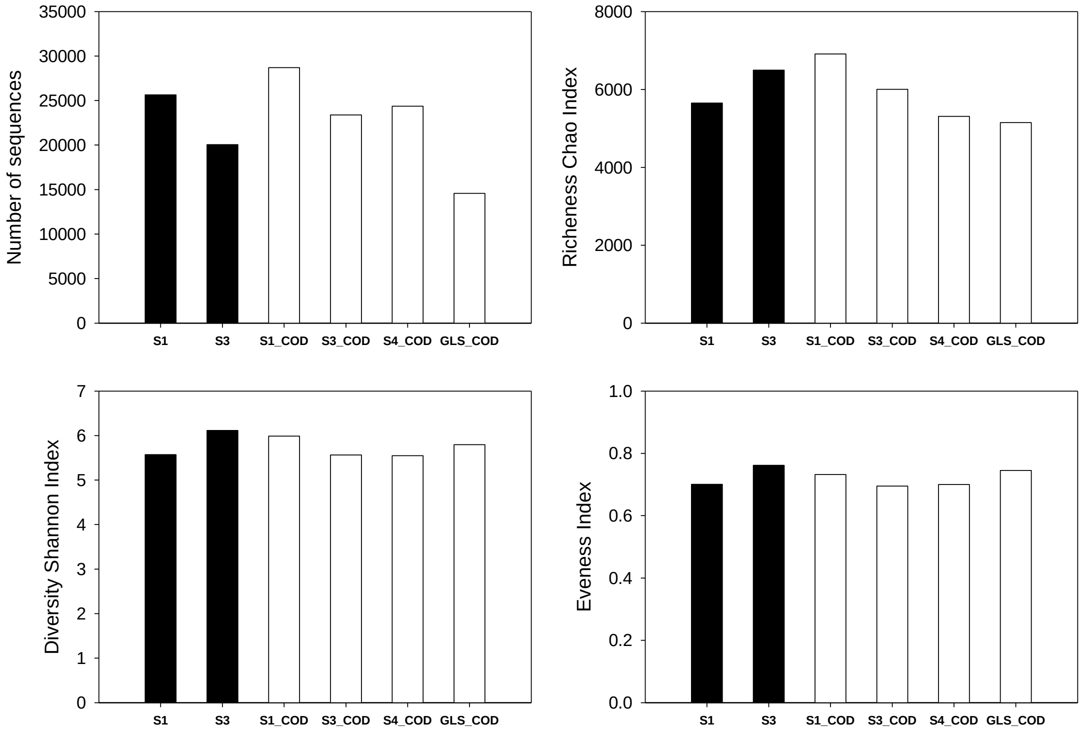

Fig. A9. Number of sequences and biological diversity indices (Chao, Shannon and Evenness) for libraries S1 and S3 before acetate addition (day 42) (black columns) and for libraries S1, S3, S4 and gas-liquid-solid (GLS) separator phase after ca. 2 months of acetate addition (day 97) (white columns).

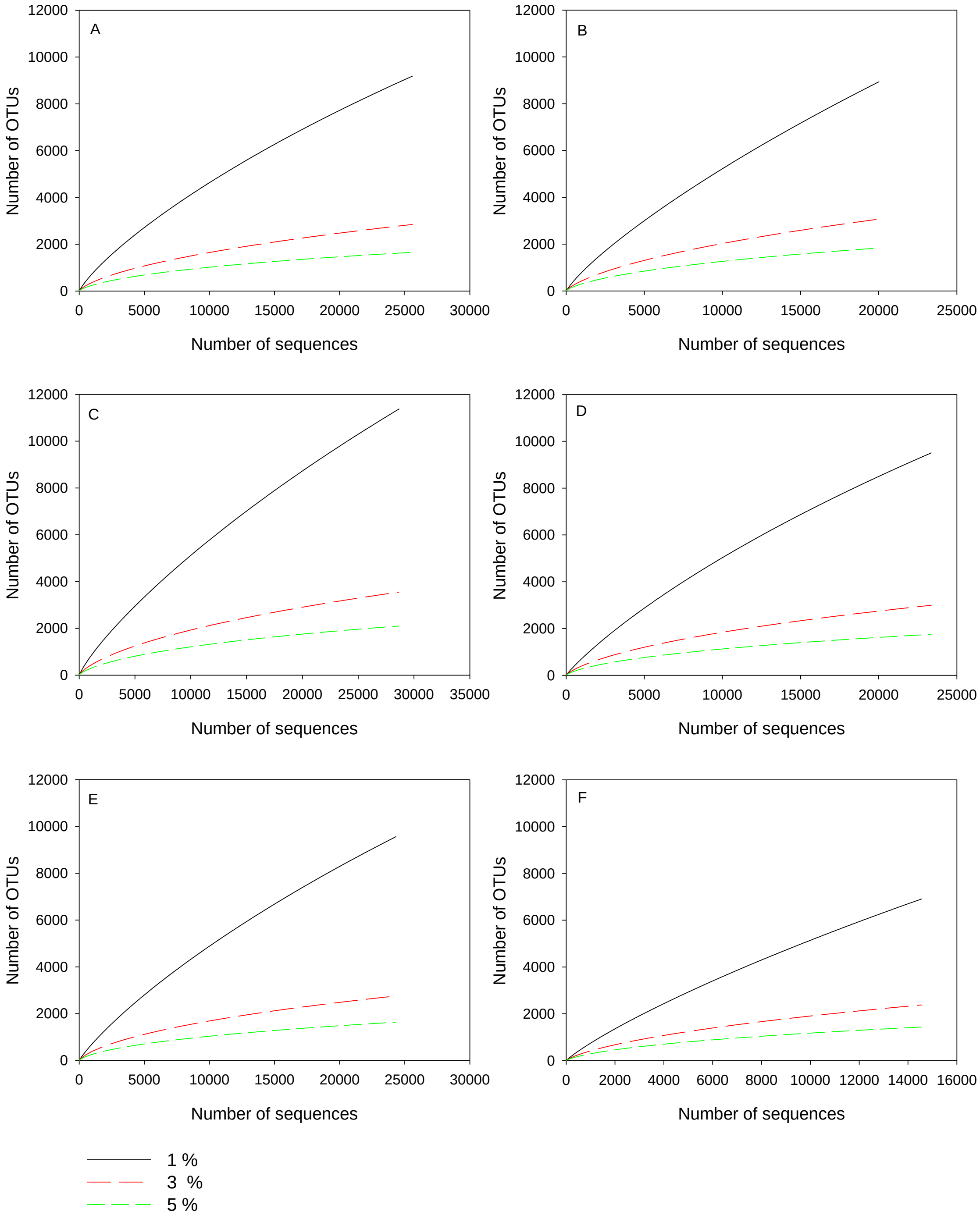

Fig. A10. Rarefaction curves for libraries S1 and S3 before acetate addition (day 42) (A and B, respectively) and for libraries S1, S3, S4 and gas-liquid-solid (GLS) separator phase after ca. 2 months of acetate addition (day 97) (C, D, E and F, respectively). OTUs were defined at 1 %, 3 % and 5 % distances, respectively.

Table A1. Jaccard Index at 97 % of similitude for libraries S1 and S3 before acetate addition (day 42) and for libraries S1, S3, S4 and gas-liquid-solid (GLS) separator phase after ca. 2 months of acetate addition (day 97). Colour intensity represents the degree of similitude among libraries. Green and red indicate the maximum and lowest degree of similitude, respectively.

	S1	S1-COD	S3	S3-COD	S4-COD	GLS-COD
S1	0.00					
S1-COD	0.12	0.00				
S3	0.16	0.13	0.00			
S3-COD	0.68	0.25	0.63	0.00		
S4-COD	0.63	0.23	0.56	0.09	0.00	
GLS-COD	0.57	0.24	0.51	0.12	0.10	0.00

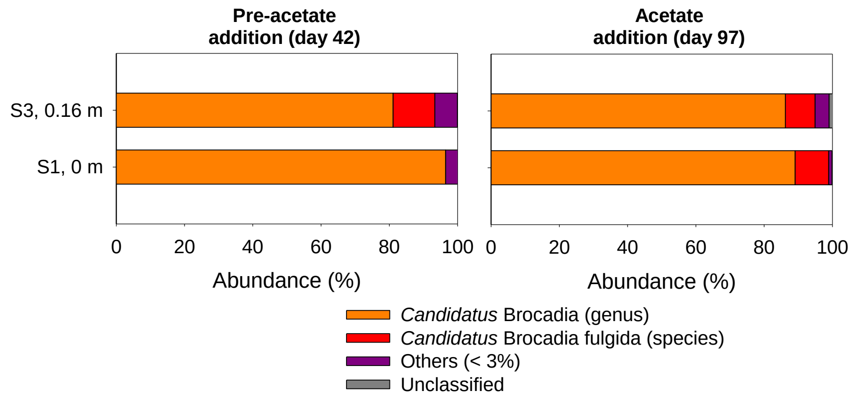

Fig. A11. Microbial diversity using the 368F-820R primer for libraries before and after ca. two months of acetate addition (day 42 and 97, respectively) at different CANDLE reactor heights: 0 m (S1) and 0.16 m (S3). Relative abundance was calculated only considering those microorganisms in which the number of 16S copies was higher than 3 % of the total copies.

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