Universitat Autònoma de Barcelona

AUTOTROPHIC BIOLOGICAL NITROGEN REMOVAL IN A TWO-STAGE SYSTEM AT MAINSTREAM CONDITIONS

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Que l'enginyera química **CLARA REINO SÁNCHEZ** ha realitzat sota la meva direcció el treball titulat: **"AUTOTROPHIC BIOLOGICAL NITROGEN REMOVAL IN A TWO-STAGE SYSTEM AT MAINSTREAM CONDITIONS"**, el qual es presenta en aquesta memòria, i que constitueix la seva Tesi per a optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

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Bellaterra, 9 de Setembre de 2016

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A Vito, Naso e Ana.

CONTENTS

SUMMARY

Urban wastewater treatment plants (WWTPs) are widely implemented over the industrialized world since urban wastewater treatment is needed before the discharge of the wastewater into the environment. In conventional activated sludge systems, nitrogen compounds are biologically removed through autotrophic nitrification and heterotrophic denitrification with a good effluent quality. Nevertheless, such treatment requires a lot of energy due to the aeration needed for nitrification and uses most of the organic matter for denitrification instead of producing biogas. For achieving an energy-neutral or even energy-positive urban WWTP the implementation of autotrophic biological nitrogen removal (BNR) in the mainstream has been proposed. Hence, the urban wastewater treatment would consist of a first step (A-Stage), where all the organic matter is removed and derived to biogas production, and a second step (B-Stage), where the nitrogen is removed through the autotrophic BNR process.

Autotrophic BNR is a two-step process. First, half of the ammonium contained in the wastewater is oxidized to nitrite under aerobic conditions in a process called partial nitritation (PN); and secondly, the rest of the ammonium and nitrite generated are converted to dinitrogen gas through the anammox process without the need of oxygen and organic matter. Autotrophic BNR has been successfully applied for treating some industrial wastewaters and reject water from digested sludge (high-strength and warm wastewaters). However, it has never been applied in the mainstream of an urban WWTP (low-strength and cold wastewater) since mainstream conditions are more disadvantageous for the process. So far researchers have focused on implementing the autotrophic BNR in one-stage systems, where the whole process takes place in one single reactor. However, in most cases PN fails in the long-term operation and destabilization of the anammox process eventually occurs; even those systems which succeed on achieving stable operation showed low nitrogen conversion rates.

Here, a two-stage system is proposed as an alternative for a better implementation of autotrophic BNR in the mainstream of an urban WWTP. Thus, the thesis aimed at demonstrating the stability of PN and anammox processes in two separated reactors treating wastewater at mainstream conditions.

Firstly, a granular sludge airlift reactor performing the PN process was successfully operated in continuous mode. A synthetic influent mimicking the effluent of the A-Stage was treated at temperatures as low as 10 °C. Not only stable operation was achieved in the long-

term operation, but also high nitrification rates and a suitable effluent for a subsequent anammox reactor. Moreover, N_2O gas emissions were determined in the reactor and, furthermore, a medium-term study to assess the temperature effect on the N_2O emissions associated to the PN process was performed.

Secondly, an Upflow Anaerobic Sludge Blanket (UASB) reactor performing the anammox process was successfully operated in continuous mode at mainstream conditions. On one hand, the feasibility of using an UASB reactor to implement the anammox process at mainstream conditions was demonstrated by achieving high nitrogen removal rates and high nitrogen removal efficiencies at 26 ºC treating a synthetic influent. In addition, an in depth study of the effect of the upflow velocity on the performance of the anammox UASB reactor was done. Furthermore, an exhaustive study of the effect of decreasing temperature on anammox activity was performed and an adaptation of anammox bacteria after long-term operation at low temperatures was observed. On the other hand, a successful long-term operation of the anammox reactor treating a real urban wastewater was achieved at high nitrogen removal rates and a temperature as low as 11 ºC.

Moreover, a detailed study of the biomass developed in the above-mentioned reactors from the microbiological, kinetic and physicochemical points of view, was performed aiming at correlating such characteristics to the reactor's operation at mainstream conditions.

RESUMEN

Actualmente las estaciones depuradoras de aguas residuales (EDAR) urbanas están ampliamente implantadas en los países industrializados, puesto que es necesario realizar un tratamiento de las aguas residuales antes de verterlas al medioambiente. En los sistemas de tratamiento convencionales, los compuestos nitrogenados se eliminan mediante un tratamiento biológico de nitrificación autotrófica y desnitrificación heterotrófica, que garantiza una buena calidad del efluente. En estos tratamientos se necesita gran cantidad de aireación para la nitrificación y la mayoría de la materia orgánica del agua residual se destina a la desnitrificación en lugar de a la producción de biogás. Por tanto, las EDAR urbanas presentan un gran consumo energético y el principal reto en la actualidad es conseguir una depuradora urbana autosuficiente energéticamente. Una alternativa para conseguirlo es la implementación de la eliminación biológica autotrófica de nitrógeno (BNR) en la línea principal de aguas. El tratamiento consistiría en una primera etapa (*A-Stage*) en la cual se eliminaría toda la materia orgánica destinándola a la producción de biogás, y una segunda etapa (*B-Stage*) en la que se eliminaría el nitrógeno mediante el proceso BNR.

El proceso BNR es un proceso en dos etapas. Primero, la mitad del amonio presente en el agua residual se oxida a nitrito mediante el proceso de nitritación parcial (PN), y a continuación el amonio restante y el nitrito generado se convierten en N_2 mediante el proceso anammox, sin necesidad de oxígeno ni materia orgánica. El proceso BNR se ha aplicado al tratamiento de aguas con altas cargas de nitrógeno y temperaturas cálidas, pero nunca se ha implementado en el tratamiento de aguas residuales urbanas (bajas cargas y temperaturas). Recientemente, la investigación se ha centrado en el desarrollo del proceso BNR en un único reactor. Sin embargo, muchos de los estudios publicados mostraron el fallo del proceso PN en la operación a largo plazo al tratar agua urbana, e incluso aquellos sistemas con una operación estable alcanzaron bajas velocidades de eliminación de nitrógeno.

En esta tesis se propuso la utilización de un sistema en dos etapas como alternativa para una mejor implementación del proceso BNR en la línea principal de aguas de una EDAR urbana. Así, el principal objetivo fue demostrar la estabilidad de los dos procesos implicados, PN y anammox, en dos reactores independientes tratando un agua residual urbana.

En primer lugar se operó en continuo un reactor *airlift* con biomasa granular tratando un influente sintético que simulaba el efluente de la etapa *A-Stage*. Se trabajó a bajas temperaturas (hasta los 10 °C) y no solo se consiguió una operación estable a largo plazo sino

que se obtuvieron altas velocidades de nitrificación y un efluente adecuado para un reactor anammox contiguo. Además, se determinaron las emisiones de N_2O producidas en el reactor y se realizó un estudio del efecto de la temperatura sobre éstas.

En segundo lugar se operó en continuo un reactor UASB (*Upflow Anaerobic Sludge Blanket*) realizando el proceso anammox a largo plazo. Por una parte, se presentó el reactor UASB como una buena alternativa para realizar el proceso anammox en la línea principal de aguas de la depuradora. Además se realizó un estudio exhaustivo del efecto de la velocidad ascensional en el reactor y del efecto de la baja temperatura en la actividad anammox. Por otra parte, el reactor UASB anammox no sólo mostró una operación estable a largo plazo tratando un agua residual urbana real, sino que también se alcanzaron altas velocidades de eliminación de nitrógeno a una temperatura de 11 °C.

Conjuntamente, se realizó un estudio detallado de la biomasa desarrollada en ambos reactores desde los puntos de vista microbiológico, cinético y físico-químico, con el objetivo de relacionar estas características con la operación.

LIST OF ACRONYMS AND ABBREVIATIONS

A	Cross-sectional area of the reactor
AMO	Ammonia monooxygenase
Anammox	Anaerobic ammonium-oxidizing
AOB	Ammonia Oxidizing Bacteria
AOR	Ammonium Oxidation Rate
BLAST	Basic Local Alignment Search Tool
BNR	Biological Nitrogen Removal
C	Carbon
CANON	Completely Autotrophic Nitrogen removal Over Nitrite
CAS	Conventional Activated Sludge
CLSM	Confocal Laser Scanning Microscopy
COD	Chemical Oxygen Demand
D_F	Diffusional coefficient
d_g	Granule diameter
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
E_a	Activation energy
EF_{gas}	Gas emission factor
EPS	Extracellular Polymeric Substances
EU	European Union
EGSB	Expanded Granular Sludge Bed
FISH	Fluorescence *in situ* hybridization
GHG	Greenhouse Gas
HAO	Hydroxylamine oxidoreductase
H-MBBR	Hybrid Moving Bed Biofilm Reactor

HRT	Hydraulic Retention Time
IFAS	Integrated Fixed film Activated Sludge
k_c	External mass transfer coefficient
k_La	Mass transfer coefficient
$K_{S,TAN}$	TAN affinity constant
L_L	External boundary layer
MBBR	Moving Bed Biofilm Reactor
N	Nitrogen
N/D	Nitrification/Denitrification
NLR	Nitrogen Loading Rate
NOB	Nitrite Oxidizing Bacteria
NRR	Nitrogen Removal Rate
OLAND	Oxygen-Limited Autotrophic Nitrification/Denitrification
OTU	Operational Taxonomic Unit
OUR	Oxygen Uptake Rate
PBS	Phosphate Buffered Saline
PF_{liq}	Liquid production factor
PF_{tot}	Total production factor
PN/A	Partial Nitritation and Anammox
Q	Flow rate
RBC	Rotating Biological Contactor
Re	Non-dimensional Reynolds number
rRNA	Ribosomal Ribonucleic Acid
sAOR	Specific Ammonium Oxidation Rate
SAA	Specific Anammox Activity
SBR	Sequential Batch Reactor
Sc	Non-dimensional Schmidt number

Sh	Non-dimensional Sherwood number
SHARON	Single reactor High activity Ammonia Removal Over Nitrite
SMP	Soluble Microbial Products
sNLR	Specific Nitrogen Loading Rate
SRT	Solids Retention Time
SVI	Sludge Volumetric Index
T	Temperature
TAN	Total Ammonia Nitrogen
TNN	Total Nitrite Nitrogen
TS	Total Solids
TSS	Total Suspended Solids
UASB	Upflow Anaerobic Sludge Blanket
UAnSB	Upflow Anammox Sludge Blanket
UMABR	Upflow Membrane-Aerated Biofilm Reactor
V	Volume
VS	Volatile Solids
VSS	Volatile Suspended Solids
Vup	Upflow velocity
WWTP	Wastewater Treatment Plant

GREEK SYMBOLS

ρ_w	Density of water
μ	Specific growth rate
μ_{max}	Maximum specific growth rate
μ_w	Viscosity of water
θ	Temperature coefficient

Chapter 1

MOTIVATIONS AND THESIS OVERVIEW

1.1. MOTIVATIONS

Since last century, urban wastewater treatment plants (WWTPs) were widely implanted all over the industrialized world and all developments were aimed at improving the quality of water. Therefore, nowadays a good effluent quality is guaranteed in the developed countries and the new challenge is to achieve an advanced and sustainable urban WWTP. Since urban wastewater treatment plants are very energy-demanding facilities, one of the main challenges to face is the achievement of an energetic self-sufficient urban WWTP, with an improved energy balance and more efficient control strategies and operational procedures, as pointed out by the WATER 2020 EU project in the frame of the EU project HORIZON 2020. Several theoretical studies have revealed that the only way of reducing the costs of operation of an urban WWTP, and even to turn it into a producing energy facility, is implementing the autotrophic Biological Nitrogen Removal (BNR) in the main water line (Kartal et al., 2010; Verstraete and Vlaeminck, 2011). As in any process, before the implementation at real-scale, an extensive labour of research is needed at smaller scale such as lab-scale and pilot scale. Thus, this thesis is focused on achieving a stable operation at lab-scale of the process comprising the autotrophic BNR (i.e. partial nitritation and anammox process) at mainstream conditions.

1.2. THESIS OVERVIEW

In the present chapter (Chapter 1) the motivations of this thesis and the thesis overview are presented. In Chapter 2 a brief introduction of the topic is presented, focused on the urban wastewater treatments already implemented in urban WWTPs and the future perspectives of the wastewater treatment field. Chapter 3 states the main objectives of the thesis. Chapter 4 describes the general materials and methods used during the experimental work of this thesis which were common to all the experiments presented in the chapters of results; thus, the more specific materials and methods used in specific chapters are described in the corresponding chapter where they were used. Chapters 5 to 9 contain the results obtained during the development of the thesis.

Chapter 5, 6 and 7 comprise the results obtained for the partial nitritation reactor. More specifically Chapter 5 describes the successful long-term operation at low temperature and mainstream conditions; Chapter 6 is focused on the determination of nitrous oxide

emissions of the partial nitritation process at mainstream conditions; and Chapter 7 deals with the destabilization of the nitritation process after a long-term stable operation.

Chapters 8 and 9 comprise the results obtained for the anammox reactor. More specifically, Chapter 8 describes the start-up of the anammox reactor in an Upflow Anaerobic Sludge Blanket (UASB) reactor and a complete study of the granulation and implementation of anammox process in UASB reactors; and Chapter 9 describes the successful long-term operation of the UASB anammox reactor at mainstream conditions.

Chapter 10 states the main conclusions extracted from this thesis and, finally, Chapter 11 presents all the references used during this writing.

Chapter 2

GENERAL INTRODUCTION

2.1. THE NEED OF WASTEWATER TREATMENT

The industrialization, increasing urbanization, modern life standards and irrigation degree led to a huge wastewater production in the developed countries (25–150 m^3 p^{-1} $year^{-1}$ in EU countries in 2011, Eurostat 2015), which brought up the need of a wastewater treatment before the discharge into the environment. Urban wastewater is characterized for presenting anthropogenic origin, low levels of pollutants (more commonly organic matter, nitrogen and phosphorous) and low heat energy content. Overall, urban wastewater is transported to a wastewater facility, where it is treated in an energy-demanding dissipative way before being returned to a natural system (Verstraete and Siegfried, 2011).

Since its presentation at the beginning of last century (Ardern and Lockett, 1914), the conventional activated sludge (CAS) system continues being the most commonly used technology for urban wastewater treatment, although significant improvements have been developed since the first version of CAS system was implemented. Activated sludge is a mixture of inert solids combined with a microbial population growing on the biodegradable substrates present in the sewage (van Loosdrecht and Brdjanovic, 2014). CAS system allows the conversion of roughly half of the chemical oxygen demand (COD) of wastewater into sludge and the other half to CO_2, and the resulted water can be decanted and discharged into the environment (Verstraete and Siegfried, 2011).

Most current urban wastewater treatment plants (WWTPs) only treat organic matter through CAS system, however during the past several decades nutrients as nitrogen and phosphorous became an important concern since can be toxic to aquatic life and cause eutrophication and oxygen depletion in water streams. Actually, nutrients pollution has impacted many streams, rivers, lakes, bays and coastal waters resulting in serious environmental and human health issues, and impacting the economy (EPA Website, 2016). Thus, in the most developed countries, CAS systems have incorporated the biological nutrients removal through nitrification/denitrification and enhanced phosphorous removal.

2.2. NITROGEN REMOVAL IN URBAN WASTEWATER TREATMENT PLANTS

2.2.1. From activated sludge systems to the potential implementation of anammox in the mainstream

In the current CAS systems with nitrogen removal incorporated, nitrogenous compounds are removed by biological treatment through bacterial nitrification/denitrification (N/D). First, nitrification takes place under aerobic conditions; ammonia is oxidized to nitrite by ammonia oxidizing bacteria (AOB) and nitrite is oxidized to nitrate by nitrite oxidizing bacteria (NOB). Then, denitrification occurs under anoxic conditions where nitrite and nitrate are reduced by heterotrophic bacteria to nitrogen gas (N_2).

The main advantage of this conventional treatment is that guarantees a good effluent quality with a relative easy implementation, however it presents high operational costs and needs a high land availability. The major costs associated to CAS systems with biological N/D implemented are related to the energy consumption and sludge treatment. The aeration energy consumption for organic matter removal and nitrification is about 60–80% of the total energy consumption of an urban WWTP (Siegrist et al., 2008; Zessner et al., 2010); and up to 40% of the operational costs are associated to the sludge treatment and disposal (Verstraete and Siegfried, 2011). Thus, nowadays, urban WWTPs are very energy-demanding facilities and achieving an advanced and sustainable WWTP is the challenge to face by the modern society.

Some urban WWTPs incorporate the anaerobic digestion of the primary and secondary sludge produced during the CAS treatment in order to recover energy as biogas, however the energy recovery only achieves values up to 50% in the most energy-efficient plants (Verstraete and Siegfried, 2011). This is due to that a significant part of the organic matter present in the wastewater is used for denitrification instead of being used for producing biogas and, thus, only primary and secondary sludge can be destined to anaerobic digestion. If the organic matter removal and nitrogen removal processes were independent treatments, all the organic matter present in the raw wastewater could be redirected to the anaerobic digestion process and the subsequent energy recovery through biogas could be increased.

The finding of anaerobic ammonium-oxidizing (anammox) bacteria (Mulder et al., 1995) brought up the possibility of separating the organic matter and nitrogen removal processes. Anammox bacteria are chemolithoautotrophic microorganisms which grow by the oxidation of ammonium coupled to nitrite reduction, using CO_2 as the sole carbon source and

hence do not require organic carbon (Kartal et al., 2013). The appearance of anammox bacteria guaranteed the possibility of performing an autotrophic denitrification in urban WWTPs; and thus, the autotrophic biological nitrogen removal (BNR) via nitritation-autotrophic denitrification appeared as an alternative to the conventional BNR via autotrophic nitrification-heterotrophic denitrification. Autotrophic BNR is a two-step process. Firstly, half of the supplied ammonium is oxidized to nitrite (partial nitritation) by AOB under aerobic conditions; and secondly, ammonium and nitrite are directly converted to N_2 by the anammox bacteria without oxygen and organic matter consumption.

The implementation of the autotrophic BNR has been proposed for achieving an energy-neutral or even energy-positive urban WWTP (Kartal et al., 2010; Siegrist et al., 2008). As depicted in Fig. 2.1, the wastewater treatment would consist of a first step (A-Stage) where all the organic matter is removed and derived to biogas production, and a second step (B-Stage) where the nitrogen is removed through the autotrophic BNR process. Several technologies have been proposed for performing the A-Stage, such as the use of anaerobic digestion (Gao et al., 2014, 2015) or the use of a high-rate activated sludge system (Ge et al., 2013; Jimenez et al., 2015).

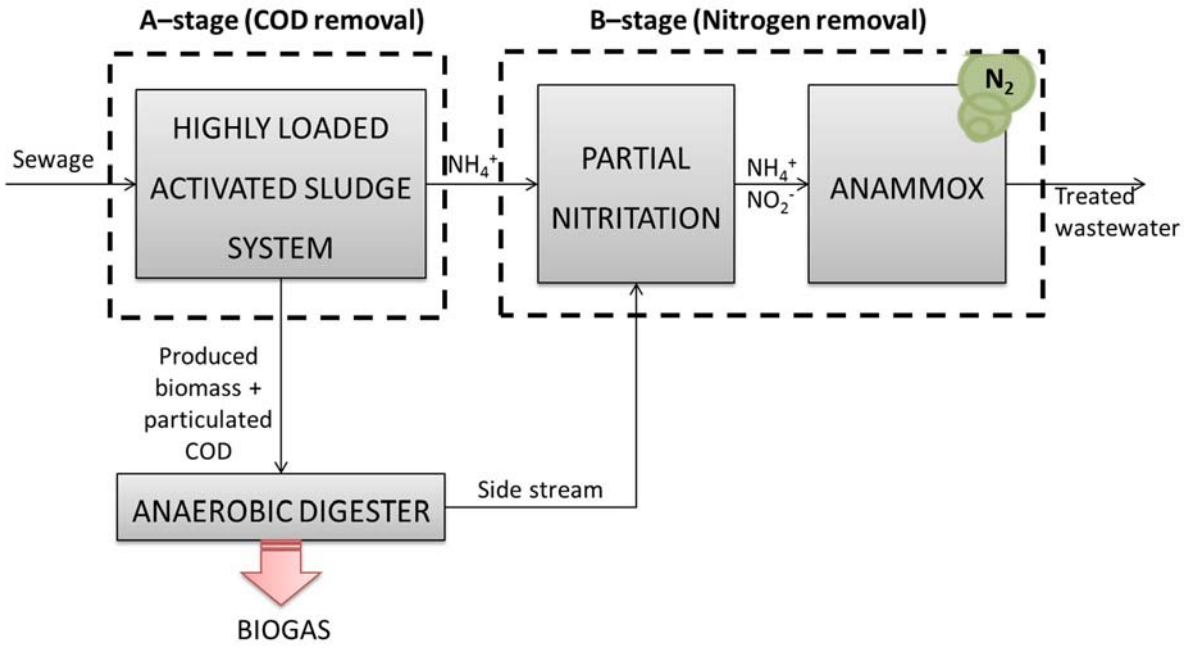

Fig. 2.1. Scheme of the implementation of autotrophic biological nitrogen removal process in the main water line of an urban wastewater treatment plant.

When autotrophic BNR is implemented, aeration costs are reduced because of the lower oxygen requirements of the process compared to conventional activated sludge treatment; and furthermore, biogas production is increased since most of the organic matter will be converted to biogas in the anaerobic digestion process, with the consequent energy recovery. In fact, according to Kartal et al. (2010) calculations, a facility where conventional CAS treatment is used presents a net energy consumption of 44 Wh p^{-1} d^{-1} but if anammox is implemented in the main water line the facility could present a net energy production of 24 Wh p^{-1} d^{-1}. Table 2.1 presents a comparison of operational requirements of the autotrophic BNR process compared to CAS systems in an urban WWTP.

Table 2.1. Comparison of the main operational requirements of the autotrophic BNR process implemented in the mainstream and/or in the sidestream of an urban WWTP in reference to a CAS treatment. Data obtained from Kartal et al. (2010) and Morales et al. (2015).

	Autotrophic BNR process in the sidestream	Autotrophic BNR process in the mainstream
Aeration requirements (including COD and N removal)	- 16–26 %	- 50 %
Energy recovery through biogas production	+ 18–34 %	+ 67–84 %
Sludge generation	+ 17 %	+ 9 %
Nitrous oxide emissions	- 22 %	- 83 %
Pumping/Mixing energy	0 %	- 25 %

2.2.2. Implementation of anammox-based technologies for nitrogen removal in urban WWTPs: from sidestream to mainstream

Autotrophic BNR can be implemented as a one-stage system, where partial nitritation and anammox processes (PN/A) take place in the same reactor, or as a two-stage system, when partial nitritation and anammox processes are separated in two different reactors. In one-stage systems a co-culture of AOB and anammox bacteria is established under microaerobic conditions to avoid inhibition of anammox bacteria by oxygen and to achieve appropriated conditions to obtain partial nitritation (Van Hulle et al., 2010). The use of one single reactor leads to lower capital costs than two-stage systems; however difficulties in dissolved oxygen regulation can appear triggering to the eventually growth of NOB and the destabilization of the process (Hao et al., 2002). Two-stage systems present higher capital

costs than one-stage systems but make possible a more stable performance and control. In addition, the use of a two-stage system avoids the negative effects of organic compounds present in the influent on anammox bacteria and, besides, allows complete anoxic conditions in the anammox reactor.

Autotrophic BNR has been successfully applied for treating some industrial wastewaters and reject water from digested sludge (sidestream in urban WWTPs), since they present more advantageous conditions for autotrophic BNR. The high ammonium concentrations and the elevated temperatures typical of sidestream (500–1500 mg N L^{-1} and temperature higher than 30 ºC) allow a more efficient NOB repression and process stability. During the last decade, numerous full scale facilities have implemented autotrophic BNR in the sidestream by using different technologies: sequencing batch reactors (SBRs) (Wett, 2007), granular reactors (Castro-Barros et al., 2015), moving bed biofilm reactors (Rosenwinkel and Cornelius, 2005), rotating biological contactors and even activated sludge systems (Desloover et al., 2011). Two-stage systems were used in the first place for implementing the autotrophic BNR, because allowed a better control of partial nitritation and, in addition, the use of the already existing nitritation systems like SHARON (Single reactor High activity Ammonia Removal Over Nitrite); however, with more knowledge gained about the process, one-stage systems became the most implemented systems at full-scale with the 88% of all the installations and the 75% of the installations implemented in urban WWTPs (Lackner et al., 2014) due to the lower investment costs and effectiveness.

Recently, several technologies have been patented and widely used in European and North American countries for treating industrial and sidestream wastewater, differing mainly in: the type of system (one- or two-stage system), the state of biomass (suspended or attached), the feeding strategy, the control of aeration, etc. One of the first autotrophic BNR systems implemented at full scale was the two-stage system SHARON/ANAMMOX® from Paques in Rotterdam, The Netherlands. However, since 2006, Paques changed its perspective and aimed for the implementation of one-stage installations, which guaranteed good results with lower investment costs. Technologies as DEMON® (aerobic/anoxic deammonification), OLAND® (Oxygen Limited Autotrophic Nitrification and Denitrification), CANON® (Completely Autotrophic Nitrogen removal Over Nitrite), SNAP® (Single-stage Nitrogen removal using Anammox and Partial nitritation), ELAN® (Eliminación Autótrofa de Nitrógeno), ANITAMox™ or DeAmmon® are some of the patented technologies for the one-stage systems performing autotrophic BNR. Besides of those patented technologies, some

facilities have implemented their own strategies for achieving autotrophic BNR in the sidestream. The high variety of technologies already implemented at full-scale highlights the success of the autotrophic BNR implementation when a high-strength and warm stream is treated.

Nevertheless, despite of the successful implementation of autotrophic BNR in the sidestream, it has never been applied in the main water line of an urban WWTP (low-strength and cold wastewater). The typical conditions of the mainstream, with temperatures as low as 8 ºC during winter (De Clippeleir et al., 2013), nitrogen concentrations lower than 80 mg N L^{-1} (Clippeleir et al., 2011) and the possible input of organic matter from the A-Stage, are not advantageous for implementing partial nitritation and anammox process, since the NOB repression is more challenging at low temperature and, furthermore, a competition between autotrophic and heterotrophic bacteria can appear. Hence, the application of the autotrophic BNR in the mainstream of an urban WWTP with high nitrogen removal rates and a good effluent quality is nowadays a challenge.

Recently, different alternatives have been proposed for implementing the autotrophic BNR at mainstream conditions. Initially, studies focused on the use of one-stage systems due to the successful results obtained at sidestream conditions and the lower investment costs associated. Table 2.2 shows the most recently studies which implemented partial nitritation and anammox process in one-stage systems at mainstream conditions. Most systems focused on the use of oxygen-limiting conditions to achieve an efficient NOB repression and maintain the anammox bacteria activity. Thus, innovative systems such as the use of integrated fixed film activated sludge (IFAS) reactors, where a combination of moving bed biofilm (mainly responsible for anammox activity) and suspended sludge (mainly responsible of aerobic ammonium oxidation) are integrated and operated under transient oxygen and anoxic conditions (Malovany et al., 2015); or the use of most commonly studied systems such as SBRs operating with suspended or granular biomass (Gilbert et al., 2015) and even the comparison of moving bed biofilm reactors with different types of carriers (Gilbert et al., 2015) were tested to achieve stable operation in one-stage systems. Nevertheless, most cases showed nitrate accumulation due to the decrease of anammox bacteria activity at the same time that NOB activity increased (Table 2.2), and even those systems which achieved an efficient NOB repression operated at high temperature (25 ºC, Li et al. 2016) and/or achieved low nitrogen conversion rates (0.015 g N L^{-1} d^{-1}, Gilbert et al., 2014).

Overall, data from Table 2.2 shows that the main challenge for the implementation of PN/A in one-stage systems at mainstream conditions is achieving an efficient NOB repression at low temperature and high nitrogen removal. Recently, the two-stage systems have been presented as an alternative of great interest to overcome the drawbacks associated to one-stage systems (Ma et al., 2011; Pérez et al., 2015) and numerous studies tried to demonstrate the feasibility of the two-stage implementation.

On the one hand, different strategies were reported to achieve a stable partial nitritation at mainstream conditions in a single reactor with an efficient NOB repression and a suitable effluent for a subsequent anammox reactor. For instance, Gao et al. (2014) reported stable NOB repression at room temperature (12-27 ºC) in a lab-scale SBR by applying a control strategy which controlled the duration of the aeration phase needed to enable half-ammonia oxidation according to the ammonium and COD concentrations in the influent and the temperature of operation. At pilot scale, Regmi et al. (2014) proposed an operational and process control strategy based on optimizing the COD concentration in the influent, imposing transient anoxia and aggressive solids retention time operation (close to AOB washout) and operating at high DO in a continuously stirred tank reactor at 25 ºC. Regarding stable operation at long-term, stable partial nitritation with efficient NOB repression was reported by Isanta et al. (2015a) in a granular sludge airlift reactor operating at 12.5 ºC by applying a control strategy based on maintaining a low ratio between oxygen and ammonium concentrations in the bulk liquid of the reactor.

On the other hand, the successful operation of a single anammox reactor at such adverse mainstream conditions was also assessed. Different reactors were chosen to prove the stability of the anammox process at mainstream conditions. Thus, Ma et al. (2013) reported successful anammox operation in an upflow anaerobic sludge blanket reactor treating a synthetic influent at mainstream conditions with high nitrogen removal rates at 16 ºC. Similarly, Lotti et al. (2014b) also reported stable long-term operation in an upflow fluidized granular sludge reactor operating between 20 ºC and 10 ºC treating a real effluent from an A-Stage. And more recently, Laureni et al. (2015) reported successful treatment of an aerobically pre-treated municipal wastewater at 29 ºC by using suspended-growth anammox biomass in SBRs and also demonstrated the feasibility of operating a SBR anammox reactor at 12.5 ºC although with a marked decrease in activity.

Table 2.2. Studies performing partial nitritation and anammox process as a one-stage system at mainstream conditions. T: temperature; NRR: Nitrogen Removal Rate; NRE: Nitrogen Removal Efficiency; RBC: Rotating Biological Contactor; COD: Chemical Oxygen Demand; N: Nitrogen; SBR: Sequencing Batch Reactor; MBBR: Moving Bed Biofilm Reactor; H-MBBR: Hybrid Moving Bed Biofilm Reactor; IFAS: Integrated Fixed film Activated Sludge; UMABR: Upflow Membrane-Aerated Biofilm Reactor; NOB: nitrite-oxidizing bacteria; AMX: anammox bacteria.

Scale	Influent	Biomass	Reactor	T (ºC)	NRR (g N L^{-1} d^{-1})	NRE (%)	Comments	Reference
Lab-scale	Synthetic	Biofilm	RBC	25	0.44	46	OLAND technology NOB activity developed	Clippeleir et al. (2011)
Lab-scale	Synthetic	Biofilm	RBC	15	0.50	36	OLAND technology NOB activity developed Destabilization when COD/N=1	De Clippeleir et al. (2013)
Lab-scale	Synthetic	Suspended	SBR	12	0.03	> 90	Nitrate highly accumulated when temperature decreased to 9 ºC	Hu et al. (2013)
Lab-scale	Synthetic	Biofilm	MBBR	10	0.015	60	NOB activity decreased causing a nitrite accumulation when temperature decreased from 13°C to 10 ºC	Gilbert et al. (2014)
Lab-scale	Synthetic	Granules	Airlift	10	0.20	48	NOB activity developed	Lotti et al. (2014a)

Table 2.3. Continuation.

Scale	Influent	Biomass	Reactor	T (ºC)	NRR (g N L^{-1} d^{-1})	NRE (%)	Comments	Reference
Lab-scale	Synthetic	Granules and biofilm	UMABR	25	0.08	81	Efficient NOB repression Pure oxygen through membrane	Li et al. (2016)
Lab-scale	Real effluent from A-Stage	Suspended	SBR	10	0.01	< 25	Nitrate highly accumulated when temperature was below 12 ºC	Lackner et al. (2015)
Lab-scale	Real effluent from A-Stage	Biofilm	MBBR	10	0.04	< 50	Nitrate highly accumulated when temperature was below 12 ºC.	Lackner et al. (2015)
Lab-scale	Real effluent from A-Stage	Biofilm and biofilm + suspended	MBBR and H-MBBR	15	0.02–0.04	70–90	Destabilization at 11 ºC	Laureni et al. (2016)
Pilot-scale	Real effluent from A-Stage	Biofilm + suspended sludge	IFAS	25	0.05	52	Inefficient NOB repression Higher NRE when COD/N=1.8 due to the heterotrophic denitrification	Malovany et al. (2015)
Pilot-scale	Real effluent from A-Stage	Granules	Plug-flow	19	0.18	46	Minimum $[NO_3^-]/[NH_4^+]$ ratio achieved of 0.35, which indicated NOB activity	Lotti et al. (2015a)

2.3. THE FUTURE OF URBAN WWTPS

During the last decades, since a good effluent quality was guaranteed, the attempts for reducing the energy associated costs and increasing the energy recovery in urban WWTPs have been considered the most important issue on the race for achieving energetic self-sufficient facilities. This is the reason why the development of a process such as the autotrophic biological nitrogen removal, which could guarantee an energy-positive urban WWTP, has appeared as a hot topic in current wastewater research. However, in the race for achieving a sustainable urban WWTP not only the energy requirements need to be considered but also the environmental impact of the facility. Thus, in addition to the development of energy-efficient processes for the removal of pollutants, other subjects, as the described below, should be taken into account:

- **Greenhouse gases emissions**

Nitrous oxide (N_2O), carbon dioxide (CO_2), and methane (CH_4) are greenhouse gases (GHGs) whose emissions are the most commonly emissions which can be produced during wastewater treatment in urban WWTPs (Kampschreur et al., 2009). Since CH_4 gas is likely not produced during the treatment in the facilities but produced in the sewerage system and during the sludge handling (e.g. anaerobic digestion), and CO_2 is produced by the biological treatment of organic matter (biogenic origin) and it does not contribute to the greenhouse effect; N_2O is the most significant GHG emitted during wastewater treatment in urban WWTPs and its emissions cannot be overlooked. The source and magnitude of N_2O emissions in WWTPs (produced during biological nitrification and denitrification) are relatively unknown and subject of debate in the literature (Kampschreur et al., 2009). For instance, the N_2O emissions associated to wastewater treatment accounted approximately the 2% of the total anthropogenic N_2O emissions in U.S in 2014, corresponding to 4.8 million metric tons of CO_2 equivalents (EPA, 2016). The N_2O gas presents a global warming potential of about 300 times higher than CO_2 on a 100 year time horizon (IPCC, 2013) and, furthermore, N_2O emissions are currently the most important ozone-depleting emissions and are expected to remain the largest throughout the 21st century (Ravishankara et al., 2009). Therefore, even low amounts of N_2O emissions should be avoided in urban WWTPs; and mitigation strategies and control of emissions are essential issues to consider in the implementation of any process in an urban wastewater facility.

- **Resources recovery**

Nowadays the equivalent of 1.6 planets are used to provide the resources used by humanity and absorb the waste produced (Global Footprint Network, 2016). This means that planet Earth needs one year and six months to regenerate what humanity use in a year. Such worrying situation brings up the need of improving the resource efficiency and the concept of using 'waste' as a resource appears as an important alternative to build a more sustainable society. Hence, urban WWTPs should be thought as potential facilities of resource recovery both from wastewater and from sludge. In fact, phosphate recovery from wastewater and the production of other valuable materials from sludge are emerging (e.g. the recovery of cellulose fibers and the production of bioplastics and biopolymers) at quantities and costs that match the current market demand and prices (van Loosdrecht and Brdjanovic, 2014). Thus, the implementation of new processes for wastewater treatment should consider the potential resource recovery, rather than just pollutants removal.

- **Decentralized technology**

WWTPs are usually the central topic when talking about sustainable wastewater treatment, however the potential energy recovery from wastewater in sewers and households should be also taken into account. For instance, it is known that the largest energy content of wastewater is found as heat, with about 85% of the energy contained in urban wastewater (Larsen, 2015). Decentralized heat recovery from warm water sources at the household level holds a higher potential to extract useful energy, either with heat pumps or heat exchangers as reported by Larsen (2015). It is also known that the nitrogen content of human urine accounts for about 75% of the total nitrogen in urban wastewater, but it comprises only 1% of the volume (Luther et al., 2015). Thus, another potential application of decentralized technology is the urine separation at household level for subsequent fertilizer production (Luther et al., 2015; Udert and Wächter, 2012). Hence, the implementation of decentralized processes should be also considered a hot point to achieve a sustainable wastewater treatment.

Further research is needed to find out which is the most suitable technology for performing a sustainable wastewater treatment. In any case, the implementation of autotrophic BNR appears as an efficient alternative to achieve a sustainable urban WWTP from the energetic point of view, although other complementary alternatives cannot be overlooked. Furthermore, the most appropriated process to guarantee effluent quality with a sustainable operation can be different for each independent case, according to the land

availability, the possibility of resource recovery, the characteristics of the wastewater, the possibility of modify the actual processes in the facility or build a new plant, etc.

Chapter 3

OBJECTIVES OF THE THESIS

The main objective of this thesis was to demonstrate the feasibility of implementing the autotrophic biological nitrogen removal process as a two-stage system in the main water line of urban wastewater treatment plants. Thus, this thesis aimed at demonstrating the stability of partial nitritation and anammox processes in two separated reactors treating wastewater at mainstream conditions.

More specifically, the goals of this thesis were:

- To demonstrate the long-term stability of partial nitritation and anammox processes treating an urban influent at mainstream conditions with granular sludge reactors operated in continuous mode.

- To propose the use of Upflow Anaerobic Sludge Blanket (UASB) reactors as a good alternative for the implementation of the anammox process at mainstream conditions.

- To demonstrate that high nitrogen removal efficiencies and high nitrogen removal rates can be achieved operating a two-stage system for the autotrophic biological nitrogen removal in urban influents.

- To study in depth the biomass developed in partial nitritation and anammox reactors from the microbiological, kinetic and physicochemical points of view during the operation at mainstream conditions.
- To determine the nitrous oxide emissions (N_2O) from a partial nitritation reactor treating an influent at mainstream conditions and, in addition, to evaluate the effect of temperature on the N_2O emissions produced in the partial nitritation reactor.

Chapter 4

GENERAL MATERIALS AND METHODS

4.1. DESCRIPTION OF THE REACTORS AND EXPERIMENTAL SET-UP

4.1.1. Lab-scale airlift reactor

A lab-scale airlift reactor with a total volume of 5.2 L, with a downcomer-to-separator diameter ratio of 0.36 and a total length-to-downcomer diameter ratio of 16 was used (Fig. 4.1). Compressed air was supplied through an air diffuser placed at the bottom of the reactor and the dissolved oxygen (DO) concentration in the bulk liquid was measured on-line by means of a DO electrode (DO 60-50, Crison Instruments, Spain). The pH was measured on-line with a pH probe (pH 52-10, Crison Instruments, Spain). The temperature was measured in the bulk liquid and controlled by means of a cooling system (E100, LAUDA, Germany) and an electric heater (HBSI 0.8 m, HORST, Germany) connected to a temperature controller (BS-2400, Desin Instruments, Spain). Total ammonia nitrogen (TAN = $N\text{-}NH_4^+$ + $N\text{-}NH_3$) and nitrate concentrations in the bulk liquid were measured by using an on-line probe (AN-ISE sc probe with a Cartrical cartridge plus, Hach Lange, Germany). The range of the on-line probe for TAN and nitrate concentrations was 0-1000 mg N L^{-1} whereas the detection limit was 0.2 mg N L^{-1} for both parameters.

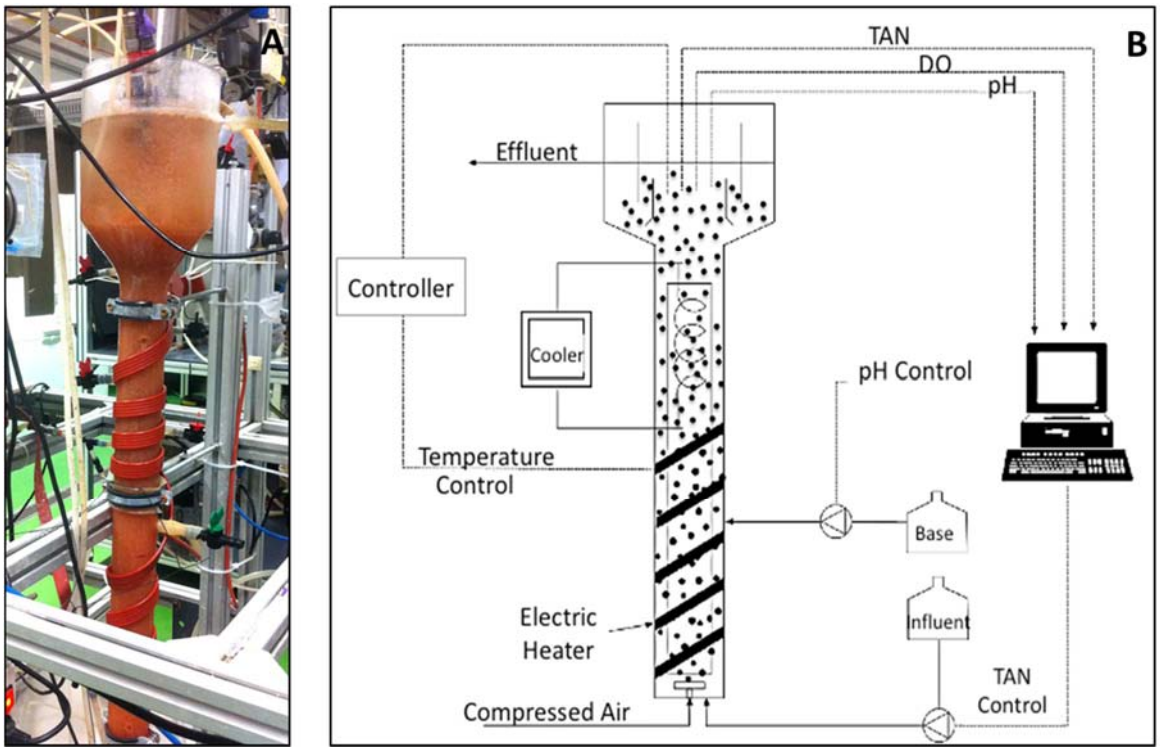

Fig. 4.1. Image of the lab-scale airlift reactor (A) and schematic diagram of the set-up showing the peripheral instrumentation and control loops (B).

4.1.2. Lab-scale UASB reactor

A lab-scale UASB reactor with a working volume of 2 L including the gas-liquid-solid separator was used for the implementation of the anammox process (Fig. 4.2). The inner diameter of the column was 51 mm and the total-reactor-height to column-diameter ratio was 12.5. The pH of the reactor bulk liquid was not controlled but measured offline, while the pH of the influent was set to values around 7.5 to avoid any shock of pH in the reactor. Influent was devoid of oxygen since influent tank was periodically flushed with dinitrogen gas (N_2) to guarantee a DO concentration lower than 0.3 mg O_2 L^{-1}. Additionally, N_2 gas was introduced into the reactor headspace. DO concentration was measured in the bulk liquid of the reactor by means of a DO electrode (DO 60-50, Crison Instruments, Spain). The temperature was measured and controlled by means of a cooling system and an electric heater (HBSI 0.8m, HORST, Germany) connected to a temperature controller (BS-2400, Desin Instruments, Spain). The cooling system consisted of a tube surrounding the column of the UASB reactor with a continuous recycling of cold antifreeze liquid at temperature c.a. -5 ºC.

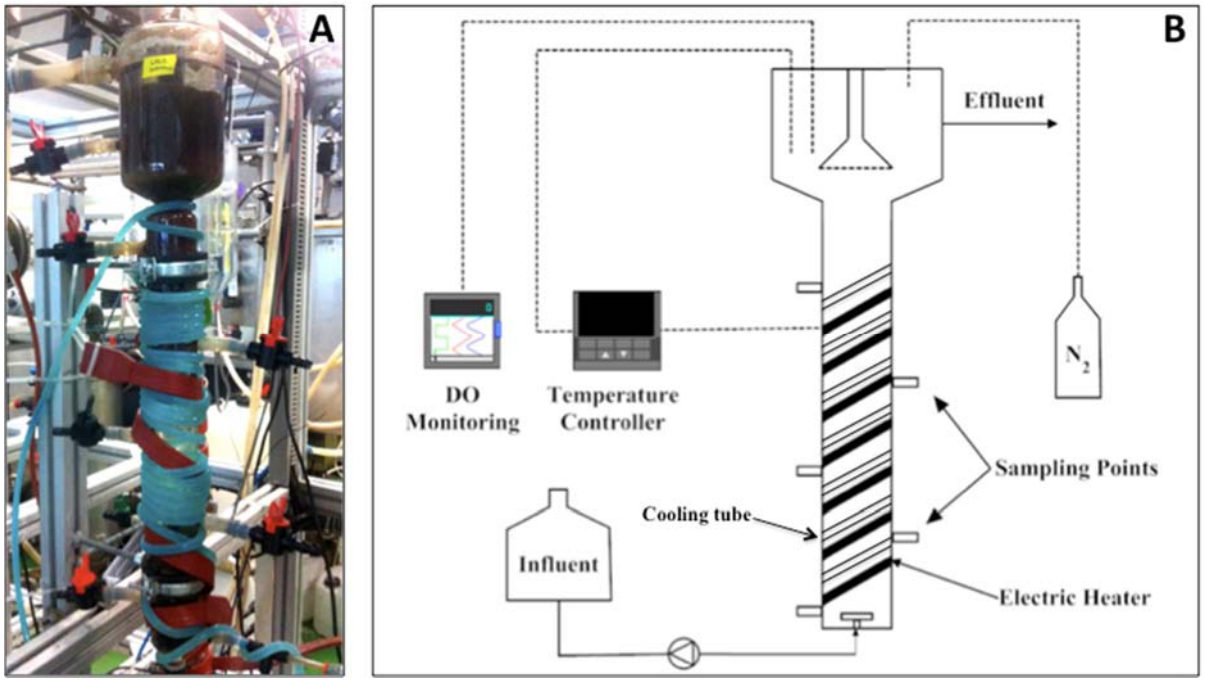

Fig.4.2. Image of the lab-scale UASB reactor (A) and schematic diagram of the set-up showing the peripheral instrumentation (B).

4.2. ANALYTICAL METHODS

The influent, effluent and biomass of the reactors were characterised throughout the different experiments performed during this thesis. Hence, different analytical methods were applied. In this section only the common analysis for all the experiments are detailed. More specific analytical methods and techniques used for each experiment will be detailed in the corresponding chapter of results.

4.2.1. Analysis of nitrogen species: ammonium, nitrite and nitrate

Samples were previously filtered by 0.22 μm. Total ammonia nitrogen concentration was analysed off-line with an ammonium analyser (AMTAX sc, Hach Lange, Germany). Nitrite and nitrate concentrations were analysed off-line with ionic chromatography using an ICS-2000 Integrated Reagent-Free IC system (DIONEX Corporation, USA).

4.2.2. Chemical oxygen demand

Chemical oxygen demand (COD) was analysed by using colorimetric Hach Lange kits (LCK314) and the DR2800 Hach Lange spectrophotometer. Total COD was directly measured by using the kits, while samples where soluble COD was analysed were previously filtered by 1.6 μm. Samples were analysed in triplicates.

4.2.3. Settling properties

Settling velocity was determined for at least 30 individual granules by dropping the individual granule in a glass cylinder containing tap water and measuring the time spent settling a known distance. Sludge volumetric index (SVI) was analysed in duplicates according to Standard Methods (APHA, 2005).

4.2.4. Total and volatile solids concentrations

Total suspended solids (TSS) and volatile suspended solids (VSS) were analysed according to Standard Methods (APHA, 2005). Samples were analysed in triplicates. First, samples were filtered through previously weighted (W_1) standard glass microfiber filters of 0.7 μm (GF/F grade, Whatman, USA) and dried at 105 ºC until constant weight (W_2) (c.a. 2.5 hours). The relation between the difference between W_1 and W_2 and the sample volume was the concentration of TSS. Then, sample was ignited at 550 ºC for about 45 min and weighted (W_3). The difference between W_1 and W_3 per volume of sample represented the concentration

of VSS. Total solids (TS) and volatile solids (VS) concentrations were calculated following the same procedure without the filtering step. In this case, samples were weighted using metallic dishes.

4.3. MICROBIAL ANALYSIS

Two molecular biology techniques were used to identify and quantify the microorganisms present in sludge samples: fluorescence *in situ* hybridization and pyrosequencing.

4.3.1. Fluorescence *in situ* hybridization (FISH)

-*Sample fixation*

Biomass samples were grabbed from the reactor and the granules were crushed by means of a mortar and a pestle in order to ease hybridization. Then, biomass was fixed by adding three volumes of 4% (v/v) paraformaldehyde solution to one volume of biomass suspension. The mixture was keep at 4 ºC for 1–3h. Afterwards, biomass suspension was washed twice with 0.01M Phosphate Buffered Saline (PBS) solution (1:30 dilution of a solution 0.3M PBS which was prepared from 77.4 g of $Na_2HPO_4.12H_2O$, 13.1 g $NaH_2PO_4.2H_2O$ and 226.2 g NaCl) and it was re-suspended in one volume of 0.01M PBS per one volume of ice-cold ethanol 98%. Fixed biomass could be spotted onto glass slides to starting the hybridization protocol or stored at -20 ºC for several months.

-*Hybridization*

The hybridization protocol was adapted from Hugenholtz et al. (2002) and Manz et al. (1992). Suspended fixed biomass was spotted onto a glass slide and dehydrated in ethanol series of 50, 80 and 98% (v/v) (3 min each).

For samples of anammox sludge three steps of membrane permeabilization were performed before the dehydration with ethanol: (i) a lysozyme solution (prepared with 1 mL 0.5 M EDTA, 1 mL 1M Tris/HCl pH 8, 8 mL Milli-Q-grade water and 100 mg lysozyme) was added to biomass and incubated at 37 ºC for 1.5 h; then (ii) fresh achromopeptidase solution was added to biomass and incubated at 27 ºC for 30 min. To prepare the achromopeptidase solution 10 mL of achromopeptidase buffer (100 µL 5M NaCl, 500 µL 1M Tris/HCl pH 8 and

50 mL of Milli-Q-grade water, pH 8) were mixed with 20 µL achromopeptidase stock solution (the lyophilized powder as come from supplier (Sigma-Aldrich, ref.: A3547-100KU) was dissolved in Milli-Q-grade water to prepare a stock solution of 3KU/ml). Finally, (iii) biomass was washed with Milli-Q-grade water before dehydrating with ethanol series.

After dehydration with ethanol and when glass slide was dry, 10 µL of hybridization buffer were added plus 1µL of (each) probe working solution (probe concentration of 50 ng μL^{-1}). Solution was mixed without scratching the slide and cell layer. Hybridization buffer contained: 360 µL of 5M NaCl (autoclaved), 40µL of 1 M Tris/HCl (autoctaved), 2µL of 10% SDS, 898 µL of Milli-Q-grade water, and the corresponding amount of formamide for each molecular probe. The slide was placed in a 50 mL Falcon tube containing a moistened tissue and the tube was closed and put in the hybridisation oven at 46 ºC for 2 hours. After hybridization, the slides were quickly transferred to the washing buffer tube by immersing the whole slide in the pre-warmed washing buffer at 48 ºC for 15 min. Washing buffer contained 80µL of NaCl 5M (autoclaved), 500µL EDTA 0.5M, 1mL Tris/HCl 1M (autoclaved), 43.8mL Milli-Q-water (autoclaved) and 50µL 10% SDS. After washing, the slide was rinsed with cold Milli-Q-grade water. Afterwards, all droplets of water were removed from the slide by directly applying compressed air to the slide. To finish, a mounting medium (specifically *Fluoprep*) was applied for the subsequent microscopic observation.

Hybridizations were carried out using at the same time the general bacteria probe and the specific probes for the specific microorganisms, which wanted to be identified. The general bacteria probe was an equal mixture of probes EUB338I, EUB338II and EUB338III for all Bacteria. All the probes used for the experiments of this thesis are shown in Table 4.1.

-Microscope observation and quantification

A Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystem Heidelberg GmbH; Mannheim, Germany) using a Plan-Apochromatic 63x objective (NA 1.4, oil) was used for biomass quantification. The quantification was performed following an automated image analysis procedure described in Jubany et al. (2009a), where at least 30 microscopic fields were analysed and a single-z position was selected based on the highest intensity for each sample.

Table 4.1. 16S rRNA-targeted oligonucleotide probes, target microorganisms, and references used in this thesis to determine the relative abundance of microbial population with the FISH technique**.**

Probe	Sequence (from 5' to 3')	5' Dye	Specificity	Reference
EUB338 I	GCTGCCTCCCGTAGGAGT	Cy5	Most bacteria	Amann et al., 1990
EUB338 II	GCTGCCTCCCGTAGGAGT	Cy5	Planctomycetales	Daims et al., 1999
EUB338 III	CGCCATTGTATTACGTGTGA	Cy5	Verrucomicrobiales	Daims et al., 1999
NSO190	CGATCCCCTGCTTTTCTCC	6FAM / ALEXA594	All AOB	Mobarry et al., 1996
NIT3	CCTGTGCTCCATGCTCCG	Cy3/Pacific Blue/Fluos	*Nitrobacter* spp.	Wagner et al., 1996
NIT3 Competitor	CCTGTGCTCCAGGCTCCG	-	-	Wagner et al., 1996
NTSPA662	GGAATTCCGCGCTCCTCT	6FAM	*Nitrospira* genus	Daims et al., 2001
NTSPA662 Competitor	GGAATTCCGCTCTCCTCT	-	-	Daims et al., 2001
NSV443	CCGTGACCGTTTCGTTCCG	Atto550	*Nitrosospira* spp.	Mobarry et. al., 1996
BAN162	CGGTAGCCCCAATTGCTT	Texas Red	*Candidatus Brocadia Anammoxidans*	Schmid et al., 2001
KST157	GTTCCGATTGCTCGAAAC	Texas Red	*Candidatus Kuenenia Stuttgartiensis*	Schmid et al., 2001
BFU613	GGATGCCGTTCTTCCGTTAAGCGG	Texas Red / ALEXA594	*Candidatus Brocadia Fulgida*	Kartal et al., 2008
AMX368	CCTTTCGGGCATTGCGAA	ALEXA 488	All anammox bacteria	Alm et al., 1996

4.3.2. Pyrosequencing analysis

Pyrosequencing was used for analysing the diversity and relative abundance of different microorganisms in the granular sludge of the reactors.

First, DNA was extracted from biomass samples by applying the the manufacturer protocol of MoBio PowerBiofilm™ DNA extraction kit (MoBio Laboratories, USA). Two modifications of the manufacturer protocol were performed: 200 µL of solution BF3 were added instead of the 100 µL recommended, and 80 µL of solution BF7, instead of the 100 µL recommended. Once the extraction was performed, NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA) was used to measure the quantity and quality of extracted DNA. A 260/280 nm ratio of 1.8 was used as quality cut-off and a minimum of 25 ng μL^{-1} of extracted DNA was guaranteed to perform pyrosequencing.

Paired-end sequencing of the extracted DNA was performed on an Illumina MiSeq platform by Research and Testing Laboratory (Lubbock, Texas, USA). Bacterial 16S rRNA variable regions V2-V4 were targeted using the primer pair 341F-907R in the studies of nitrifying population (Chapter 5) and the primer pair 315F-909R in the studies of anammox population (Chapter 9).

Bioinformatics for the biodiversity analysis and phylogenetic classification were performed as follows: The forward and reverse reads were merged together using the PEAR Illumina paired-end read merger (Zhang et al., 2014), sequence reads were then sorted by length from longest to shortest and prefix dereplication and clustering at a 4% divergence was performed using the USEARCH algorithm (Edgar, 2010). Following, the clusters were classified into operational taxonomic units (OTUs) using the UPARSE OTU selection algorithm (Edgar, 2013). Chimera checking was performed using the UCHIME chimera detection software executed in *de novo* mode (Edgar et al., 2011). The representative sequences reads were mapped to their corresponding nonchimeric cluster using the USEARCH global alignment algorithm (Edgar, 2010).

To determine the identity of each remaining sequence, the sequences were quality checked and demultiplexed using the denoised data previously generated. Sequences that passed the quality control screening were then clustered into OTUs using the UPARSE algorithm (Edgar, 2013). Each of the original reads was then assigned back to their OTUs using the USEARCH global alignment algorithm (Edgar, 2010). The centroid sequence from each cluster was run against the USEARCH global alignment algorithm along with a database

of high quality sequences derived from NCBI and maintained by Research and Testing Laboratory. 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 agree with the best match at that level and dividing that by the number of high quality sequence matches that were found. Each OTU was then assigned 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. Data obtained provided relative abundance information within and among individual samples. 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. In bacteria, the rRNA operon is frequently found in multiple copies (1 to 15; Stoddard et al., 2015). Therefore, the community structure can be biased as one may obtain sequences with a lesser abundance but high number of reads (multiples copies of the 16S gene), or a higher abundance but low number of reads (single copy of the 16S gene). To remove this bias, the relative abundances of reads by taxonomic level for each library have been normalised by the average number of copies of the rRNA operon of each taxonomic level using the database freely available at https://rrndb.umms.med.umich.edu/.

When the taxonomy of an OTU was not assigned by using the protocol mentioned above (resulted as either in "Unclassified", in "Unknown" or in "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 at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Chapter 5

KINETIC AND MICROBIOLOGICAL CHARACTERIZATION OF AEROBIC GRANULES PERFORMING PARTIAL NITRITATION OF A LOW-STRENGTH WASTEWATER AT 10 °C

A modified version of this chapter has been published as:

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. doi:10.1016/j.watres.2016.05.059

Abstract

A granular sludge airlift reactor enriched in ammonia oxidizing bacteria (AOB) was operated at 10 ºC performing stable partial nitritation in the long-term. The reactor treated a synthetic low-strength influent during 250 days with an average nitrogen loading rate of 0.63 ± 0.06 g N L^{-1} d^{-1}. Nitrate production was barely detected, being the average concentration in the effluent of 0.6 ± 0.3 mg N-NO_3 L^{-1}. Furthermore, a suitable effluent for a subsequent reactor performing the anammox process was achieved. A maximum specific growth rate as high as 0.63 ± 0.05 d^{-1} was determined by performing kinetic experiments with the nitrifying granular sludge in a chemostat and fitting the results to the Monod model. Pyrosequencing analysis showed a high enrichment in AOB (41 and 65 % of the population were identified as *Nitrosomonas* genus on day 98 and 233, respectively) and an effective repression of nitrite oxidizing bacteria in the long-term. Pyrosequencing analysis also identified the coexistence of nitrifying bacteria and heterotrophic psychrotolerant microorganisms in the granular sludge. Some psychrotolerant microorganisms are producers of cryoprotective extracellular polymeric substances that could explain the better survival of the whole consortia at cold temperatures.

5.1. INTRODUCTION

Nitrogen removal is essential in urban wastewater treatment plants (WWTPs) since nitrogenous compounds are toxic to aquatic life and cause eutrophication and oxygen depletion in receiving waters. Conventional activated sludge systems are the most frequently used systems in urban WWTPs since a good removal of pollutants is guaranteed, however the costs associated to this typical biological treatment make WWTPs as very energy-demanding facilities. For the achievement of a cost-effective (energy-neutral or even energy-positive) urban WWTP, the implementation of the autotrophic biological nitrogen removal (BNR) in the mainstream has been proposed (Jetten et al., 1997; Kartal et al., 2010; Siegrist et al., 2008). Thus, aeration costs are reduced because of the lower oxygen requirements of the process compared to conventional activated sludge treatment; and furthermore, biogas production is increased since most of the organic matter will be converted to biogas in the anaerobic digestion process, with the consequent energy recovery.

Recently, many studies were focused on the implementation of autotrophic BNR in one-stage systems, such as CANON (Completely Autotrophic Nitrogen removal Over Nitrite) and OLAND (Oxygen-Limited Autotrophic Nitrification/Denitrification) technologies. Nevertheless, at low temperature and low-strength wastewaters most of these systems showed the failure of nitritation in the long-term operation, due to the growth of nitrite oxidizing bacteria (NOB) triggering the production of nitrate and the destabilization of the subsequent anammox process (De Clippeleir et al., 2013; Hu et al., 2013; Wett et al., 2013; Winkler et al., 2011). Even though Gilbert et al. (2014) reported stable operation at 10 ºC with synthetic low-strength wastewater in a one-stage system, the achieved ammonium conversion rate resulted as low as 0.015 g N L^{-1} d^{-1}. Hence, two-stage systems appear as the alternative to overcome the destabilization problems and the low conversion rates associated to one-stage systems (Ma et al., 2011; Pérez et al., 2015; Regmi et al., 2014). Separation of the partial nitritation and the anammox processes in two different reactors makes possible a more stable performance and control. In fact, stable partial nitritation at 12.5 ºC with a granular sludge reactor was reported by Isanta et al. (2015a) and long-term operation of an anammox reactor at temperatures between 20 and 10 ºC was reported by Lotti et al. (2014b). Both of these studies treated low-strength wastewater, demonstrating the feasibility of application of autotrophic BNR to mainstream conditions.

For both, one and two-stage approaches, successful implementation of autotrophic BNR at mainstream conditions relies on the stability of partial nitritation in the long-term, i.e. by achieving an effective repression of NOB activity. Previous research has shown a more sensitive temperature dependence of ammonia oxidizing bacteria (AOB) compared to that of NOB (Hunik et al., 1994; Knowles et al., 1965; Van Hulle et al., 2010). Thus, different strategies have been conducted in order to favour AOB over NOB activity at mainstream conditions. On one hand, Gao et al. (2014) proposed an aeration control strategy depending on the temperature and ammonia concentration in the influent. Efficient NOB repression was obtained at room temperature (12-27 ºC) but temperature fluctuated daily, being lower than 15 ºC less than 10 days. On the other hand, Isanta et al. (2015a) achieved stable partial nitritation for 300 days at 12.5 ºC in a granular sludge system, by maintaining an adequate ratio between oxygen and ammonium concentrations in the reactor bulk liquid. However, in northern climates, temperature can easily achieve values lower than 12.5 ºC during winter. In fact, average temperatures of wastewater in west European region are around 17 ºC, with a minimum of 8 ºC and a maximum of 29 ºC (De Clippeleir et al., 2013), and thus, a temperature gradient from 20 ºC in summer to 10 ºC in winter was presented as representative for WWTPs in moderate climates (Gilbert et al., 2015).

In the present study the first objective was to demonstrate the long-term stability of partial nitritation at 10 ºC for low-strength synthetic wastewater in a granular sludge reactor operated in continuous mode. Furthermore, a better understanding of the process through the in depth study of the nitrifying biomass of the granular sludge reactor was aimed. Thus, the second objective was to characterize the population developed at low temperature in the reactor from both microbiological and kinetic points of view. Finally, the special characteristics of the biomass with the nitrifying ability of the granular reactor at 10 ºC were correlated.

5.2. MATERIALS AND METHODS

5.2.1. Reactor set-up and operation

A lab-scale airlift reactor with a total working volume of 5.2 L was used. The detailed diagram of the reactor and set-up details are described in Section 4.1.1., Chapter 4. Compressed air was supplied through an air diffuser placed at the bottom of the reactor and

was manually manipulated to maintain the dissolved oxygen (DO) concentration in the bulk liquid in the range 0.5-2.5 mg O_2 L^{-1}. The pH was measured on-line and automatically controlled at 8.0 ± 0.1 by dosing a Na_2CO_3 0.5 M solution. The pH was controlled throughout the operation period to rule out any potential effects derived from pH changes. Since the effect of pH on nitritation rates is known to be reduced in the range 7.5-8, a pH set point of 8 was selected, as done in a previous study (Isanta et al., 2015a). The temperature was measured and controlled at 10 ºC. Total ammonia nitrogen (TAN = $N\text{-}NH_4^+$ + $N\text{-}NH_3$) and nitrate concentrations in the bulk liquid were measured on-line. TAN concentration in the bulk liquid was automatically controlled by varying the inflow rate by means of a proportional controller during the whole period of operation, except between days 93-95, 144-172 and 241-245 when the control was manually made based on the off-line bulk liquid TAN concentration measurement.

5.2.2. Inoculum and influent characteristics

The reactor treated a synthetic influent with an average TAN concentration of 70 mg N L^{-1}, which mimics a pretreated municipal wastewater coming from the mixture of the effluent of a previous A-stage plus the recirculation of the reject water of the digested sludge, as in an anammox-based WWTP (Isanta et al., 2015a; Kartal et al., 2010). The synthetic influent also contained: 45 mg L^{-1} KH_2PO_4, 784 mg L^{-1} $NaHCO_3$, 80 mg L^{-1} NaCl, 40 mg L^{-1} $CaCl_2$, 90 mg L^{-1} $MgCl_2$ and 1 mL of trace elements solution per L of influent (Guerrero et al., 2011).

The biomass was enriched in AOB and adapted to low temperature (12.5 ºC) in a reactor which was operating for more than 400 days performing stable partial nitritation (Isanta et al., 2015a). Hence, the inoculum contained around 81 ± 12 % of AOB and 1 ± 1 % of NOB as analyzed by fluorescent *in situ* hibridization (FISH).

5.2.3. Kinetic experiments

Kinetic experiments were conducted in a chemostat with a working volume of 2.9 L (Fig. 5.1). For each experiment, the chemostat was inoculated with nitrifying granules from the continuous airlift reactor to a final concentration of 83 ± 3 mg VSS L^{-1}. The same synthetic wastewater of the reactor was used as influent to carry out the kinetic experiments. DO was measured in the bulk liquid and it was maintained in excess to avoid oxygen limitations (around 9 mg O_2 L^{-1}). Biomass was mixed both by mechanical stirring at 100 rpm

(Stirrer type BS, VELP Scientifica, Italy) and bubbling of air to avoid mass transfer limitations. The pH was monitored and controlled at 7.5 by using an ON/OFF control system by automated addition of 1 M NaOH with an automatic dispensing burette (Multi-Burette 2S-D, Crison Instruments, Spain). Temperature was maintained at 10 ºC by means of a cooling system (E100, LAUDA, Germany), which provided cooled water through the jacket of the chemostat. The measurement of the particle size of the biomass of both the effluent and the reactor confirmed that biomass was not retained in the reactor and consequently the operation was as a chemostat (Fig 5.2).

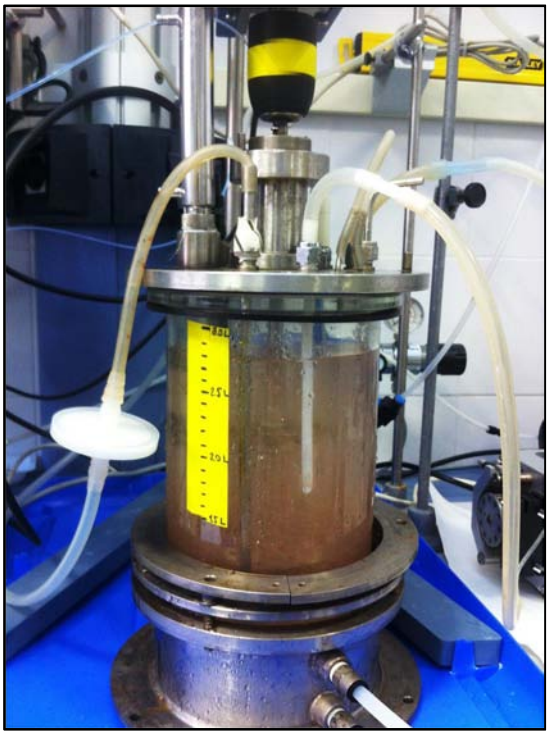

Fig. 5.1. Image of the lab-scale chemostat used for the kinetic experiments.

Taking into account that dilution rate is equal to growth rate (μ) in a chemostat, the growth rate was fixed by varying the dilution rate, and thus the inflow. The chemostat was operated continuously and experiments were finished when steady state conditions were achieved, that is, when TAN concentration in the effluent was constant. Five experiments were carried out at different growth rates ranging from 0.36 to 0.56 d^{-1}, and a value of TAN concentration at steady state conditions was obtained for each experiment.

Kinetic parameters (maximum specific growth rate, μ_{max}, and TAN affinity constant, $K_{S,TAN}$) were determined by fitting the data of the experiments to the Monod equation (Eq. 5.1).

$$\mu = \mu_{max} \frac{[TAN]}{K_{S,TAN} + [TAN]} \quad \text{(Eq. 5.1)}$$

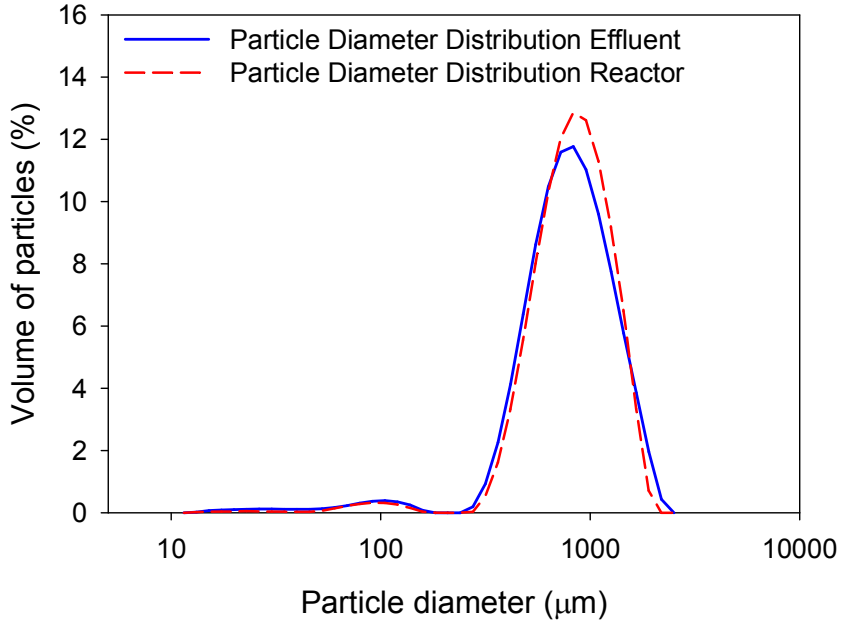

Fig. 5.2. Particle size distribution of the nitrifying biomass in the chemostat and in the effluent for one kinetic experiment at steady state conditions.

5.2.4. Fluorescence in situ hybridization (FISH)

Abundances of AOB and NOB were analysed by FISH coupled to confocal laser scanning microscopy (CLSM). Regarding AOB, specific probes for *Nitrosomonas* spp. and *Nitrosospira* spp. were 5'-6FAM-labeled and 5'-Atto550-labeled, respectively. Regarding NOB, specific probes for *Nitrobacter* spp. and *Nitrospira* spp. were 5'-Cy3-labeled and 5'-6FAM-labeled, respectively. The general probe for all microorganisms was 5'-Cy5-labeled. Hybridization protocol and probes are fully described in Section 4.3.1 of Chapter 4.

5.2.5. Pyrosequencing analysis

Identification of the microbial population was performed using next-generation sequencing at samples from days 98 and 233 of the reactor operation. DNA extraction, pyrosequencing settings and bioinformatics applied are described in Section 4.3.2, Chapter 4.

Bacterial 16S rRNA variable regions V2-V4 were targeted using the primer pair 341F-907R. For bacteria biodiversity analysis and phylogenetic classification reads shorter than 100 bps and larger than 566 bps were trimmed, and the followed methodology is explained in detail in Section 4.3.2 of Chapter 4. Relative abundances of reads were determined by taxonomic level. Indices of biological diversity (Shannon), richness (Chao), and rarefaction curves were calculated for all libraries at 97, 95 and 90% of similitude. Table AI.1.1 and Figs. AI.1.1 and AI.1.2 in the Annex I – Section I show the indices of biological diversity and rarefaction curves, respectively. All these results indicate the libraries were comparable in terms of abundance percentages and that good coverage of diversity was reached.

5.2.6. Scanning electron microscopy

A biomass sample of 8–10 granules was fixed in 2.5% (v/v) glutaraldehyde and 0.1 M phosphate buffer (pH 7.4) for 2 h at 4 °C, washed 4 times for 10 min each time in 0.1 M phosphate buffer, fixed in 1% (wt/v) osmium tetraoxide with 0.7% ferrocyanide in phosphate buffer, washed in water, dehydrated in an ascending ethanol series (50, 70, 80, 90, and 95% for 10 min each and twice with 100% ethanol), and dried at critical-point with CO_2. Then, the sample was metalized with Au-Pd and observed by using a scanning electron microscope (EVO MA10; Zeiss, Germany) at the following conditions: 20 kV, 100 pA, secondary electron detector (SE1).

5.2.7. Specific analytical methods

TAN, total nitrite nitrogen (TNN) and nitrate concentrations were measured off-line according to Section 4.2.1, Chapter 4. These measured off-line values are the ones represented in the results section. Solid retention time (SRT) was estimated by dividing the amount of VSS in the reactor by the sludge washed out with the effluent (Eq. 5.2).

$$SRT = \frac{[VSS]_{reactor} * V_{reactor}}{[VSS]_{effluent} * Q_{effluent}} \quad \text{(Eq. 5.2)}$$

where, $[VSS]_{reactor}$ and $[VSS]_{effluent}$ are the VSS concentrations in the reactor and the effluent, respectively, $V_{reactor}$ is the reactor volume and $Q_{effluent}$ is the effluent flow rate.

Average particle size was measured by a laser particle size analysis system (Malvern Mastersizer Series 2600, Malvern instruments Ltd., UK). The off-gas of the reactor was periodically collected and analyzed with gas chromatography (Agilent Technologies 6890 N Network GC system, Madrid, Spain) to measure N_2O emissions.

5.3. RESULTS AND DISCUSSION

5.3.1. Long-term operation at 10 ºC

The reactor was previously operated at 12.5 ºC, with an average NLR of 0.7 ± 0.3 g N L^{-1} d^{-1}, for more than 400 days performing stable partial nitritation before the temperature was directly lowered to 10 ºC (Isanta et al., 2015a). After the decrease in temperature (day 0), the reactor was operated during 250 days with an average nitrogen loading rate (NLR) of 0.63 ± 0.06 g N L^{-1} d^{-1}. Stable partial nitritation was maintained in the long-term at 10 ºC (Fig. 5.3), which was achieved by applying a ratio of DO/TAN concentrations in the bulk liquid of 0.04 ± 0.02 mg O_2 mg^{-1} N. Low DO/TAN concentrations ratio was reported before to maintain stable partial nitritation in granular systems (Bartrolí et al., 2010; Isanta et al., 2015a; Jemaat et al., 2013). Efficiency of the NOB repression is thought to be linked to the fact that a steep oxygen gradient is present in the granular sludge (Bartrolí et al., 2010; Isanta et al., 2015a). Therefore, direct extrapolation of this strategy to other systems, such as flocculent sludge reactors in which sludge retention is assured by other means, it is not straightforward but might be object of future research. Nitrate production was barely detected, being the average concentration in the effluent of 0.6 ± 0.3 mg N-NO_3^- L^{-1}. Furthermore, a suitable effluent for a subsequent reactor performing the anammox process was achieved, with an average TNN/TAN concentrations ratio of 1.1 ± 0.2. The ammonium oxidation rate (AOR) was maintained stable during the whole operation, with an average value of 0.34 ± 0.06 g N L^{-1} d^{-1}. This value is considerably high compared to the one obtained in one-stage biofilm systems. Thus, Gilbert et al. (2015) reported an AOR lower than 0.02 g N L^{-1} d^{-1} at 10 ºC and Hu et al. (2013) reported an AOR of 0.03 g N L^{-1} d^{-1} at 12 ºC.

Particle size was maintained stable during the whole period of operation (Fig. 5.3A) with an average value of 810 ± 70 μm. From day 50 onwards, the biomass concentration increased to an average value of 3.6 ± 0.1 g VSS L^{-1}, as shown in Fig. 5.3A. In spite of the high and constant NLR and AOR achieved in the granular airlift reactor, specific rates (specific nitrogen loading rate, sNLR; specific ammonium oxidation rate, sAOR) decreased during the first 100 days at 10 °C (Fig. 5.3C). However, sAOR remained constant from day 100 with an average value of 0.18 ± 0.03 g N mg^{-1} VSS d^{-1}. This fact demonstrated that biomass maintained the same activity during 150 days of operation at 10 ºC.

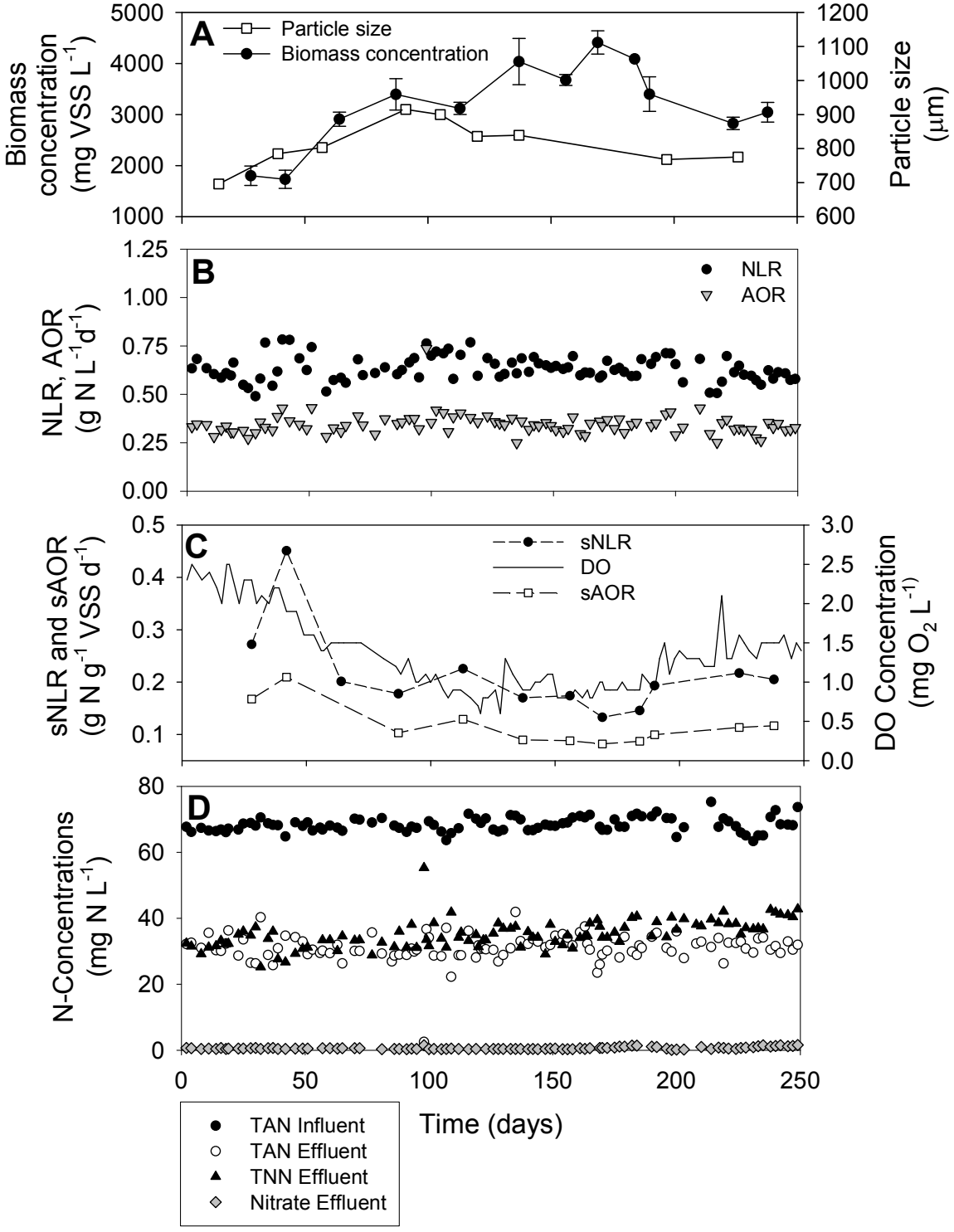

Fig. 5.3. Continuous operation of the granular airlift reactor treating a synthetic low-strength wastewater at 10 °C. (A) Biomass concentration and particle size; (B) Nitrogen loading rate (NLR) and ammonium oxidation rate (AOR); (C) Specific nitrogen loading rate (sNLR), specific ammonium oxidation rate (sAOR) and dissolved oxygen concentration (DO); (D) Nitrogen compounds concentrations throughout the operation of the granular reactor.

Between days 93–95, 144–172 and 241–245 the ammonium control was switched from automatic to manual, but the process remained stable. Moreover, on day 98 the reactor remained without feeding for 4 hours (NLR of 0 g N L^{-1} d^{-1}) which resulted in an increase of DO and complete oxidation of ammonium to nitrite by AOB. Despite of the high concentration of TNN and DO, the nitrate in the bulk liquid was only 2.2 mg N-NO_3^- L^{-1} and the next day the system was totally recovered. Hence, the successfully repression of NOB in the system was demonstrated and thus, the stability of this technology.

The balance of nitrogen during the operation of the reactor was fulfilled, with an average value of 96 ± 6% (Fig. 5.4). Hence, neither heterotrophic nor autotrophic (anammox process) denitrification was considered to take place in the granular airlift reactor.

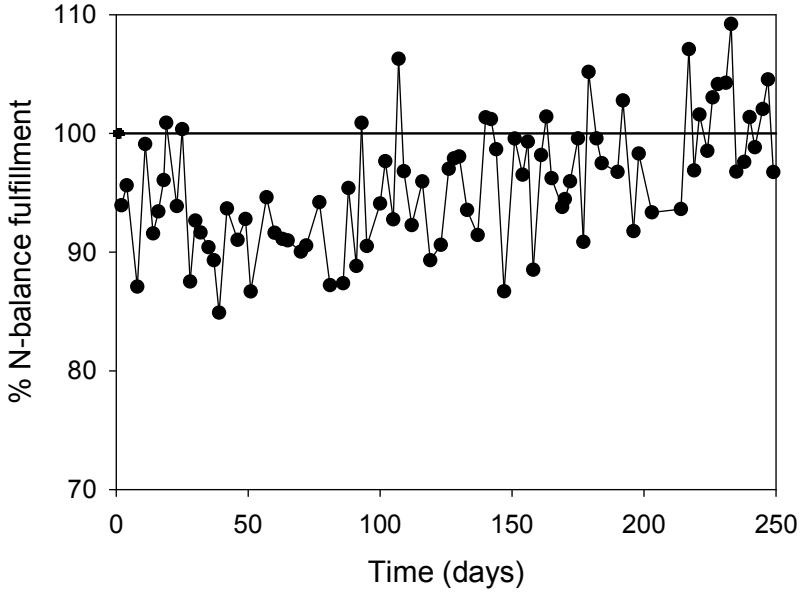

Fig. 5.4. Fulfillment of the nitrogen balance during the operation of the granular airlift reactor treating synthetic low-strength wastewater at 10 °C. The continuous line indicates the 100% fulfillment of the N-balance.

Off-gas samples from days 94, 95, 241, 242 and 245 were analyzed in order to calculate the N_2O emission factor of the reactor. As it is shown in Table 5.1, less than 0.35% of the TAN in the influent was emitted as N-N_2O. Furthermore, from the converted nitrogen the average emitted as N-N_2O was 0.36 ± 0.07%. Thus, the N_2O emissions from the granular airlift reactor performing partial nitritation of a low-strength wastewater at 10 °C were very low, even at low DO concentrations (1.3 ± 0.3 mg O_2 L^{-1}) which is known to trigger high N_2O emissions (Kampschreur et al., 2009). Applying the same control strategy but treating a reject

water from the dewatering of digested sludge (high-strength wastewater) at 30ºC, the N_2O emission factor was as high as 6% of the TAN oxidized at a DO of 1 mg O_2 L^{-1} (Pijuan et al., 2014), which means more than one order of magnitude higher than the reported in this study. Hence, the temperature could be an important factor affecting N_2O emissions, the lower the temperature the lower the emissions. Nevertheless further experiments are necessary to confirm this hypothesis, since other factors could cause the difference between the results of Pijuan et al. (2014) and this study.

Table 5.1. N_2O emission factors during the operation of the granular airlift reactor treating a synthetic low-strength wastewater at 10 °C.

Day	N-N_2O (% of N-influent)	N-N_2O (% of N-oxidized)
94	0.13 ± 0.01	0.23 ± 0.02
95	0.32 ± 0.04	0.58 ± 0.08
241	0.23 ± 0.04	0.40 ± 0.08
242	0.17 ± 0.06	0.30 ± 0.10
245	0.14 ± 0.02	0.28 ± 0.04

5.3.2. Kinetics

As it was mentioned before, the granular airlift reactor not only achieved stable partial nitritation at 10 ºC but also operated at higher NLR than other similar systems. Nitrifying capacity is related to the growth rate of AOB community and, hence, a nitrifying sludge with an unusually high maximum growth rate could explain the high activity in this airlift reactor.

The results of the five kinetic experiments carried out in a chemostat reactor are presented in Table 5.2. An increase in the applied growth rate caused an increase in the TAN concentration at steady state conditions, which follows satisfactorily (R^2 = 0.97) the Monod kinetic model (Fig. 5.5). Hence, the maximum specific growth rate and TAN affinity constant were obtained by fitting the data achieved in each experiment to the Monod kinetic model. Thus μ_{max} and $K_{S,TAN}$ resulted in 0.63 ± 0.05 d^{-1} and 2.1 ± 0.7 mg N L^{-1}, respectively.

Table 5.2. Operational parameters of the kinetic experiments in the chemostat at steady state conditions. All the experiments were conducte with the same influent of the main reactor at pH = 7.5 ± 0.1, DO = 9.3 ± 0.1 mg O_2 L^{-1} and T = 10 °C.

Number of Experiment	Experiment duration (days)	μ (d^{-1})	Inflow (L d^{-1})	$[TAN]_{effuent}$ (mg N L^{-1})	$[TNN]_{effluent}$ (mg N L^{-1})	$[N\text{-}NO_3^-]_{effluent}$ (mg N L^{-1})
1	13	0.55	1.60 ± 0.01	26.3 ± 0.7	49.0 ± 2.0	1.9 ± 0.1
2	10	0.34	1.00 ± 0.01	3.4 ± 0.2	30.0± 4.0	36.0 ± 1.0
3	13	0.53	1.53 ± 0.01	10.2 ± 0.6	47.0 ± 2.0	10.8 ± 0.4
4	9	0.56	1.62 ± 0.01	10.0 ± 1.0	56.0 ± 2.0	12.0 ± 1.0
5	16	0.45	1.30 ± 0.01	4.3 ± 0.4	34.0 ± 9.0	31.0 ± 7.0

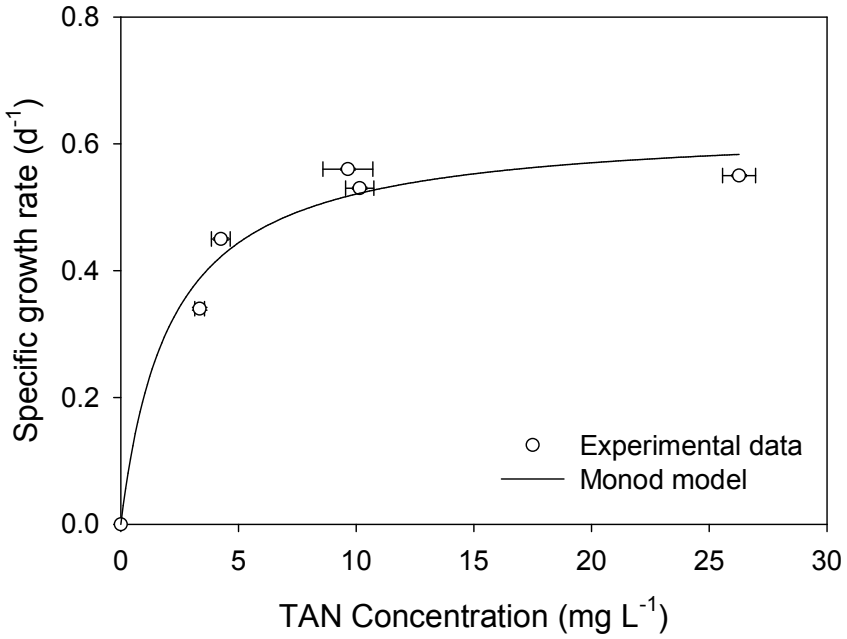

Fig. 5.5. TAN oxidation kinetics for the nitrifying granules. (○) TAN concentration at steady state conditions for each specific growth rate imposed.

Different values of maximum growth rate have been reported up to now, being most of them determined at high temperatures (20–30 ºC) (Blackburne et al., 2007; Esquivel-Rios et al., 2014; Vadivelu et al., 2006). However, large discrepancies were found between different studies. The large variety of parameter values found in literature lies in the differences of systems evaluated, operational conditions applied, biomass growth types and the techniques used to determine the parameters themselves. Vannecke and Volcke (2015) presented a literature review on microbial characteristics of nitrifiers and reported μ_{max} in the range of 0.34–3.40 d^{-1} for attached growth of AOB at 30 ºC and pH 7.5. For suspended growth, Farges et al. (2012) used the flow cytometry technique to study the growth of *Nitrosomonas europaea* in pure cultures at 26 ºC and pH 8 and μ_{max} resulted in the range of 0.13–0.23 d^{-1}. On the other hand, Chandran et al. (2008) obtained higher values (μ_{max} = 0.24–0.74 d^{-1}) by using respirometric batch tests and substrate depletion assays in continuous reactors for an enriched nitrifying culture at 25 ºC and pH 7.4.

It is well known that growth rate decreases considerably with decreasing temperature. Knowles et al. (1965) reported a decrease in the μ_{max} from 1.5 to 0.2 d^{-1} when temperature decreased from 27 to 8.3 ºC for *Nitrosomonas* sp. in samples from Thames estuary, London, England; and afterwards, Sözen et al. (1996) determined μ_{max} in the range of 0.10-0.17 d^{-1} for

a nitrifying mixed culture treating real urban wastewater at 10 ºC. In spite of this, little has been published about kinetic parameters of nitrifying mixed cultures at low temperature, and in any case, the μ_{max} values reported were much lower than the one achieved in the current study (μ_{max} = 0.63 ± 0.05 d^{-1} at 10 ºC). In fact, to the best of the authors' knowledge, this is the highest growth rate achieved by a nitrifying sludge enriched in AOB at 10 ºC. Furthermore, an estimation of the μ_{max} of the nitrifying sludge at higher temperatures was calculated by considering an Arrhenius-type equation ($\mu_{1,T_1} = \mu_{2,T_2}\ \theta^{(T_1-T_2)}$) and a temperature coefficient of θ = 1.13 ± 0.03 which was determined in Isanta et al. (2015a) for the inoculum of the current airlift reactor. The values obtained for μ_{max} were 2.1 ± 0.2, 3.9 ± 0.3 and 7.3 ± 0.6 d^{-1} at 20, 25 and 30 ºC, respectively. Thus, the nitrifying biomass of the granular airlift reactor presented the higher μ_{max} than has been reported hitherto at any temperature. A nitrifier culture with such a high μ_{max} could explain the high NLR and AOR achieved in the operation of the granular airlift reactor at 10 ºC. A second implication is that the enrichment of an AOB population with such a high μ_{max} would be an advantage for NOB repression at low temperatures, since it would help to keep AOB growth rate higher than that of NOB.

Along with the high μ_{max} obtained, a high value for the TAN affinity constant was determined from the kinetic experiments ($K_{S,TAN}$ = 2.1 ± 0.7 mg N L^{-1}) compared to the previously reported by Chandran et al. (2008) at 25 ºC ($K_{S,TAN}$ = 0.21–0.69 mg N L^{-1}), Knowles et al. (1965) at 8.3 ºC ($K_{S,TAN}$ = 0.2 mg N L^{-1}) and the proposed in the Activated Sludge Model 2d at 10 ºC ($K_{S,TAN}$ = 1 mg N L^{-1}; Henze et al, (2000)).

From the ecological concept, a microorganism showing quick growth on easily available substrate is defined as r-strategist microorganism (Andrews and Harris, 1986), which applied to the kinetic context represents a microorganism with high maximum specific growth rate and high substrate affinity constant (Andrews and Harris, 1986; Martín-Hernández et al., 2009). Therefore, the nitrifier population of the granular airlift reactor can be considered as r-strategist. The enrichment of a r-strategist AOB population has been reported when high residual ammonium concentrations are used (Terada et al., 2013). Hence, the high residual ammonium concentration may be also a key factor for the enrichment of r-strategist AOB population in two-stage partial nitritation/anammox reactor systems, like the one presented in this study.

5.3.3. Microbial characterization

FISH-CSLM was used to evaluate the enrichment in AOB and the presence of NOB in the granular sludge performing partial nitritation at 10 ºC. On day 233, 92 ± 4% of the population was quantified as AOB, and less than 1 ± 1% as NOB (specifically *Nitrobacter* spp.*)*. Since the inoculum contained 81 ± 12% of AOB and 1 ± 1% of *Nitrobacter* spp., a high enrichment in AOB and an effective repression of NOB was maintained in the long-term at 10 ºC although NOB were always present in the biomass.

On the other hand, neither *Nitrosospira* spp. (species belonging to AOB) nor *Nitrospira* spp. (species belonging to NOB) hybridizations were detected in the sludge. This fact was expected since they are k-strategist microorganisms and, consequently, they are not favored at high TAN and TNN concentrations (Kim and Kim, 2006), such as those in the reactor of this study.

Moreover, pyrosequencing technique was used to examine the microbial community through the operation of the granular reactor at 10 ºC. With that purpose, samples on days 98 and 233 were analyzed.

On sample from day 98, *Betaproteobacteria* was clearly the most abundant class of the total reads, with a relative abundance of 52% (Fig 5.6). It is widely known that *Betaproteobacteria* class comprises autotrophic nitrifying microorganisms, such as AOB and NOB, and also denitrifying bacteria and organic matter decomposing bacteria. Thus, it is expected that *Betaproteobacteria* was the most abundant class in an AOB enriched sludge, such as the one of this study. *Alphaproteobacteria* was the second class in order of abundance, with a value of 23%, following by *Actinobacteria* and *Gammaproteobacteria* representing the 7 and 5% of total reads, respectively, among other classes of heterotrophs less abundant in the sample. These values of heterotrophic classes are in the range of the observed by Kindaichi et al. (2004), with 23% of *Alphaproteobacteria* and 13% of *Gammaproteobacteria* quantified in an autotrophic nitrifying biofilm system operating at 25 ºC and with a high-strength synthetic wastewater.

On sample from day 233, corresponding to long-term operation of the granular reactor at 10 ºC, *Betaproteobacteria* increased their relative abundance to 68%, followed by the increasing of *Cytophagia* with a 15%; while *Alphaproteobacteria* sharply decreased to 4% (Fig. 5.6). There was a 10% of reads not identified at class level, and most of them comprised

the phylum *Bacteroidetes*. In fact, *Bacteroidetes* abundance increased significantly compared to day 98, being the phylum with the highest increase (Fig. 5.7).

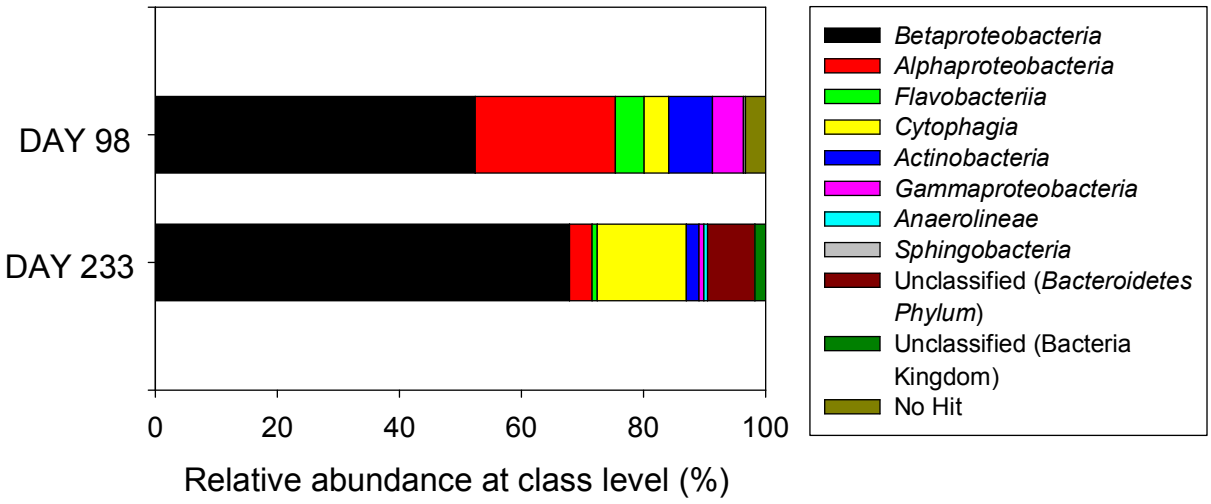

Fig. 5.6. Microbial diversity at class level. Relative abundance was calculated only considering those microorganisms in which the number of 16S copies was higher than 0.5% of the total copies. (See Annex I for detailed results and rarefaction curves).

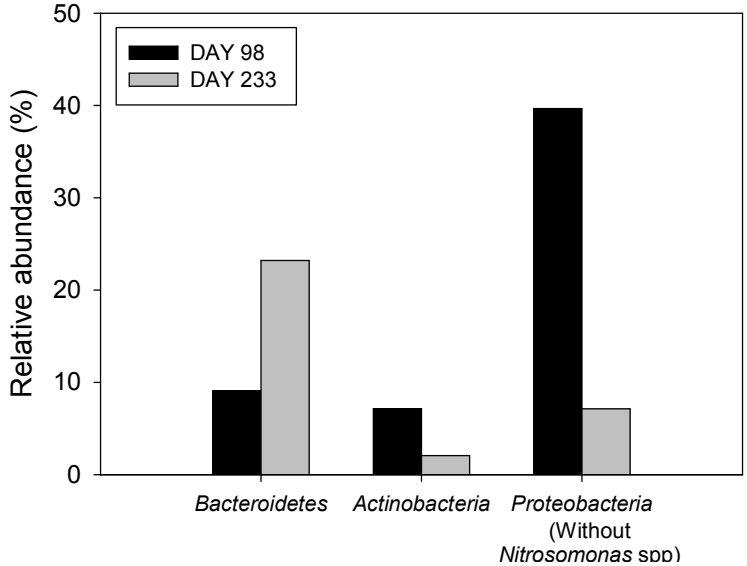

Fig. 5.7. Microbial dynamics at phylum level between days 98 and 233. Proteobacteria includes all the phylum except the corresponding to *Nitrosomonas* genus which is the genus enriched in the sludge.

At genus level, in the biomass community of day 98 (Fig. 5.8), *Nitrosomonas* was the most abundant genus, which was expected since the sludge was enriched in AOB and *Nitrosomonas* is the most frequently genus of AOB found in wastewater treatment systems (Wagner et al., 2002; Wang et al., 2012). Thus, *Nitrosomonas* genus counted up the 41% of the total population, indicating a majority of AOB in the sludge. Since *Nitrosomonas* genus comprises r-strategist microorganisms (Terada et al., 2013), their high abundance in the nitrifying sludge agreed with the high values of μ_{max} and $K_{S,TAN}$ obtained from kinetic experiments. Besides, *Nitrosospira* genus was also detected in the sample with a 7% of relative abundance, in spite of *Nitrosospira* spp. were never detected by FISH technique. This may be due to the fact that FISH technique points toward the abundance of rRNA in samples, while pyrosequencing points toward the abundance of DNA (Wittebolle et al., 2005). Thus, *Nitrosospira* spp. could be not detected by FISH because their probably low or null activity in the reactor, but their DNA could be still detected. Regarding NOB, 1.4% of the total population was identified as *Nitrobacter* genus, which agrees with the production of nitrate in the reactor and with the result of the FISH analysis. Thus, *Nitrobacter* spp. were not abundant in the reactor, but active. Finally, *Nitrospira* genus was not detected in the sample, in agreement with the FISH analysis.

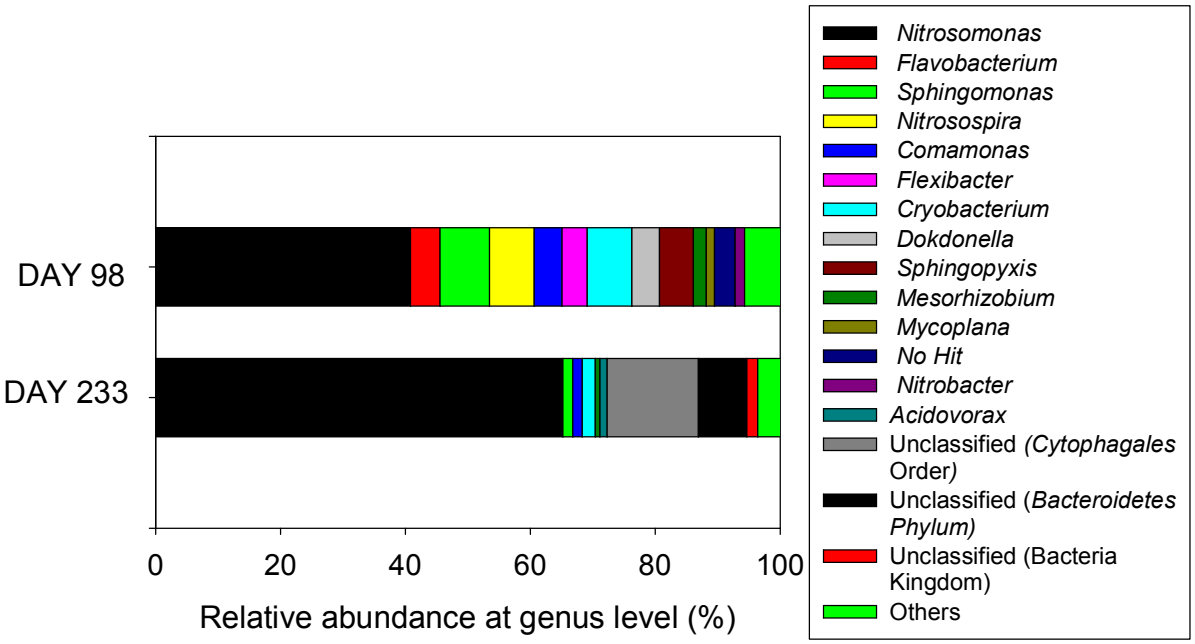

Fig. 5.8. Microbial diversity at genus level. Relative abundance was calculated only considering those microorganisms in which the number of 16S copies was higher than 0.5% of the total copies. (See Annex I for detailed results and rarefaction curves).

Other genera were detected in a low abundance, but in a more or less equal proportion between them. High bacteria richness is expected in mixed cultures and the coexistence and interaction of heterotrophic bacteria and autotrophic nitrifiers were reported before (Ducey et al., 2010; Kindaichi et al., 2004; Okabe et al., 2005). In this sense, genera as *Sphingomonas* and *Dokdonella*, with a relative abundance in the sample of 8 and 4% respectively, were reported as heterotrophic, or even autotrophic nitrifiers (Fitzgerald et al., 2015). Moreover, two other genera, *Cryobacterium* and *Flavobacterium*, with several species known to be either psychrotolerant (*Cryobacterium psychrotolerans*, Zhang et al. (2007); *Flavobacterium gelidilacus*, Van Trappen et al. (2003)) or even psychrophilic (*Cryobacterium* sp. MLB-32, Singh et al. (2015)) microorganisms, were identified in the sample with 7 and 5 % of the total reads, respectively.

On day 233 at genus level (Fig. 5.8), enrichment in *Nitrosomonas* genus was observed to the detriment of the rest of genera. Moreover, *Nitrosomonas* genus counted the 65% of the total reads in the sludge sample, being at day 233 the 96% of all the *Betaproteobacteria* while at day 98 this percentage was of 78%. Thus, the sludge was much more enriched in AOB at day 233 than at the beginning of the operation at 10 ºC. *Nitrosospira* genus was present in the sample in less than 0.5% of abundance, so it was not considered for the data treatment. Since *Nitrosospira* was found on day 98 (7% of the reads), pyrosequencing analysis confirmed its wash-out from the granular airlift reactor operating at 10 ºC. Furthermore, the absence of *Nitrosospira* genus at day 233 confirms that *Nitrosospira* were not active on day 98 (no detected by FISH) and, hence, they were washed out of the reactor. Besides, since the reactor was operated at high solid retention time (80 ± 20 days) the wash-out was slow. There may be two reasons for the wash-out of *Nitrosospira* genus in the granular airlift reactor. The first one is that *Nitrosospira* spp. were not favoured at the operating conditions of the reactor since they are k-strategist microorganisms (low TAN affinity constant and low specific growth rate) while *Nitrosomonas* spp. are r-strategists (high TAN affinity constant and high specific growth rate). The second possible explanation is that *Nitrosospira* spp. are more sensitive to temperature than *Nitrosomonas* spp. (Hoang et al., 2014; Park et al., 2008).

Neither *Nitrobacter* nor *Nitrospira* genera were identified by pyrosequencing in the sample of day 233, in spite of being detected with the FISH analysis (with an abundance of 1 ± 1 %). Probably this could be due to poor or null amplification of a low DNA content of these bacteria in the sample. In any case, successful NOB repression in the granular airlift reactor operating at 10 ºC was demonstrated.

As it is shown in Fig. 5.8, the second genus in order of abundance with a 15% of the total reads was unclassified at genus level (classified at order level as *Cytophagales*), however its DNA sequence was ran against BLAST and matched the one found by Larose et al. (2010) in bacteria present in snow and melt water samples from Svalbard, Norway. Therefore, the corresponding microorganism will probably be a cold-adapted microorganism (psychrotolerant or psychrophilic species) and its presence in cold waters would fit with the presence in the 10 ºC system of this study.

In general terms, in addition to the enrichment in AOB, three main points can be extracted from the results obtained by pyrosequencing in this study. The first one is that the diversity of the bacterial community decreased in the long-term of operation at 10 ºC. The second one is that despite the fact that the influent of the reactor was devoid of an organic carbon source, a considerable part of the population in both samples was composed by heterotrophic bacteria. Finally, the third one is the presence of psychrotolerant microorganisms in the sludge performing partial nitritation at 10 ºC.

As shown in Table 5.3, there were more genera with abundances higher than 5% on day 98 than on day 233. The decrease in bacterial diversity with cold temperature was reported before (Karkman et al., 2011). Thus, not only non-adapted microorganisms to cold temperatures diminished in the long-term operation, but also diversity of psychrotolerant genera (the unclassified microorganism mentioned before appears to the detriment of *Cryobacterium* and *Flavobacterium*). Only *Nitrosomonas* and the unclassified genera (one of them corresponding to the cold-adapted microorganism mentioned before) were identified with abundance superior to 5% on day 233.

The coexistence of nitrifying and heterotrophic bacteria in absence of an external organic carbon source has also been reported before (Ducey et al., 2010; Hoang et al., 2014; Karkman et al., 2011). It is known that nitrifiers produce organic matter from biomass decay and substrate metabolism which is used by heterotrophs to survive. Nogueira et al. (2005) correlated the presence of heterotrophs in nitrifying biofilm reactors with the hydraulic retention time (HRT) and determined that values of HRT in the range of the one used in this study (2.5 ± 0.3 h) guarantees enough soluble microbial products (SMP) available for heterotrophic growth. There are also studies focused on the determination of these SMP derived from nitrifiers that can be used by heterotrophs (Kindaichi et al., 2004; Okabe et al., 2005).

Table 5.3. Genera with relative abundance higher than 5 % in samples of sludge on day 98 and 233.

Day 98		**Day 233**	
Nitrosomonas	(41%)	*Nitrosomonas*	(65%)
Nitrosospira	(7%)	Unclassified (*Cytophagales* Order)	(15%)
Sphingomonas	(8%)	Unclassified (*Bacteroidetes Phylum*)	(8%)
Sphingopyxis	(5%)		
Cryobacterium	(7%)		
Flavobacterium	(5%)		
Comamonas	(5%)		

Interaction between nitrifiers and heterotrophs is not only profitable for heterotrophic bacteria, but also for nitrifiers, becoming a synergic system as it was suggested by Ducey et al. (2010) and Hoang et al. (2014). Some psychrotolerant and psychrophilic bacteria have been identified as producers of cryoprotective extracellular polymeric substances (EPS) that allow a better survival of the whole consortium at cold temperatures (Ducey et al., 2010). Therefore, this protection would affect in the same way to AOB, which could maintain the nitritation even when conditions were not ideal for their growth. Psychrotolerant microorganisms were found in both samples of the granular sludge and thus, they were present during the whole operation of the granular reactor at 10 ºC. Hence, although in this system the heterotrophic population was less significant than that of nitrifiers, its presence could be essential for the maintenance of the nitritation in the granular reactor. Fig. 5.9 shows a SEM image of the surface of a granule where bacteria seem to be embedded in a high amount of extracellular polymeric substances, which could be the cryoprotective EPS.

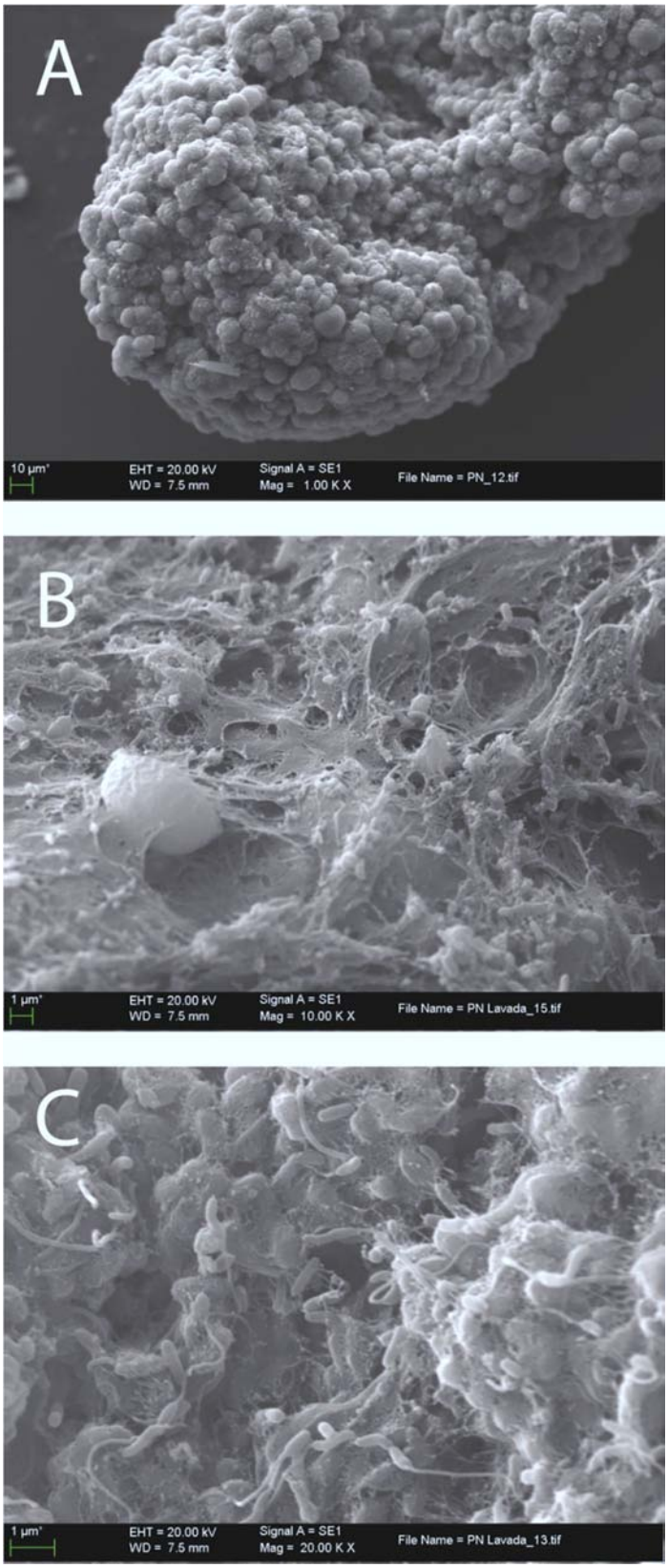

Fig. 5.9 Scanning electron microscopy (SEM) images of a granule surface. Granule sample was taken on day 45 of operation at 10 °C. (A) 1,000x magnification; (B) 10,000x magnification; (C) 20,000x magnification.

This study revealed high nitrifier ability for performing partial nitritation at 10 ºC: stable operation was maintained in the long-term in the granular airlift reactor and kinetic experiments showed the higher μ_{max} than has been hitherto determined for a nitrifying sludge at low temperature. Two hypothesis could explain it: (i) AOB were cultivated in the long-term under low temperatures which could lead to a metabolic adjustment of the biomass and thus, to improve the ability to nitrify under this condition; (ii) granules comprised a consortium of microorganisms which included producers of cryoprotective EPS that give an adaptive advantage to AOB, protecting them from low temperatures.

5.4. CONCLUSIONS

Stable partial nitritation at 10 ºC was maintained in the long-term in a granular airlift reactor operating at high NLR.

The nitrifier culture enriched in AOB presented a significantly high μ_{max} compared to other studies which allowed the operation at high nitritation rates, being advantageous for NOB repression.

The microbial community was dominated by AOB (specifically *Nitrosomonas* genus) throughout the whole operation of the reactor; while NOB genera were barely detected, demonstrating their effective repression from the system. Effective NOB repression was not only achieved, but also it was obtained a suitable effluent for a subsequent reactor performing the anammox process.

The operation of the granular reactor in the long-term at 10 ºC with a high residual ammonium concentration decreased the microbial diversity and further enriched the granular sludge in AOB.

Partial nitritation at 10 ºC can be operated with low N_2O emissions since less than 0.35 % of the TAN in the influent was emitted as $N\text{-}N_2O$.

Chapter 6

EFFECT OF TEMPERATURE ON N_2O PRODUCTION FROM A HIGHLY ENRICHED NITRIFYING GRANULAR SLUDGE PERFORMING PARTIAL NITRITATION AT MAINSTREAM CONDITIONS

A modified version of this chapter is being prepared for publishing as:

Reino, C., van Loosdrecht M.C.M., Pérez, J., Carrera, J., 2016. Effect of temperature on N_2O production from a highly enriched nitrifying granular sludge performing partial nitritation at mainstream conditions. *In preparation*.

Abstract

In the race to achieve a sustainable urban wastewater treatment plant, not only the energy requirements have to be considered but also the environmental impact of the facility. Thus, nitrous oxide (N_2O) emissions are a key-factor to pay attention to, since N_2O emissions can dominate the total greenhouse gases emissions from biological wastewater treatment. In this study, N_2O production factors were calculated during the operation of a granular sludge airlift reactor performing partial nitritation at mainstream conditions, and furthermore, the effect of temperature on N_2O production was assessed. A 3-times increase was observed in N_2O gas emissions when temperature increased from 10 to 20 ºC, mainly due to the increase of N_2O stripping; but also an increase in the N_2O production was observed in the bulk liquid of the airlift reactor. Thus, average gas emission factors of 1.5 ± 0.3% and 3.7 ± 0.5% and liquid production factors of 0.5 ± 0.1% and 0.7 ± 0.1% (% N-oxidized) at 10 and 20 ºC were obtained, respectively. Hence, the higher the temperature was, the higher the N_2O production by the nitrifying sludge was. The reasons why high temperatures favoured the N_2O emissions remained unclear, but different hypothesis were suggested such as the accumulation of hydroxylamine or the enhancing of the nitrifier denitrification pathway caused by the lower oxygen penetration into the granules at high temperature compared to low temperature.

6.1. INTRODUCTION

The implementation of the autotrophic biological nitrogen removal (BNR) in the mainstream has been proposed as the most promising solution for achieving energy-neutral or even energy-positive urban wastewater treatment plants (WWTPs) (Kartal et al., 2010; Siegrist et al., 2008). Significant efforts have been made to implement such a treatment as a one-stage system, where partial nitritation and anammox process (PN/A) are integrated in one single reactor (De Clippeleir et al., 2013; Gilbert et al., 2014; Lotti et al., 2014a; Wang et al., 2016a; Wett et al., 2013). This is based on the practise of implementing this processes for sidestream treatment (Lackner et al., 2014). However the different conditions of low required effluent concentrations, lower temperature and much larger hydraulic loading relative to nitrogen loading might make a different process design more feasible. Two-stage systems have been reported as a successful alternative to face the challenges of efficient autotrophic BNR at mainstream conditions (Isanta et al., 2015a; Ma et al., 2011; Pérez et al., 2015).

In the race to achieve a sustainable urban WWTP not only the energy requirements have to be considered but also the environmental impact of the facility. Thus, greenhouse gases emissions of the wastewater treatment are a key-factor to pay attention to (Kampschreur et al., 2009). Nitrous oxide (N_2O) is produced in conventional urban WWTPs during the autotrophic nitrification and heterotrophic denitrification and, actually, N_2O emissions can dominate the total greenhouse gases emissions from biological wastewater treatment (Wunderlin et al., 2012). N_2O is an important greenhouse gas with a global warming potential of about 300 times higher than CO_2 on a 100 year time horizon (IPCC, 2013) and a substantial ozone-depleting compound in the stratosphere. Hence, mitigation strategies and control of emissions is an essential issue to consider in the implementation of the autotrophic BNR in the mainstream of urban WWTPs.

It is well known that N_2O production in WWTPs is associated to nitrification by ammonia oxidizing bacteria (AOB) and to denitrification by heterotrophic bacteria (Kampschreur et al., 2009; Wunderlin et al., 2012). Furthermore, N_2O emissions can be also produced by abiotic chemical reactions (Harper et al., 2015; Kampschreur et al., 2011; Soler-Jofra et al., 2016). AOB produce N_2O by two different pathways: (i) from intermediates of the biological oxidation of hydroxylamine (NH_2OH), which is an intermediate during the ammonia oxidation until nitrite and (ii) the nitrifier denitrification pathway, which is the reduction of nitrite to N_2O with ammonia, hydrogen or pyruvate as possible electron donors

(Wunderlin et al., 2012). Heterotrophic denitrifiers produce N_2O as intermediate in the denitrification so it can be released due to an imbalanced metabolic activity, a nitrite accumulation or a limited availability of biodegradable organic compounds and incomplete denitrification (Kampschreur et al., 2009; Wunderlin et al., 2012).

In the autotrophic BNR process, N_2O emissions will mainly occur in the partial nitritation step since anammox bacteria are not supposed to produce N_2O as it is not involved in the anammox metabolism (Kartal et al., 2011). Actually, very low N_2O emissions were reported in anammox reactors and they were associated to side reactions independent of anammox bacteria (Lotti et al., 2014c), or to abiotic reactions (Kampschreur et al., 2011). In recent years, N_2O gas emissions were widely studied for partial PN/A systems (either in one-stage systems or in a single partial nitritation reactor) treating high-strength nitrogen wastewaters, mainly reject water (Castro-Barros et al., 2015; Desloover et al., 2011; Kampschreur et al., 2008; Mampaey et al., 2016; Okabe et al., 2011; Pijuan et al., 2014). There was a huge variability on N_2O emissions values reported in literature, ranging from 1.5% (Rathnayake et al., 2013) to 11% (Desloover et al., 2011) of the ammonium oxidized emitted as N_2O. This variability was due to differences in reactor configurations, type of influent, conditions applied and even the methodology used for quantifying emissions (Bollon et al., 2016). In the case of PN/A systems at mainstream conditions, to the best of the author's knowledge, only Wang et al. (2016b) and Reino et al. (2016) reported N_2O gas emissions of a nitritation reactor treating a low-strength synthetic influent. Reino et al. (2016) reported very low values (0.36 ± 0.07% of the ammonium oxidized) in a granular sludge reactor performing partial nitritation at 10 °C, compared to N_2O gas emissions reported by Pijuan et al. (2014) (6% of the ammonium oxidized) which used the same control strategy but treating a reject water at 30 °C, and it was suggested that temperature could be an important factor affecting N_2O emissions.

The effect of temperature on N_2O emissions was never deeply studied since, as mentioned before, most studies were performed for systems treating reject water, which is characterized by a high temperature (30–35 °C). However, wastewater temperature is a key parameter in the nitrification process which affects to mass transfer, chemical equilibrium and growth rate (Van Hulle et al., 2010), so it could be also an important parameter affecting N_2O emissions. Furthermore, N_2O solubility decreases when temperature increases which affects N_2O stripping from wastewater to gas phase resulting in the enhancement of N_2O gas emissions.

Hence, the objective of the present study was to investigate the effect of temperature on the N_2O gas emissions from a granular sludge airlift reactor performing partial nitritation of a low-strength synthetic influent. Hereto, the reactor was operated at three different temperatures: 10, 15 and 20 °C.

6.2. MATERIALS AND METHODS

6.2.1. Configuration and operation phases of the reactor

A lab-scale granular sludge airlift reactor with a working volume of 1.5 L was used. The downcomer-to-separator diameter was 0.57 and the total length-to-downcomer diameter ratio was 8 (Fig. 6.1). Compressed air was supplied through an air diffuser placed at the bottom of the reactor and was manually manipulated to maintain the dissolved oxygen (DO) concentration in the bulk liquid in 1.6 ± 0.4 mg O_2 L^{-1}. The DO was measured online by means of a DO electrode (Mettler Toledo, USA) and the pH was controlled and maintained at 8.0 ± 0.1 by the addition of 0.5M Na_2CO_3. DO monitoring and pH control was done by a biocontroller (ADI 1030, Applikon, The Netherlands). The reactor temperature was controlled by means of a cryostat connected to the jacket of the reactor.

Continuous operation of the reactor was divided in four different periods. The period I (days 0–7) corresponded to the start-up period when the nitrogen loading rate (NLR) was increased until achieving an average value in period II (days 8–40). Periods II, III (days 41–58) and IV (days 59–65) corresponded to the stable reactor operation at the different temperatures studied: period II at 10 ºC, period III at 20 ºC and period IV at 15 ºC. During the transition between these periods temperature was directly changed. The sequence of temperatures tested was not consecutive (from the lowest temperature to the highest temperature) to minimize the effect of changing conditions on the possible increase of N_2O emissions.

6.2.2. Inoculum and influent characteristics

The airlift reactor was inoculated with 1L of granular sludge (approximately 2 g VSS) from a granular sludge reactor operated in the long-term performing partial nitritation of a low-strength synthetic wastewater at 10 ºC (Reino et al., 2016). The operational

characteristics of the granular reactor at the moment when the inoculum was withdrawn are shown in Table 6.1. The inoculum was highly enriched in ammonia oxidizing bacteria (AOB) with more than 90% of abundance of AOB and 1 ± 1% of NOB (specifically *Nitrobacter* spp.) quantified through fluorescence *in situ* hybridization (FISH). *Nitrospira* spp. (NOB-type bacteria) were not detected in the inoculum.

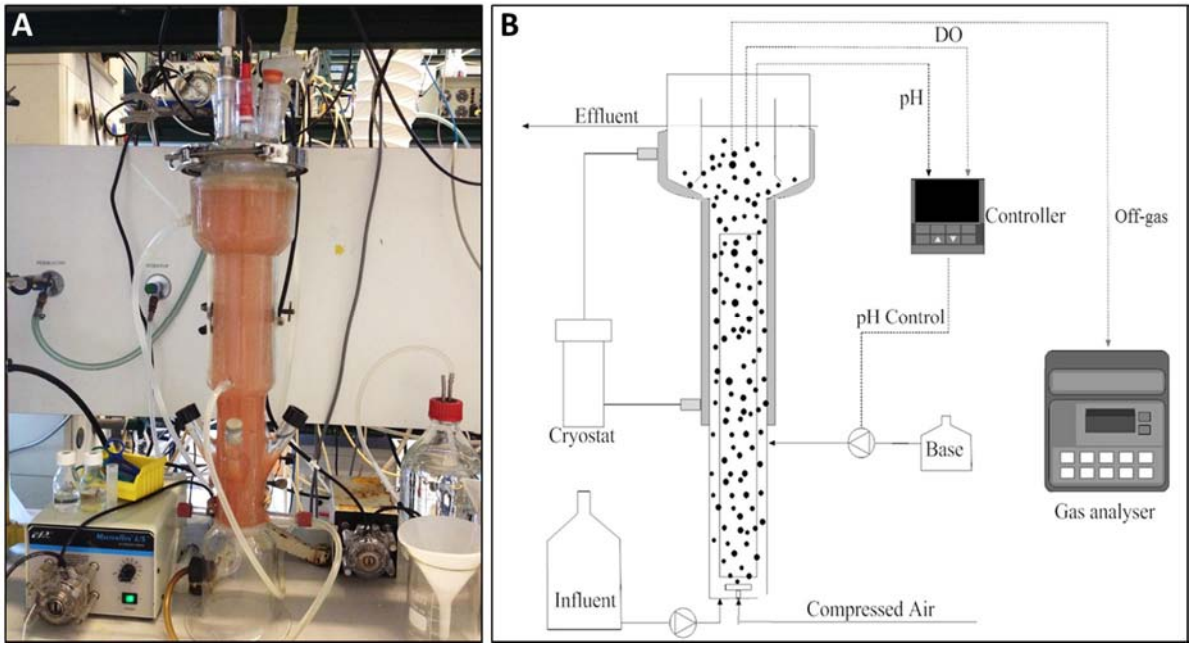

Fig. 6.1. Image of the airlift reactor and (A) and schematic diagram of the reactor set-up showing the peripheral instrumentation and control loops (B). DO: dissolved oxygen.

The granular airlift reactor was fed with a synthetic influent mimicking the pretreated municipal wastewater coming from the mixture of the effluent of a previous A-stage plus the recirculation of the reject water of the digested sludge, as in an anammox-based WWTP (Isanta et al., 2015a; Kartal et al., 2010). The resulting influent contained, in average, 70 mg $N\text{-}NH_4^+$ L^{-1}, 45 mg KH_2PO_4 L^{-1}, 784 mg $NaHCO_3$ L^{-1}, 80 mg NaCl L^{-1}, 40 mg $CaCl_2$ L^{-1}, 90 mg $MgCl_2$ L^{-1} and 1 mL of trace elements solution per L of influent consisting of 1.5 g $FeCl_3.6H_2O$ L^{-1}, 0.18 g KI L^{-1}, 0.15 g $CoCl_2.6H_2O$ L^{-1}, 0.12 g $ZnSO_4.7H_2O$ L^{-1}, 0.12 g $MnCl_2.4H_2O$ L^{-1}, 0.06 g $Na_2MoO_4.2H_2O$ L^{-1}, 0.03 g $CuSO_4.5H_2O$ L^{-1} and 10 g EDTA L^{-1}.

6.2.3. Specific analytical methods and N_2O measurements

Concentrations of ammonium, nitrite and nitrate in influent and effluent were regularly measured off-line with Dr. Lange test kits (Hach Lange, Germany) in previously filtered (0.22 μm pore) samples.

Measurements of N_2O concentration in the off-gas were analysed by means of an online analyser (Emerson Rosemount NGA 2000). Off-gas was collected continuously from the reactor headspace and conducted via a gas tube to the online analyser. A moisture filter was installed at the gas inlet of the analyser and a t-shaped tubing joint was fitted on the gas tube connecting the gas outlet of the reactor and the gas analyser, allowing the excess of gas to escape and thus avoiding overpressure in the line. Data were logged every minute for a period of at least 4 hours for each test. In period II (10 °C), two sets of tests were done: (i) during days 17, 18 and 21 of operation, and (ii) during days 30, 31, 32 and 33 of operation. In period III (20 °C) the N_2O measurements were performed during days 44, 56 and 57. And finally, in period IV (15 °C) measurements were performed during days 63 and 64 of operation.

Table 6.1. Operational characteristics of the granular sludge airlift reactor which provided the inoculum of the granular sludge airlift reactor of the present study. (NLR = Nitrogen Loading Rate; NRR = Nitrogen Removal Rate; sNLR = specific Nitrogen Removal Rate)

Parameter	Value	Units
Volume	5.2	L
Temperature	10	°C
Dissolved Oxygen	1.3 ± 0.5	g L^{-1}
pH	8.0 ± 0.1	
NLR	0.63 ± 0.06	g N L^{-1} d^{-1}
AOR	0.34 ± 0.06	g N L^{-1} d^{-1}
sNLR	0.18 ± 0.03	g N g^{-1} VSS d^{-1}
$[N\text{-}NH_4^+]_{effluent}$	31 ± 4	g $N\text{-}NH_4^+$ L^{-1}
$[N\text{-}NO_2^-]_{effluent}$	35 ± 4	g $N\text{-}NO_2^-$ L^{-1}
$[N\text{-}NO_3^-]_{effluent}$	0.6 ± 0. 3	g $N\text{-}NO_3^-$ L^{-1}

6.2.4. Calculation of the N_2O production factors

Two different N_2O production factors were calculated. One was based on the total amount of N_2O produced in relation to the total ammonium oxidized to nitrite, and the other one was based on the total amount of N_2O produced in relation to the total ammonium of the influent. The way of calculating the N_2O production factors is important to compare different nitrifying systems since, as explained by Pijuan et al. (2014), the production factor relative to the total ammonium oxidized to nitrite is the most adequate factor to compare the N_2O production when the reactor is oxidizing only a certain fraction of the ammonium load (e.g. either full or partial nitritation).

Moreover, in the present study N_2O production factors were also divided in: N_2O gas emission factors and N_2O liquid production factors, depending on the phase where N_2O was present. The N_2O emitted in the gas phase was quantified with N_2O gas emission factors (EF_{gas}), while the dissolved N_2O in the reactor bulk liquid was quantified with N_2O liquid production factor (PF_{liq}). Finally, a total N_2O production factor (PF_{tot}), comprising production in both gas and liquid phases, was calculated by the sum of the EF_{gas} and PF_{liq}. All the calculations used are described below:

$$PF_{tot} = EF_{gas} + PF_{liq} \quad \text{(Eq. 6.1)}$$

$$EF_{gas}(per\ NH_4^+\ oxidized) = \frac{[N - N_2O]_{gas} \times Q_{gas}}{[N - NH_4^+]_{oxidized} \times Q_{influent}} \times 100 \quad \text{(Eq. 6.2)}$$

$$PF_{liq}(per\ NH_4^+\ oxidized) = \frac{[N - N_2O]_{liq} \times Q_{influent}}{[N - NH_4^+]_{oxidized} \times Q_{influent}} \times 100 \quad \text{(Eq. 6.3)}$$

$$EF_{gas}(per\ NH_4^+\ in\ the\ influent) = \frac{[N - N_2O]_{gas} \times Q_{gas}}{[N - NH_4^+]_{influent} \times Q_{influent}} \times 100 \quad \text{(Eq. 6.4)}$$

$$PF_{liq}(per\ NH_4^+\ in\ the\ influent) = \frac{[N - N_2O]_{liq} \times Q_{influent}}{[N - NH_4^+]_{influent} \times Q_{influent}} \times 100 \quad \text{(Eq. 6.5)}$$

where,

$$[N-N_2O]_{gas}(mg\ N-N_2O\cdot L^{-1}) \quad \text{(Eq. 6.6)}$$

$$= \frac{[N-N_2O](ppmv)\times P(atm)\times 28(g\cdot mol^{-1})}{0.082\left(\frac{atm\cdot l}{mol\cdot K}\right)\times T(K)\times 1000}$$

$$[N-NH_4^+]_{influent}(mg\ N-NH_4^+\cdot L^{-1}) = N-NH_4^+\ concentration\ in\ the\ influent$$

$$[N-NH_4^+]_{oxidized}(mg\ N-NH_4^+\cdot L^{-1}) \quad \text{(Eq. 6.7)}$$

$$= \left([N-NH_4^+]_{influent}-[N-NH_4^+]_{effluent}\right)$$

$$Q_{gas}(L\cdot d^{-1}) = flow\ rate\ of\ the\ aeration$$

$$Q_{influent}(L\cdot d^{-1}) = flow\ rate\ of\ the\ wastewater\ influent$$

$[N-N_2O]_{liq}$ *was calculated based on mass transfer balances as explained below in section* 6.2.4.1

6.2.4.1. Calculation of the N_2O liquid concentration

Concentration of N_2O in the liquid was estimated based on the mass transfer coefficient $\left(k_La_{N_2O}\right)$ and the maximum solubility of N_2O at the system conditions, by using the equation Eq. 6.8. $k_La_{N_2O}$ was calculated with the equation Eq. 6.9 according to the Higbie's penetration model as described in Marques et al. (2016) and values obtained are presented in Table 6.2.

$$N_{N-N_2O} = k_La_{N_2O}\times\left([N-N_2O]_{liq}-[N-N_2O]^*_{liq}\right) \quad \text{(Eq. 6.8)}$$

$$k_La_{N_2O} = k_La_{O_2}\times\sqrt{\frac{D_{F,N_2O}}{D_{F,O_2}}} \quad \text{(Eq. 6.9)}$$

where,

$$N_{N-N_2O}(mg\ N-N_2O\cdot L^{-1}\cdot d^{-1}) = \frac{[N-N_2O]_{gas}\ (mg\ N-N_2O\ L^{-1})\cdot Q_{gas}(L\ d^{-1})}{V_{REACTOR}(L)} \quad \text{(Eq. 6.10)}$$

$[N-N_2O]_{liq}(mg\ N-N_2O\cdot L^{-1}) = N-N_2O\ concentration\ in\ the\ bulk\ liquid$

$[N-N_2O]_{gas}(mg\ N-N_2O\cdot L^{-1}) = N-N_2O\ concentration\ in\ the\ off-gas$

$$[N-N_2O]^{*}_{liq}(mg\ N-N_2O\cdot L^{-1}) = [N-N_2O]_{gas}(mg\ N-N_2O\cdot L^{-1})\cdot H_{adim} \quad \text{(Eq. 6.11)}$$

$D_{F,N_2O}(cm^2\cdot s^{-1}) = diffusion\ coefficient\ of\ N_2O\ in\ water\ (Table\ 6.2)$

$D_{F,O_2}(cm^2\cdot s^{-1}) = diffusion\ coefficient\ of\ O_2\ in\ water\ (Table\ 6.2)$

$Q_{gas}(L\ d^{-1}) = gas\ flow\ rate\ of\ aeration$

$V_{REACTOR}(L) = volume\ of\ the\ granular\ airlift\ reactor$

$H_{adim}(dimensionless) = Henry's\ \ constant\ (Table\ 6.2)$

The mass transfer coefficient for oxygen $\left(k_L a_{O_2}\right)$ was calculated based on the oxygen uptake rate (OUR) and the DO concentration in the bulk liquid of the granular airlift reactor as described by equation Eq. 6.12. OUR was calculated based on the stoichiometric oxygen requirement for the oxidation of ammonium by AOB and the production of nitrate by NOB.

$$OUR = k_L a_{O_2} \times \left([O_2]^{*}_{liq} - [O_2]\right) \quad \text{(Eq. 6.12)}$$

where,

$$[O_2]^{*}_{liq}(mg\ O_2\cdot L^{-1}) = [O_2]_{air}(mg\ O_2\cdot L^{-1})\cdot H_{adim} \quad \text{(Eq. 6.13)}$$

$[O_2]_{air}(mg\ O_2 \cdot L^{-1}) = O_2\ concentration$ in the air used for aeration

Table 6.2. Main parameters used for the determination of the concentration of N_2O in the bulk liquid for the three temperatures of operation of the granular sludge airlift reactor.

Temperature	Henry's constant O_2[1]	Henry's constant N_2O[1]	$k_La_{O_2}$	D_{F,O_2}[2]	D_{F,N_2O}[3]	$k_La_{N_2O}$
(°C)	(mol m^{-3} Pa^{-1})		(d^{-1})	(cm^2 s^{-1})		(d^{-1})
10	$3.81 \cdot 10^{-4}$	$1.70 \cdot 10^{-5}$	76 ± 6	$1.60 \cdot 10^{-5}$	$1.27 \cdot 10^{-5}$	68 ± 5
15	$3.25 \cdot 10^{-4}$	$1.55 \cdot 10^{-5}$	97 ± 28	$1.80 \cdot 10^{-5}$	$1.28 \cdot 10^{-5}$	82 ± 25
20	$2.78 \cdot 10^{-4}$	$1.42 \cdot 10^{-5}$	146 ± 8	$1.98 \cdot 10^{-5}$	$1.27 \cdot 10^{-5}$	117 ± 7

*References: [1](Sander, 2015); [2](Ferrell and Himmelblau, 1967) and [3](Tamimi et al., 1994).

6.2.5. Fluorescence *in situ* hybridization (FISH)

Abundances of AOB and NOB were analysed by FISH technique at the beginning (day 0) and at the ending of the operation (day 61). Specific probes for AOB and NOB (specifically *Nitrobacter* spp.) were 5'-Cy3-labeled and 5'-Fluos-labeled, respectively. Hybridizations were performed with the specific and general (5'-Cy5-labeled) probes described in Section 4.3.1 of Chapter 4. The general probe for all microorganisms was 5'-Cy5-labeled. Hybridization protocol was performed according to Section 4.3.1, Chapter 4. Slides were observed with an epifluorescence microscope (Axioplan 2; Zeiss), and image acquisition was performed with a Leica D350F camera.

6.3. RESULTS AND DISCUSSION

6.3.1. Operation of the reactor

The airlift reactor was inoculated with granular sludge from another granular sludge airlift reactor which performed stable partial nitritation of a low-strength influent for 250 days

at 10 ºC. More details about this operation can be found in Chapter 5. The operation of the granular sludge airlift reactor in the present study was divided in four periods (Fig. 6.2). Continuous operation took place from the start (inoculation at day 0) with an initial nitrogen loading rate (NLR) of 0.21 ± 0.03 g N L^{-1} d^{-1} and a temperature of 10 ºC. During the period I or start-up period (days 0–7), the NLR was gradually increased until achieving an average NLR of 0.60 ± 0.07 g N L^{-1} d^{-1} in period II (days 8–40). From day 15 onwards the nitrate concentration in the effluent started to increase and nitrite concentration decreased. This meant that nitratation activity developed in the granular airlift reactor despite of maintaining a low DO/TAN concentrations ratio (0.06 ± 0.02 mg O_2 mg^{-1} N during periods I and II) which was previously reported to maintain stable partial nitritation with efficient NOB repression in granular systems (Bartrolí et al., 2010; Isanta et al., 2015a). Table 6.3 shows the average concentrations of TAN, TNN and nitrate in the effluent of the airlift reactor during the different periods of operation. Nitrite and nitrate concentrations stabilized at the end of period II (days 30–40) at 10 °C with effluent values of 6 ± 2 mg N-NO_2^- L^{-1} and 11 ± 4 mg N-NO_3^- L^{-1}, between these days. In period III (days 41–58) temperature was increased until 20 ºC and stable operation was achieved with an average NLR of 0.78 ± 0.10 g N L^{-1} d^{-1}. The concentration of the different nitrogen species in the effluent was also maintained stable (Fig. 6.2B and Table 6.3), even when temperature was decreased again until 15 ºC in period IV (days 59–65) and the NLR decreased until an average value of 0.72 ± 0.08 g N L^{-1} d^{-1}. Specific nitrogen removal rate (sNLR) increased from 0.31 ± 0.04 g N g^{-1} VSS d^{-1} in period II (10 ºC) until 0.40 ± 0.06 g N g^{-1} VSS d^{-1} in period III (20 ºC), which was expected since biomass activity increase with temperature.

Biomass concentration in the reactor was maintained stable during the four different periods of operation and resulted in 1.9 ± 0.2 g VSS L^{-1}. Settling properties of the granules were also maintained during the whole operation of the granular airlift reactor, with an average settling velocity of 23 ± 5 m h^{-1} and an average SVI of 76 ± 4 ml g^{-1} SST, which are typical values of granular biomass (an image of the nitrifying granules is depicted in Fig. 6.3). The hydraulic retention time (HRT) was maintained at 2.4 ± 0.5 hours and the solid retention time (SRT) was kept at an average value of 23 ± 10 days.

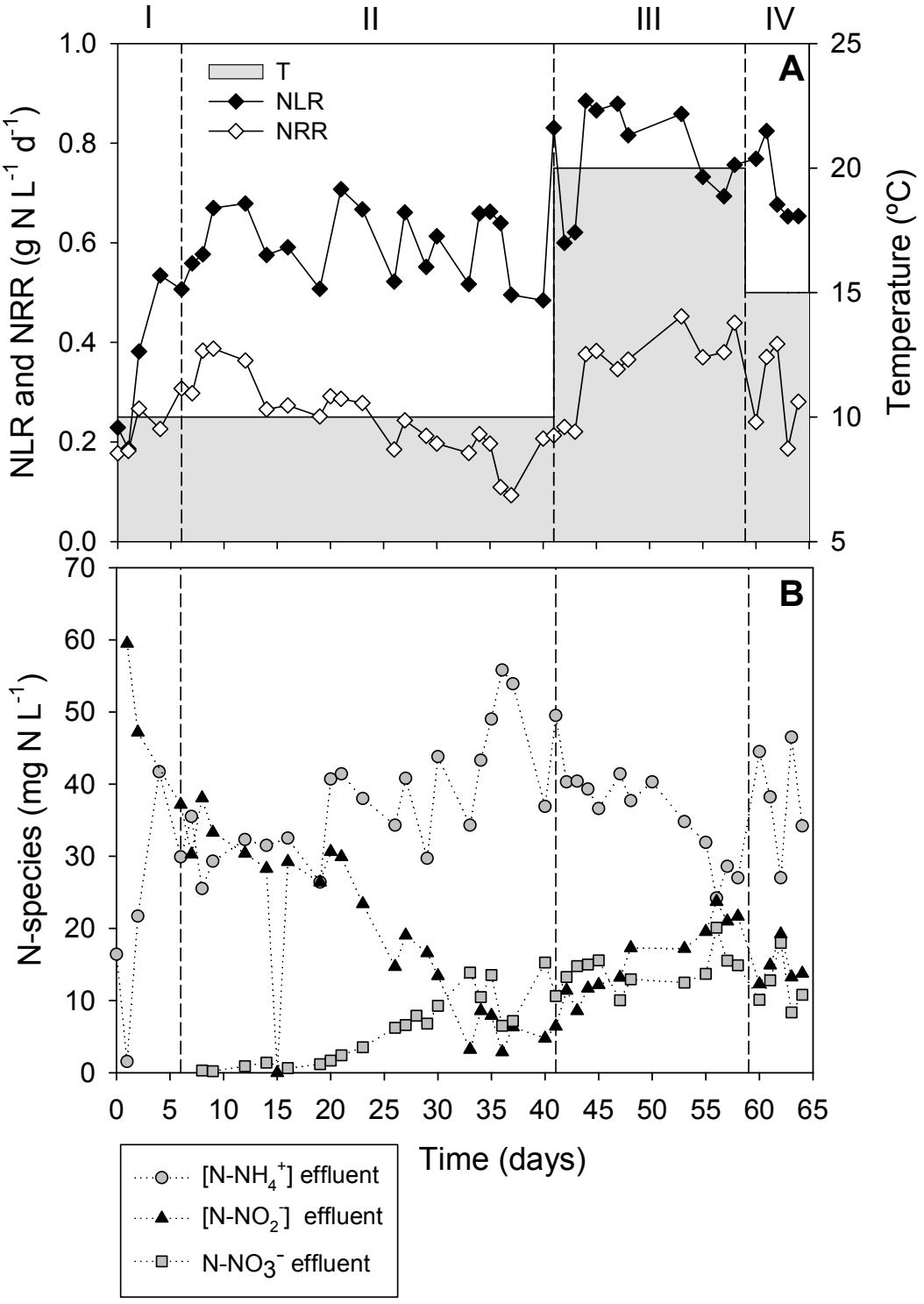

Fig. 6.2 Continuous operation of the granular sludge airlift reactor treating a low-strength synthetic influent at different temperatures. Operation was divided in four different periods: period I (start-up), period II (operation at 10 °C), period III (operation at 20 °C) and period IV (operation at 15 °C). (A) Nitrogen Loading Rate (NLR), Nitrogen Removal Rate (NRR) and temperature (T); (B) Nitrogen compounds concentrations throughout the operation of the granular sludge reactor.

Table 6.3. Nitrogen loading rate (NLR) achieved and concentration of the nitrogen species present in the effluent during the different periods of operation of the granular sludge airlift reactor. n.a.: not analysed.

Period	Temperature (°C)	NLR (g N L^{-1} d^{-1})	$[TAN]_{eff}$ (mg N L^{-1})	$[TNN]_{eff}$ (mg N L^{-1})	$[N\text{-}NO_3^-]_{eff}$ (mg N L^{-1})
I	10	0.23–0.56	25 ± 10	42 ± 10	n.a.
II	10	0.60 ± 0.07	38 ± 9	18 ± 10	6 ± 4
III	20	0.78 ± 0.10	35 ± 9	14 ± 7	14 ± 2
IV	15	0.72 ± 0.08	38 ± 8	15 ± 3	12 ± 4

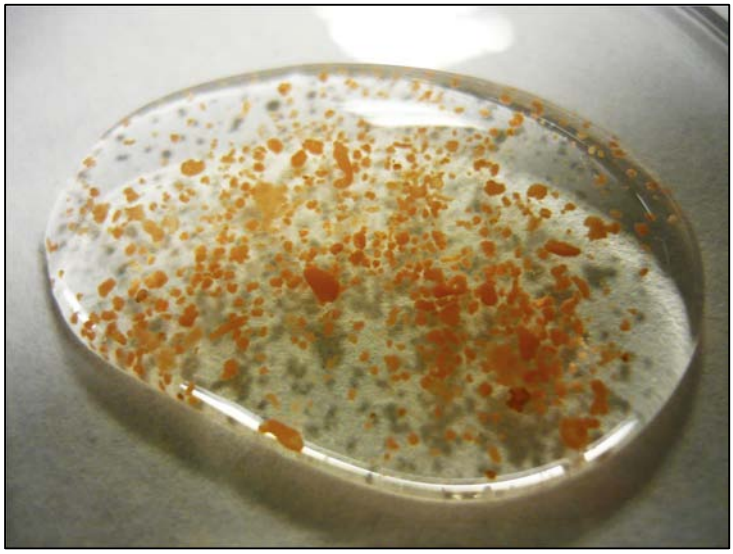

Fig. 6.3. Image of the granules of the granular sludge airlift reactor.

The measured nitrogen compounds balanced well during the whole operation of the reactor, with an average value of 99 ± 6% detection of the nitrogen load from the influent in the effluent (Fig. 6.4). Thus, neither heterotrophic nor autotrophic (anammox process) denitrification was considered to take place in the granular sludge airlift reactor, and consequently the contribution of heterotrophic denitrification to N_2O emissions can be neglected.

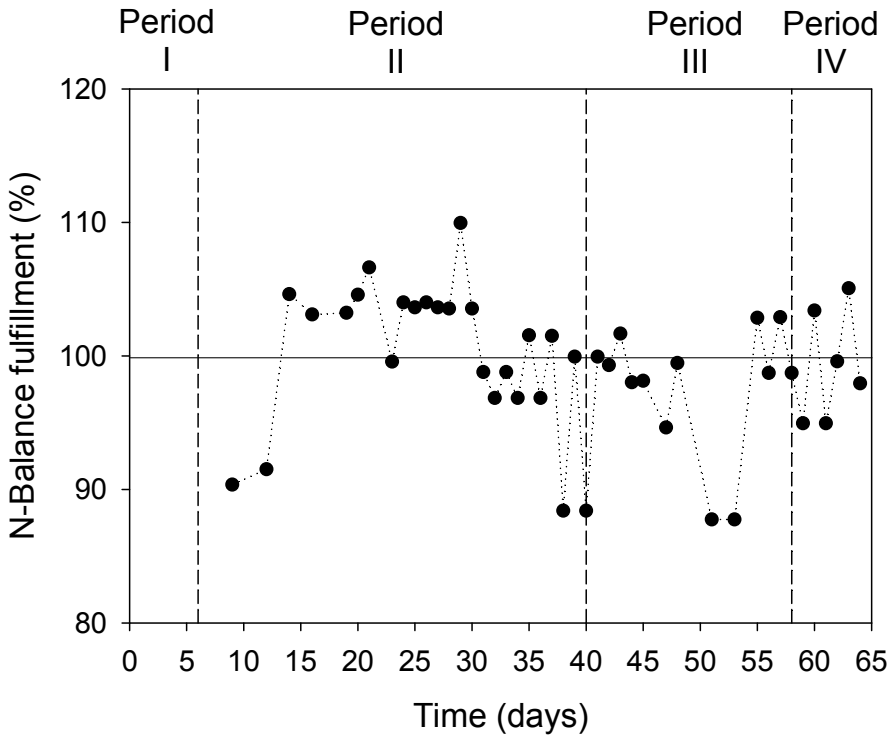

Figure 6.4. Fulfillment of the nitrogen balance during the operation of the granular sludge airlift reactor treating synthetic low-strength wastewater.

6.1.1. Microbial characterization of the granular sludge

Biomass samples from days 0 and 61 were analysed by FISH technique to assess the enrichment in AOB in the granular sludge during the whole operation of the reactor. Qualitative evaluation of the results of FISH indicated that granules were composed predominantly by AOB while *Nitrobacter* spp. (NOB-type bacteria) were not detected, as shown in Fig. 6.5. NOB were not detected even at the end of the operation, when about 10 mg $N\text{-}NO_3^-$ L^{-1} were produced in the reactor. Reasons why the stability of nitritation was lost remained unclear. The presence of *Nitrospira* spp. (NOB-type bacteria) could explain the nitratation activity developed in the granular airlift reactor, however *Nitrospira* spp. were never detected by FISH throughout the operation of the reactor.

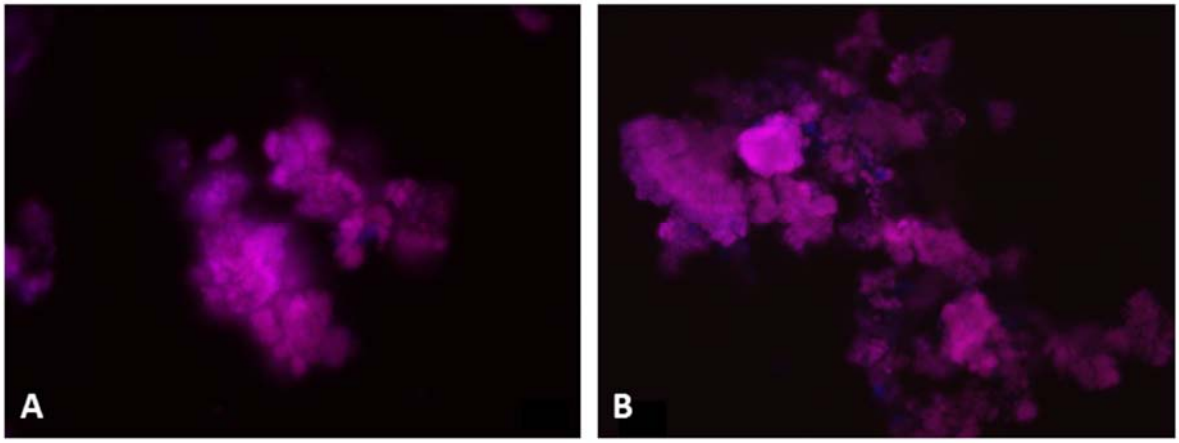

Fig. 6.5. FISH analysis performed on the granular sludge depicting AOB (red) and NOB (green) over the universal probe (blue) on A) day 0 and B) day 61 of operation.

6.1.2. Nitrous oxide production

Off-gas was monitored during the periods II, III and IV of operation of the granular sludge airlift reactor in order to calculate the N_2O gas emission factors (EF_{gas}) of the nitrifying sludge at different temperatures. During these three periods, the operation was maintained stable with average ammonium, nitrite and nitrate concentrations of 37 ± 8 mg $N\text{-}NH_4^+$, 19 ± 9 mg $N\text{-}NO_2^-$ and 10 ± 5 mg $N\text{-}NO_3^-$. DO concentration was stable (1.6 ± 0.4 mg O_2 L^{-1}) during these periods to avoid a strong influence on the N_2O production as previously reported by Pijuan et al. (2014). Since the granular sludge airlift reactor operated performing partial nitritation, a discussion of the nitrous oxide emission factors based on the N_2O emitted in relation to the total ammonium oxidized by AOB is more appropriate.

Table 6.4 shows the N_2O gas emission factors obtained during the operation of the granular sludge airlift reactor at 10, 15 and 20 ºC. A 3-times increase was measured in N_2O gas emissions when temperature increased from 10 to 20 ºC in period III. It could be argued that after a long period operating at 10 ºC there is an acclimation of the biomass at that temperature, and changing the operation at higher temperature acted as a disturbance triggering higher N_2O emissions. Nevertheless, N_2O gas measurements were performed at the beginning and end of the period III and no diminishment in emissions after operating at 20 °C was observed (Fig. 6.6). In addition, a decrease in N_2O EF_{gas} was observed when temperature decreased again until 15 ºC in period IV, reaching roughly the same value obtained for 10ºC.

Table 6.4. N_2O gas emission factors (EF_{gas}) from the granular sludge airlift reactor treating a low-strength influent at different temperatures. DO was maintained in 1.6 ± 0.4 mg O_2 L^{-1} during all the N_2O measurements.

Period	Days	T (°C)	Gas flow ($L\ h^{-1}$)	EF_{gas} (% of N-influent)	EF_{gas} (% of N-oxidized)
II	8–40	10	2.9 ± 0.2	0.6 ± 0.2	1.5 ± 0.3
III	41-58	20	4.6 ± 0.7	1.9 ± 0.6	3.7 ± 0.5
IV	59-65	15	2.4 ± 0.3	0.5 ± 0.2	1.5 ± 0.5

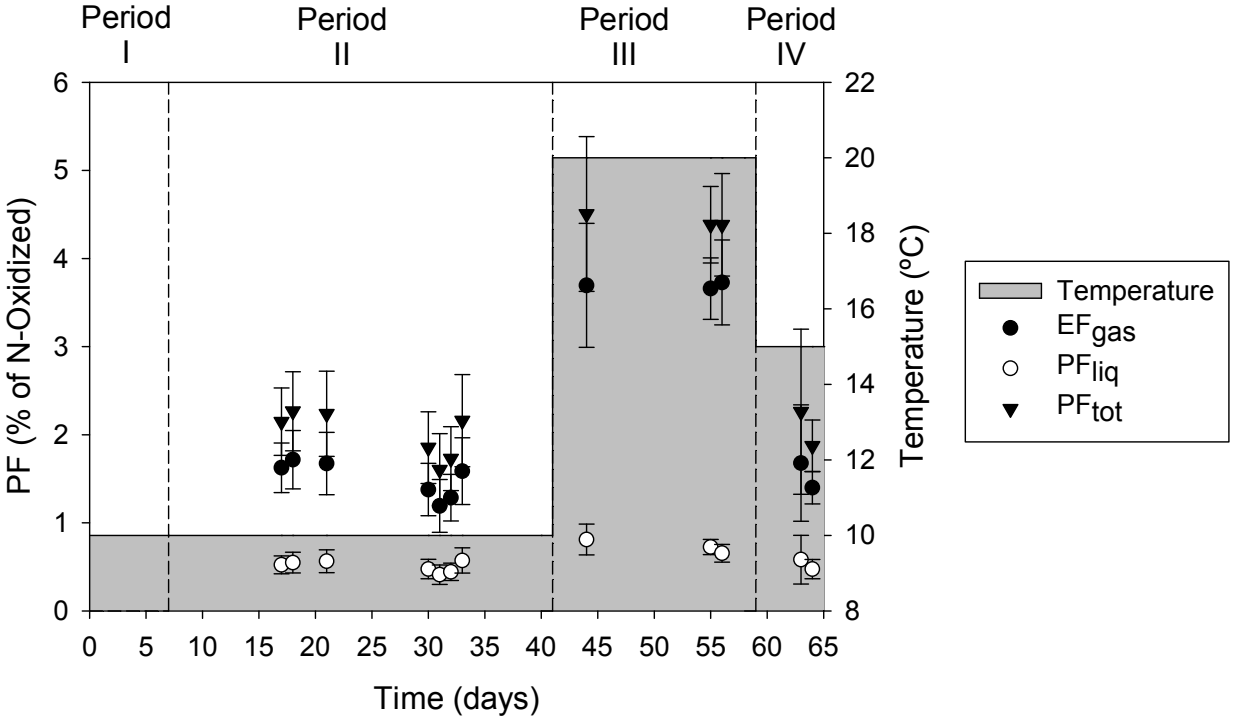

Figure 6.6. N_2O production factors relative to the ammonium oxidized to nitrite during the operation of the granular airlift reactor at 10, 15 and 20 °C. DO was maintained in 1.6 ± 0.4 mg O_2 L^{-1} during all the N_2O measurements. EF_{gas}: N_2O gas emission factors; PF_{liq}: N_2O liquid production factors; PF_{tot}: N_2O total N_2O production factors.

Hence, a clear increase of N_2O gas emissions was found for temperatures higher than 15 °C. Two reasons could explain this trend: (i) high temperature led to high N_2O production in the granular sludge airlift reactor or (ii) N_2O gas emissions increased because of the

increment of stripping of N_2O, since aeration was not constant during the three periods, being the highest aeration at 20 °C (Table 6.4).

To distinguish between the increment of N_2O emissions due to the increment of the production or due to the increment of the stripping, the N_2O liquid production factors (PF_{liq}) were calculated. Fig. 6.7 shows both gas and liquid N_2O production factors at different temperatures, together with the total production factors calculated as the sum of both contributions (liquid and gas).

Fig. 6.7 shows that PF_{liq} increased when temperature decreased despite of N_2O solubility decreases with temperature. N_2O is a very soluble gas (solubility: 1260 mg L^{-1} at 20 °C, (Weiss and Price, 1980)) and, actually, N_2O concentrations in the liquid phase were always higher than in the gas phase, and, in addition, increased when temperature increased (Table 6.5) despite of N_2O solubility decreases with temperature. Furthermore, both PF_{liq} and EF_{gas} slightly increased from 10 °C to 15 °C although gas flow decreased (gas flow of 2.9 ± 0.2 L h^{-1} at 10°C and 2.4 ± 0.3 L h^{-1} at 15 °C). These observations indicated that EF_{gas} increased not only because of the stripping but also because of an increase in the production when temperature was increased.

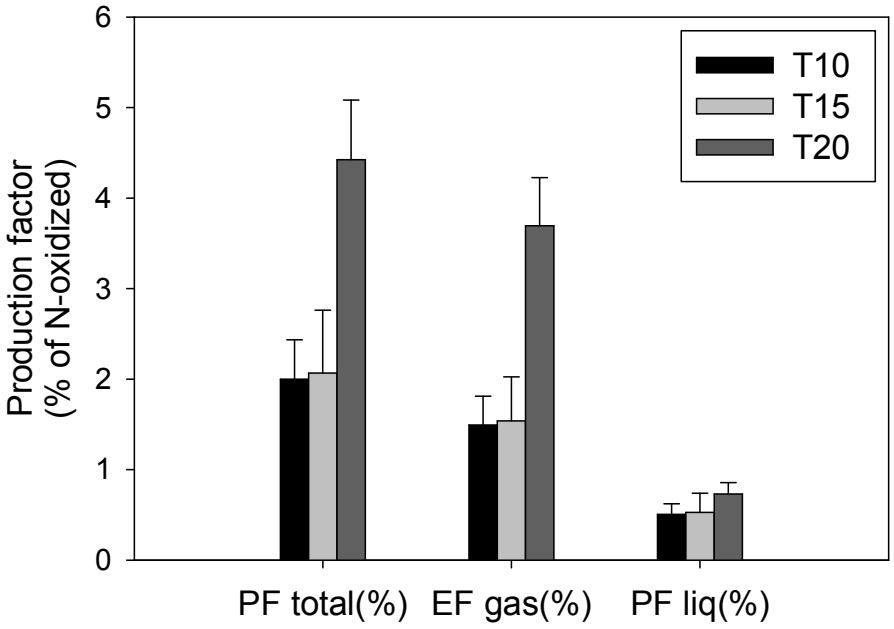

Fig. 6.7. N_2O production factors relative to the total NH_4^+ oxidized to NO_2^- at different temperatures. EF gas: N_2O gas emission factor; PF liq: N_2O liquid production factor; PF total: sum of liquid and gas N_2O production factors; T10, T15 and T20: operation at 10, 15 and 20 °C, respectively.

Table 6.5. Concentrations of N_2O in the off-gas and liquid phase during the different periods of the operation of the granular sludge airlift reactor. Concentration in the off-gas was directly measured in the reactor, while the concentration in the liquid was calculated as explained in the materials and methods section.

Period	Days	T (°C)	[N-N_2O] in the off-gas (mg L^{-1})	[N-N_2O] in the liquid (mg L^{-1})
II	8–40	10	0.08 ± 0.01	0.12 ± 0.02
III	41-58	20	0.18 ± 0.01	0.24 ± 0.03
IV	59-65	15	0.09 ± 0.02	0.11 ± 0.04

The difference in N_2O production found at 20, 15 and 10 °C in the reactor suggests that there is a kinetic deactivation of N_2O emissions at low temperatures. In this sense two possible explanations for this observation were hypothesised: (i) the kinetic dependency with temperature of the ammonia monooxygenase (AMO) enzyme catalysing the oxidation of ammonium to hydroxylamine is different than that for the hydroxylamine oxidoreductase (HAO) enzyme catalysing the oxidation of hydroxylamine to nitrite, in such a way that at 20 °C the intermediate hydroxylamine slightly accumulates because the oxidation of hydroxylamine is the limiting step. However, at lower temperature, this situation would be reversed, and the oxidation of hydroxylamine would be faster than the oxidation of ammonia. The decrease in hydroxylamine accumulation could reduce considerably the N_2O emissions because hydroxylamine is the precursor of N_2O in both pathways (hydroxylamine oxidation and nitrifier denitrification) in granular sludge reactors performing partial nitritation (Sabba et al., 2015). (ii) A second possibility to take into account is the temperature dependency of the acid-base equilibrium ammonium-ammonia. The true substrate of AOB is ammonia rather than ammonium (Suzuki et al., 1974). There is an impact of the temperature in the half-saturation coefficient expressed in ammonium concentration units (Suzuki et al., 1974). Since the residual ammonium concentration is kept rather constant among the different temperature tests (37 ± 8 mg TAN L^{-1}), the fraction of ammonia is significantly decreasing with the decrease in temperature at a pH of the bulk of 8 (free ammonia concentration of 0.81, 1.2 and 1.7 mg NH_3 L^{-1} at 10, 15 and 20 °C, respectively). Additionally, a gradient of pH is expected in the biofilm, because ammonia oxidation produces protons (de Beer et al., 1993; Gieseke et al., 2006; Schreiber et al., 2009) which will decrease even more the ammonia concentration in

the deeper layers of the granule. With the decrease in temperature if a good fraction of the cells is not saturated in ammonia, the accumulation of hydroxylamine could decrease considerably, and consequently, also the N_2O production, as already discussed.

A third possible explanation which should not be overlooked is the effect of oxygen concentration on the N_2O emissions. It was mentioned before that DO concentration in the bulk liquid was maintained stable during the three periods operating at different temperatures. However, the oxygen penetration into the granules was expected to be different at different temperatures: higher temperatures led to low oxygen penetration into the granules compared to lower temperatures. Hence, a limiting DO concentration in the granule at high temperature could be responsible of enhancing the nitrifier denitrifaction pathway of N_2O production, and thus increasing N_2O emissions.

As previously discussed, N_2O production at 20 °C, either in the gas phase or in the liquid phase, was higher than production at the lower temperatures, being the gas phase emissions the more affected, i.e. stripping was the main contributor to enhance N_2O gas emissions. Thus, lower aeration could be proposed to reduce N_2O emissions at temperatures higher than 15 °C. However, nitrifying activity increased with temperature and NLR was increased to maintain the ammonium oxidation throughout the reactor operation. This increase led to high oxygen consumption and, thus, a high aeration was needed to maintain the DO concentration in the granular airlift reactor. If NLR was not increased, more ammonium would be oxidized and effluent characteristics would be not maintained in the reactor. The high gas flow needed to maintain the DO, together with the decrease in gas solubility, increased the fraction of N_2O stripped to the gas phase. In any case, if lower aeration flowrates were used at temperatures higher than 15 °C to avoid the influence of stripping, N_2O gas emissions would be considerably reduced in the gas phase but maintained in the same range in the liquid phase, which could transpose the emissions problem to the effluent. Though, the reactor of partial nitritation is not the last reactor before effluent discharge and N_2O could be denitrified in a subsequent reactor resulting in an overall emissions reduction.

The average values of N_2O EF_{gas} obtained for a granular sludge enriched in AOB were in the same order of magnitude of other partial nitritation systems (Table 6.6). However, most of the studies available in literature reported EF_{gas} in partial nitritation systems treating high-strength wastewater (e.g. reject water) and little has been published for partial nitritation of

low-strength wastewater (e.g. urban wastewater). Overall, Table 6.6 shows that nitritation systems treating high-strength wastewater presented higher emission factors than the systems treating low-strength influents, although there is a huge variability.

The effect of temperature on N_2O production in a granular sludge enriched in AOB performing partial nitritation has not been reported before. There are few studies that characterize N_2O emissions from full-scale nitrifying reactors, which present winter and summer campaigns and, thus, high and low temperatures appear as a side parameter. Nevertheless these studies were not specifically focused on the temperature and other side parameters affected the emissions. On the one hand, Bollon et al. (2016) found that PF_{tot} (based on the N-oxidized) from full-scale nitrifying biofilters performing complete nitrification were higher in the winter campaign (PF_{tot} = 4.9 ± 0.5% at 15 °C) than in the summer campaign (PF_{tot} = 2.3 ± 0.5% at 22.5 °C), which contrasts with the results of the present study. However, they suggested that the negative influence of temperature on emissions was not related to the temperature itself, but to the low DO and high nitrite concentrations occurred in winter, which enhance N_2O production. In this sense, it could be thought that the variations of the nitrite concentration in the granular airlift reactor of the present study (Fig. 6.2) could affect N_2O emissions, however the two sets of measurements on period II were performed at different nitrite concentrations (days 17, 18, 21, 30, 31, 32 and 33 of operation, Fig. 6.6) and still they resulted in a very similar values (EF_{gas} of 1.5 ± 0.3%). On the other hand, Daelman et al. (2015) reported N_2O emission factors in the long-term operation of a full-scale urban WWTP and did not find any correlation with nitrous oxide emissions and water temperature. In the same way, Ahn et al. (2010) did not directly correlated temperature with N_2O emissions from activated sludge processes of 12 WWTPs across the United States, but expected that emissions were indirectly governed by temperature trough manifestation in ammonium, nitrite or DO concentrations. In any case, the present work is an interesting medium-term study to assess the N_2O emissions in a partial nitritation reactor at low temperature and to assess if there is an impact of the temperature in the emission factors. Added value comparing to the values obtained in Reino et al. (2016) is that online measurements were done for longer periods of time at different temperatures.

Table 6.6. N_2O gas emission factors (based on the nitrogen oxidized) for different systems performing partial nitritation in a single-reactor.

Influent	Type of reactor	EF_{gas} (% of N-oxidized)	DO Concentration (mg O_2 L^{-1})	T (°C)	Reference
Reject water	Full-scale	7.4	2	30	Mampaey et al. (2016)
Reject water	Full-scale	3.4	2.5	33	Kampschreur et al. (2008)
Reject water	Pilot-scale	2.2–6.1	1.1–4.5	30	Pijuan et al. (2014)
Reject water	Lab-scale	9.6 ± 3.2	1–2	35	Okabe et al. (2011)
Synthetic high-strength wastewater	Lab-scale	1.5 ± 0.8	1.5–1.9	35	Rathnayake et al. (2013)
Real high-strength wastewater	Full-scale	8–11	0.4–1	36	Desloover et al. (2011)
Synthetic Low-strength wastewater	Lab-scale	1.3-3.3	0.3–3	22	Wang et al. (2016b)
Synthetic Low-strength wastewater	Lab-scale	0.36 ± 0.07	1.3 ± 0.3	10	Reino et al. (2016)
Synthetic Low-strength wastewater	Lab-scale	3.7 ± 0.5	1.6 ± 0.4	20	This study
Synthetic Low-strength wastewater	Lab-scale	1.5 ± 0.3	1.6 ± 0.4	10	This study

6.2. CONCLUSIONS

A granular sludge airlift reactor performing partial nitritation at mainstream conditions was operated at high NLR with low N_2O emissions.

The production of N_2O by an enriched nitrifying granular sludge at 10, 15 and 20 ºC was determined, resulting the higher the temperature the higher the N_2O production.

Temperatures of operation higher than 15 ºC caused a negative effect on the N_2O gas emissions due to a higher production but specially due to the higher stripping occurred.

The accumulation of hydroxylamine at high temperature was suggested to be the responsible of the increase of N_2O production.

Chapter 7

DEVELOPMENT OF NITRATATION ACTIVITY AFTER THE LONG-TERM STABLE PARTIAL NITRITATION OF A LOW-STRENGTH WASTEWATER IN A GRANULAR AIRLIFT REACTOR

Abstract

A stable long-term operation of the partial nitritation process at mainstream conditions appears as a prerequisite for implementing a two-stage system for the autotrophic biological nitrogen removal in the main water line of urban wastewater treatment plants. In the present study, a lab-scale granular sludge airlift reactor was operated for 700 days performing partial nitritation of a low-strength wastewater. Stable operation with efficient repression of nitratation activity was achieved for 400 days at 10 °C, which less than 2 mg $N\text{-}NO_3^-$ L^{-1} produced in the reactor. However, nitratation activity appeared from day 401 onwards with periods ranging from stable to unstable operation at different temperatures and operational conditions. Solid retention time (SRT) was demonstrated to be a key parameter to pay attention to, because when the reactor was not purged, SRT achieved values as high as 600 ± 400 days, which led to the growth of anammox bacteria which contributed to a destabilization of the partial nitritation. In addition, a biofilm with a high content of *Nitrospira* spp. (NOB-type bacteria) developed in the wall of the riser of the airlift reactor leading to a complete destabilization of the partial nitritation, which indicated the importance of the cleaning and maintenance of the reactor walls.

7.1. INTRODUCTION

The implementation of the biological autotrophic nitrogen removal (BNR) process in the main water line of urban wastewater treatment plants (WWTPs) can make these facilities as energy-neutral or even energy-positive (Kartal et al., 2010; Siegrist et al., 2008). The current interest on implementing a two-stage system for the autotrophic BNR process in the mainstream of urban WWTPs (Ma et al., 2011; Pérez et al., 2015) appeared after the unsuccessfully attempts of such implementation in one-stage systems. Most of one-stage systems working at mainstream conditions showed the failure of partial nitritation in the long-term operation due to the growth of nitrite oxidizing bacteria (NOB) (De Clippeleir et al., 2013; Hu et al., 2013; Winkler et al., 2011); and even those systems which succeed on achieving an effective NOB repression showed considerably low nitrogen conversion rates (Gilbert et al., 2014; Laureni et al., 2016; Malovanyy et al., 2015).

A stable long-term operation of the partial nitritation with effective NOB repression appears as a prerequisite for implementing a two-stage system for autotrophic BNR in the main water line of urban WWTPs, and thus, it has been under continuous research (Isanta et al., 2015a; Pérez et al., 2014; Wett et al., 2013). The main challenge was enhancing the growth of ammonia oxidizing bacteria (AOB) in detriment of the growth of NOB at mainstream conditions (low temperature and low nitrogen concentrations), however NOB are reported to grow faster than AOB at temperatures lower than 25 °C (Hellinga et al., 1998).

Operational parameters such as pH, temperature, free ammonia and free nitrous acid concentrations, sludge retention time (SRT) and dissolved oxygen (DO) concentration affect to the kinetics of AOB and NOB (Van Hulle et al., 2010), and thus, different strategies based on these parameters or combinations thereof have been applied to favour AOB over NOB at mainstream conditions. On the one hand, strategies based on the free ammonia and free nitrous acid inhibitions were often difficult to apply due to the low nitrogen concentrations typical of mainstream. Still, Wang et al. (2016a) recently reported a NOB repression strategy which combined the sludge treatment using free nitrous acid with the DO control in a nitritation reactor treating a low-strength wastewater. On the other hand, it was generally known that NOB spp. present higher oxygen affinity than AOB spp. (Sin et al., 2008) and strategies based on the application of low concentrations of DO in the bulk liquid were highly used (Blackburne et al., 2008; Gilbert et al., 2014; Hu et al., 2013). However, it was recently demonstrated that NOB (specifically *Nitrospira* spp.) presented higher oxygen affinity than

AOB (Regmi et al., 2014) and, thus, high DO concentrations should be applied instead in systems presenting *Nitrospira* spp. to provide competitive advantage for AOB over NOB. Likewise, Gao et al. (2014) proposed an aeration control strategy depending on the temperature and ammonia concentration in the influent to effectively repress NOB at room temperature (12–27 ºC) operating at DO concentrations as high as 2–7 mg L^{-1}. Moreover, despite of operating at DO concentrations higher than 1.5 mg L^{-1}, Regmi et al. (2014) also reported the use of an online aeration and SRT control strategy which guaranteed an operation close to the critical AOB wash out and favoured NOB repression at mainstream conditions at 25 ºC. In this way, strategies based on limiting the overall SRT were also surmised, however in some cases the SRT applied was too low (2.4 days in Blackburne et al. (2008); 3 days in Ahn et al. (2008)) leading to a challenging implementation in practice, since the biomass levels and reaction rates achieved could lead to a problematic real scale implementation. It was also previously reported that stable partial nitritation was achieved if an adequate ratio between DO and total ammonium concentrations (DO/TAN) in the bulk liquid was applied in granular systems (Bartrolí et al., 2010; Isanta et al., 2015a; Jemaat et al., 2013). A low DO/TAN ratio ensured oxygen limiting conditions and a residual concentration of ammonium in the bulk liquid, which guaranteed the repression of NOB activity even at high DO concentrations. Overall, either in one and two-stage systems, further study of the AOB and NOB competition would be still needed to define the operational conditions that enable effective NOB repression and stable partial nitritation in the long-term operation.

The aim of the present study was not exploring the competition between AOB and NOB, but trying to explain the reasons why a previously successful partial nitritation achieved at mainstream conditions (described in the Chapter 5 of this thesis) was destabilized. Stable partial nitritation with efficient NOB repression was achieved in a granular sludge airlift reactor treating a low-strength influent at 10 ºC and the operation successfully prevailed in the long-term. However, nitratation activity appeared with apparently no reason. Thus, two main objectives were defined: (i) study the reasons why the destabilization occurred and (ii) explore how to avoid/confront such destabilization in a further real-scale implementation perspective.

7.2. MATERIALS AND METHODS

7.2.1. Reactor set up and wastewater

A lab-scale airlift reactor with a total working volume of 5.2 L was used. The detailed diagram of the reactor and set-up details are described in Section 4.1.1, Chapter 4. Compressed air was supplied through an air diffuser placed at the bottom of the reactor and was manually manipulated to maintain the DO concentration in the bulk liquid in the range 0.3–3.3 mg O_2 L^{-1}. The pH was measured on-line and automatically controlled at 8.0 ± 0.1 by dosing a Na_2CO_3 0.5 M solution. The temperature was measured and controlled at different set points depending on the experimental period: 10, 15, 20, 25 and 30 ºC. Total ammonia nitrogen (TAN = $N\text{-}NH_4^+$ + $N\text{-}NH_3$) and nitrate concentrations in the bulk liquid were measured on-line. TAN concentration in the bulk liquid was automatically controlled by varying the inflow rate by means of a proportional controller during the whole period of operation.

The operation of the lab-scale airlift reactor last for almost two years and was divided in three different periods, according to the stable or unstable operation. Thus, period I (days 0–400) corresponded to the stable operation performing partial nitritation at 10 °C (detailed operation from day 0 to 250 is described in Chapter 5). Period II (days 401–540) corresponded to the destabilization of partial nitritation and increase of nitratation activity. Period III (541–700) corresponded to a brief recovery of the process followed by the definitive destabilization of partial nitritation and further increase of nitratation activity.

7.2.2. Inoculum and influent characteristics

As previously described in Chapter 5, the initial biomass of the airlift reactor was enriched in AOB and adapted to low temperature (12.5 ºC) in a reactor which was operating for more than 400 days performing stable partial nitritation (Isanta et al., 2015a). The inoculum contained around 81 ± 12% of AOB and 1 ± 1% of NOB as analysed by fluorescence in situ hybridization (FISH).

The granular sludge airlift reactor treated a synthetic influent with an average TAN concentration of 70 mg N L^{-1}, which mimics a pretreated municipal wastewater coming from the mixture of the effluent of a previous A-stage plus the recirculation of the reject water of the digested sludge, as in an anammox-based WWTP (Isanta et al., 2015a; Kartal et al., 2010).

The synthetic influent also contained: 45 mg KH_2PO_4 L^{-1}, 784 mg $NaHCO_3$ L^{-1}, 80 mg NaCl L^{-1}, 40 mg $CaCl_2$ L^{-1}, 90 mg $MgCl_2$ L^{-1} and 1 mL of trace elements solution per L of influent (Guerrero et al., 2011).

7.2.3. Anammox activity batch test

A batch test to determine the anammox activity was performed in the lab-scale airlift reactor on day 603 (period III). First, influent flow was stopped and aeration was maintained until TAN concentration was around 10 mg $N\text{-}NH_4^+$ L^{-1}. Then (minute 98), air supply was changed by nitrogen gas (N_2) supply through the air diffuser placed at the bottom of the reactor. When DO concentration was lower than 0.03 mg O_2 L^{-1} a pulse of ammonium and nitrite was done in a concentrations ratio adequate for anammox biomass (i.e. approximately 1.32 g $N\text{-}NO_2^-$ g^{-1} $N\text{-}NH_4^+$, Strous et al. (1998)). pH was maintained at 8 ± 0.3 by dosing H_2SO_4 10% (v/v). Bulk liquid samples were periodically withdrawn from the reactor to measure TAN, total nitrite nitrogen (TNN) and nitrate off-line. The batch test was finished when TAN concentration was c.a. 10 mg $N\text{-}NH_4^+$ L^{-1}.

The nitrite and ammonium consumption rates and nitrate production rate were calculated from the slope of the plots depicting ammonium, nitrite and nitrate concentrations versus time, respectively. Thus, the nitrite to ammonium consumption ratio and the nitrate produced to ammonium consumed ratio were calculated by dividing the nitrite consumption and nitrate production rates by the ammonium consumption rate.

7.2.4. Fluorescence *in situ* hybridization (FISH)

Abundances of AOB, NOB and anammox bacteria were analysed by FISH technique coupled to confocal laser scanning microscopy (CLSM) as described in Section 4.3.1 of Chapter 4. Regarding AOB, specific probes for *Nitrosomonas* spp. were 5'-6FAM-labeled and 5'-ALEXA594-labeled. Regarding NOB, specific probes for *Nitrobacter* spp. were 5'-Cy3-labeled and 5'-PacificBlue-labeled and a specific probe for *Nitrospira* spp. was 5'-6FAM-labeled. For anammox bacteria, general anammox probes were 5'-ALEXA488-labeled and 5'-Cy3-labeled. The different labelled probes were used depending on the different populations analysed on each sample. Hybridization protocol and probes are fully described in Section 4.3.1 of Chapter 4.

7.2.5. Specific analytical methods and calculations

TAN, TNN and nitrate concentrations were measured off-line according to Section 4.2.1, Chapter 4. These measured off-line values are the ones represented in the results section. Sludge retention time (SRT) was calculated according to equation Eq. 5.2. described in Section 5.2.4, Chapter 5.

The amount of nitrate theoretically produced by anammox was calculated according to the anammox stoichiometry considering that 0.26 moles of nitrate are produced for each 1.02 moles of N_2, as described by Strous et al. (1998). For calculating the N_2, it was considered that the nitrogen disbalance occurred in the granular airlift reactor was only due to the production of N_2 by anammox.

7.3. RESULTS AND DISCUSSION

7.3.1. Reactor operation

The lab-scale airlift reactor was operated for almost 2 years treating a low-strength synthetic influent (Fig. 7.1). During period I (days 0–400) a stable partial nitritation at high nitrogen loading rate (NLR = 0.59 ± 0.09 g N L^{-1} d^{-1}) with effective NOB repression was achieved at 10 ºC (operation between days 0 and 250 is more detailed in Chapter 5). Between days 0 and 200 less than 1 mg N-NO_3^- L^{-1} was produced in the reactor and between days 201 and 400 only 2 ± 2 mg N-NO_3^- L^{-1} were produced in the bulk liquid of the granular sludge airlift reactor. Furthermore, an adequate effluent for a subsequent anammox reactor was achieved (1.1 ± 0.2 mg N-NO_2^- mg^{-1} N-NH_4^+, Fig. 7.2). The success of such operation was based on the maintenance of a low DO/TAN concentrations ratio in the bulk liquid (0.05 ± 0.04 mg O_2 mg^{-1} N), which was previously reported to maintain stable partial nitritation in granular systems (Bartrolí et al., 2010; Jemaat et al., 2013). Hence, DO/TAN concentrations ratio was maintained at low values during the entire operation of the airlift reactor (Fig 7.2).

However, in period II (days 401–540) nitrate concentration increased while nitrite concentration decreased in the bulk liquid. Nitrate accumulated until achieving very similar values to those of nitrite concentrations. Since NOB were reported to show a more sensitive temperature dependence than AOB (Hunik et al., 1994), temperature was increased on day

455 from 10 to 20 ºC to favour AOB over NOB with the aim of enhancing nitritation activity in detriment of nitratation activity. Operation was maintained stable for some weeks, however one month after the increase of temperature, the nitrate concentration sharply increased until achieving a value of 30.7 mg $N\text{-}NO_3^-$ L^{-1} and nitrite concentration resulted as low as 9.7 mg $N\text{-}NO_2^-$ L^{-1} on day 500. Nevertheless, on day 500 the abrupt increase stopped and nitrate concentration was stabilized in 25 ± 3 mg $N\text{-}NO_3^-$ L^{-1} for the next month. During period II, DO/TAN concentrations ratio was considerably lower than the applied in the rest of the operation (Fig. 7.2) but still the nitritation activity was the highest achieved.

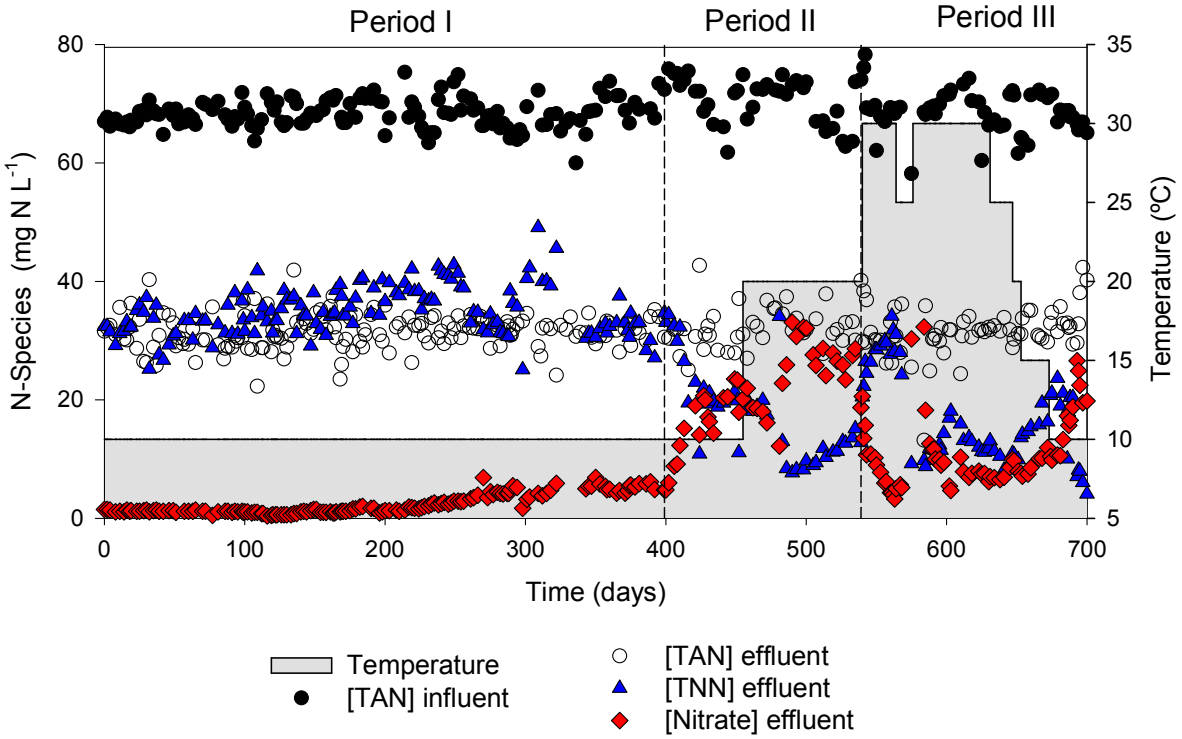

Fig. 7.1. Long-term operation of the lab-scale granular airlift reactor treating a low strength synthetic influent.

At the beginning of period III (days 541–700) temperature was directly increased from 20 to 30 °C with the aim of recovering the partial nitritation. It was reported that AOB growth rate becomes higher than NOB growth rate at temperatures above 25 ºC (Hellinga et al., 1998; Van Hulle et al., 2010). Actually, this is the principle used in the SHARON (Single reactor High activity Ammonia Removal Over Nitrite) process, where a chemostat operated at high temperature (30–40°C) and an appropriate SRT guarantees that AOB are maintained in the reactor, while NOB are washed out (Hellinga et al., 1998). Hence, during the first month of period III the partial nitritation was recovered and stabilized, with an immediate decrease of

nitrate concentration once temperature increased to 30 °C and, in addition, with a nitrite to ammonium (TNN/TAN) concentrations ratio adequate for a subsequent anammox reactor.

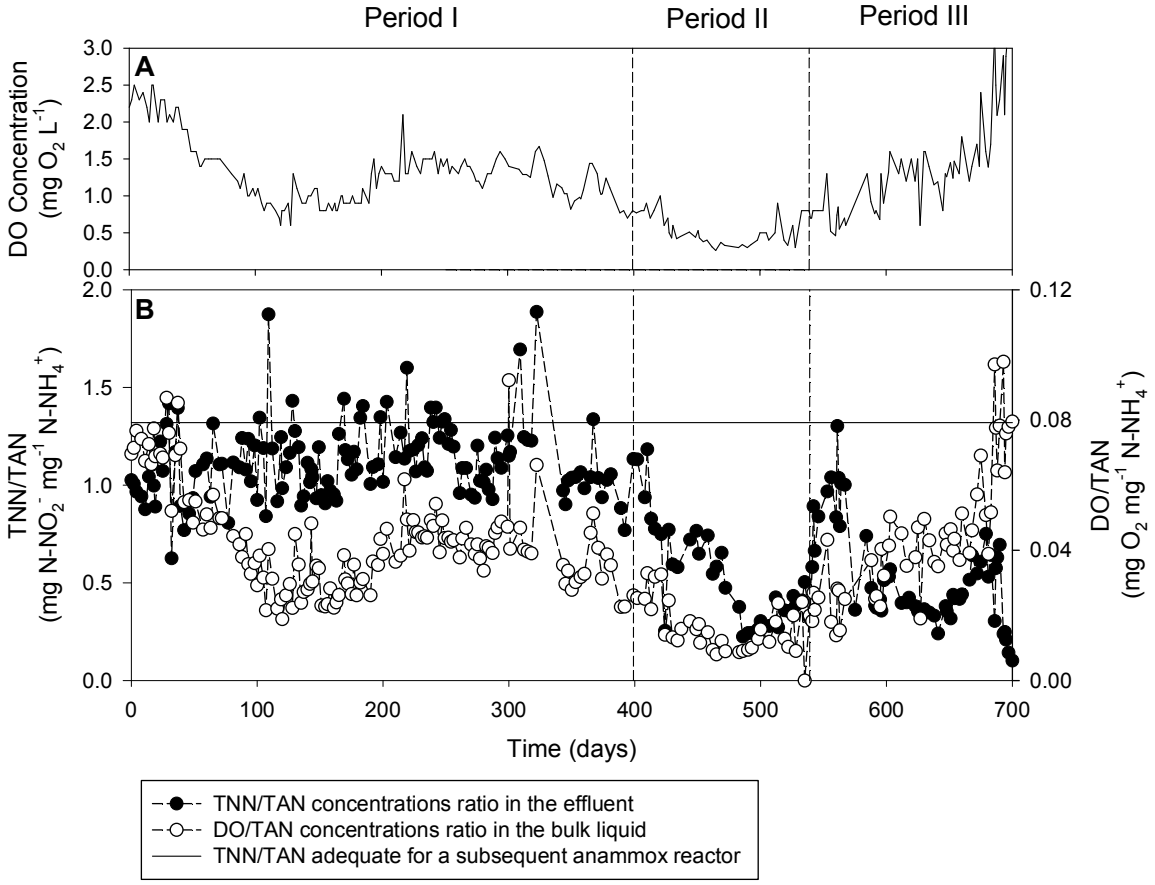

Fig. 7.2. Evolution of the dissolved oxygen concentration (A) and the DO/TAN and TNN/TAN concentrations ratios (B) in the bulk liquid of the lab-scale airlift reactor during the long-term operation treating a low-strength synthetic influent. DO: dissolved oxygen; TAN: total ammonium nitrogen; TNN: total nitrite nitrogen.

On day 569, after the recovery of partial nitritation, an attempt of working at lower temperature was made by decreasing the temperature from 30 to 25 °C. However, after one week at 25 °C the nitrate rapidly increased and partial nitritation destabilized. For that reason, temperature was increased again to 30 °C and a diminishment of nitrate concentration occurred. Nevertheless, nitrite concentration did not increase and a partial nitritation with a TNN/TAN concentrations ratio adequate for a subsequent anammox reactor was not recovered this time. However, nitrate concentration was maintained stable in an average value of 7 ± 2 mg $N\text{-}NO_3^-$ L^{-1}, so temperature was gradually lowered until achieving 10 °C on day 673 to evaluate if operation could be still maintained at low temperature. Nonetheless, nitrate

concentration sharply increased while nitrite concentration decreased resulting in a complete destabilization of the partial nitritation process.

Granules size was maintained stable during the entire operation of the airlift reactor, with an average value of 700 ± 100 μm. Between days 50–500, the biomass concentration was maintained stable with an average value of 3.3 ± 0.8 g VSS L^{-1}, afterwards biomass concentration decreased and it was maintained stable in 0.5 ± 0.1 g VSS L^{-1} from day 550 onwards. The decrease of biomass concentration was due to the biomass purge performed from day 500 onwards.

7.3.2. Development of the nitratation activity

The airlift reactor was operated performing stable partial nitritation for 400 days at 10 °C (Fig. 7.1). Between days 0 and 200 nitrate production was barely detected, with less than 1 mg $N\text{-}NO_3^-$ L^{-1} produced in the reactor, while between days 201 and 400 the production of nitrate increased but maintained at an average value 2 ± 2 mg $N\text{-}NO_3^-$ L^{-1}. This successful NOB repression was achieved by maintaining a very low DO/TAN concentrations ratio, i.e. by imposing very strong oxygen limiting conditions in the bulk liquid of the reactor (Bartrolí et al. 2010; Jemaat et al., 2013). Actually, it was the high residual TAN concentration (c.a. 31 mg $N\text{-}NH_4^+$ L^{-1}, Fig. 7.1) rather than the DO concentration the reason why a low DO/TAN concentrations ratio was maintained, as previously demonstrated in Isanta et al., (2015a). In fact, the DO concentration continuously changed during the operation of the airlift reactor (Fig. 7.2). Since DO/TAN was maintained during period I (Fig. 7.2), there should be another reason for the slight increase of nitritation activity between days 201–400 compared to days 0–200; and also for the further destabilization of partial nitritation on period II.

7.3.2.1. Effect of solid retention time

Solid retention time (SRT) is an important parameter to consider in any biological process, and even more with autotrophic microorganisms (e.g. nitrifying bacteria) that are characterized by slow growth rates (Hunik, 1993). Thus, a high SRT is usually needed to increase the biomass concentration in the system and guarantee the biomass growth. A high SRT is guaranteed when granular sludge is used, so the exact value of SRT applied does not receive as much attention as in other biological systems such as activated sludge systems. However, in the nitritation process SRT is a parameter to pay attention to, since the NOB wash out occurs if the SRT required for NOB growth is higher than the current SRT of the

system (Hellinga et al., 1998; Jubany et al., 2009b). This means that the NOB repression will be achieved through a kinetic selection which favours AOB over NOB.

Fig. 7.3 shows the evolution of SRT during the long-term operation of the granular sludge airlift reactor. The average value of SRT during period I was as high as 70 ± 30 days and, moreover, it sharply increased in period II until 600 ± 400 days, i.e. SRT could be considered as ∞. In period II the nitratation activity drastically increased in the granular airlift reactor, once almost 6 times the SRT had past after the inoculation ((400 d)/(70 d^{-1}) = 5.7). So, it could be suggested that the development of the nitratation activity could be related to the high SRT applied.

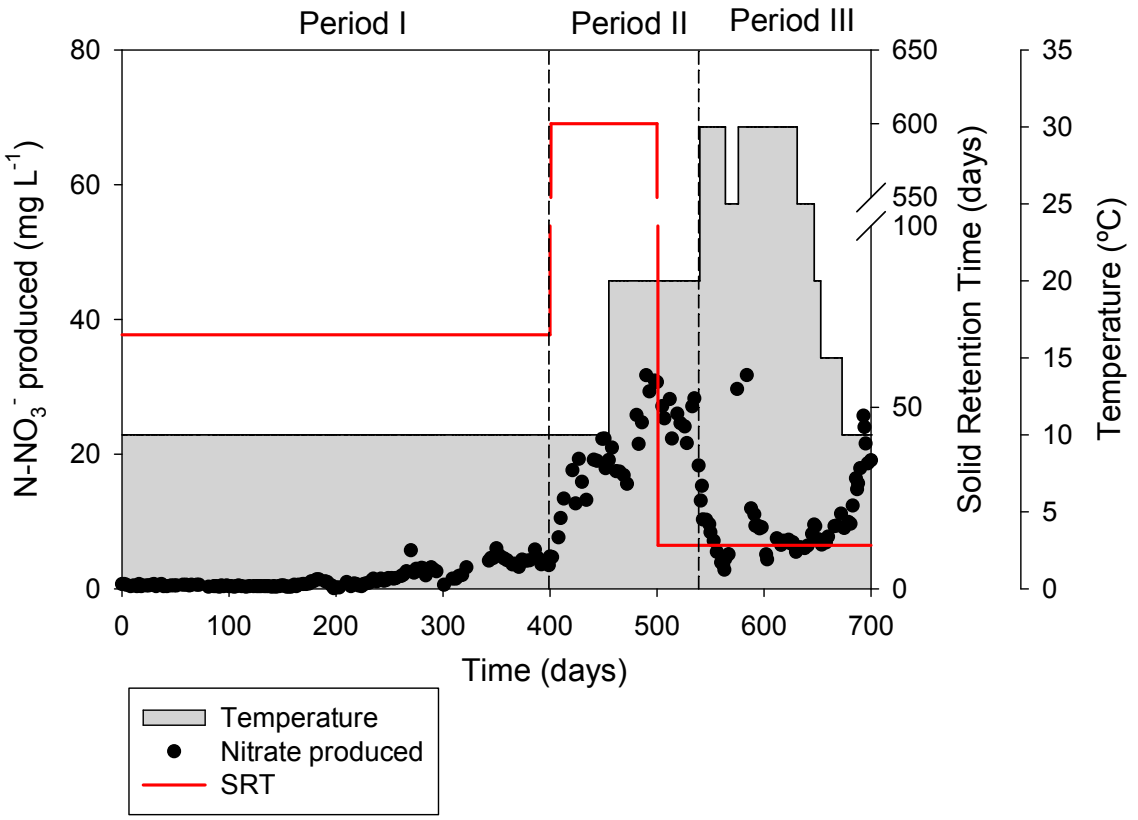

Fig. 7.3. Solid retention time versus the nitrate production in the long-term operation of the lab-scale granular sludge airlift reactor.

In period I, a stable NOB repression was maintained in the reactor for 400 days, despite of the high SRT applied (70 ± 30 days) which could be higher than the minimum SRT needed for NOB growth. The applied SRT was much higher than the applied by Isanta et al. (2015a) in the granular sludge airlift reactor which provided the inoculum of the present study, where an effective NOB repression was achieved at 12.5 °C by also applying the

DO/TAN strategy but maintaining a SRT of 8 ± 3 days. Nevertheless, during period I nitratation activity was barely detected in the reactor of the present study and it was only started to be noticed during period II. This could be due to that such high SRT applied made the nitratation activity more slowly noticeable and also to the strong effectiveness of the system. Actually, nitratation activity appeared in period II after that almost 6 times the SRT applied had past. As SRT continued being high and even drastically increased until a value considered as ∞, the high nitratation activity continued increasing rather than stabilized (Fig. 7.3). Thus, although temperature was increased to favour AOB over NOB, the SRT of the system was much higher than the minimum required for NOB and thus nitratation activity was very high. Once SRT was decreased at the end of period II (by purging the reactor from day 500 onwards), the nitrate concentration increase was stopped but maintained stable at high values (25 ± 3 mg $N\text{-}NO_3^-$ L^{-1}). Then, temperature was increased to 30 °C, SRT was maintained low (5 ± 4 days) and partial nitritation was recovered at the beginning of period III. From day 500 onwards, SRT was maintained at 12 ± 12 days by periodically purging the reactor and NOB repression was maintained when temperature was higher than 20 ºC. However, stable partial nitritation with efficient NOB repression was not recovered when temperature was lowered below 20 ºC, despite of using low DO/TAN values and low SRT. It could be hypothesised that NOB developed inside of granules when high SRT were applied and thus, even when purges were done in the airlift reactor, the NOB wash out was expected to be very slow via biomass detachment. In fact, Isanta et al. (2015a) reported a slow NOB wash out in a similar granular system where more than 300 days were needed for a decrease of NOB population from c.a. 20% to 1%. Nevertheless, in the reported work an effective NOB repression was achieved due to the application of the DO/TAN strategy even though NOB were present in the sludge, so in the reactor of the present study the reason why nitratation activity could not be repressed when SRT was lowered and DO/TAN ratio was maintained low was still unclear.

The importance of SRT in the competition of AOB versus NOB was reported before, although mainly in activated sludge systems. For instance, Ahn et al. (2008) achieved stable partial nitritation by a selective wash out of NOB through the combination of oxygen limitation, free ammonia inhibition and operation at a NOB limiting SRT of 3 days. However, an increase in SRT destabilized the partial nitritation. In addition, Jubany et al. (2009b) calculated the different SRT required for NOB growth under different conditions applied in a system treating reject water at 25 °C. The reported study achieved effective NOB wash out at

operation SRT of 30 days, demonstrating that a high SRT was not the key factor for NOB proliferation, but the different strategies applied were. Thus, an efficient NOB repression is possible at high SRT but with the adequate repression strategies, since the minimum SRT required for NOB growth changes for each specific case. In the present study the minimum SRT required for NOB growth was unknown since specific kinetic experiments should be done to calculate it.

In any case, two related implications could be extracted: (i) in addition to the DO/TAN strategy applied, the effect of SRT should not be overlooked in the system, and (ii) the importance of purging the system should be pointed out, although the NOB wash out is expected to be slow if NOB already developed inside the granule.

7.3.2.2. Growth of anammox bacteria

After recovering the stable partial nitritation at the beginning of period III by maintaining an adequate SRT (5 ± 4 days) at 30 °C, temperature was lowered to 25 °C, since at such temperature AOB growth was expected to be higher than NOB (Hellinga et al., 1998; Van Hulle et al., 2010). Nevertheless, after one week operating at 25 °C, nitrate accumulated in the reactor and temperature was increased again to 30 °C. Then, nitrate concentration decreased in the bulk liquid; however, this decreased was not associated to an increase of nitrite concentration, appearing a nitrogen disbalance in the reactor (Fig. 7.4).

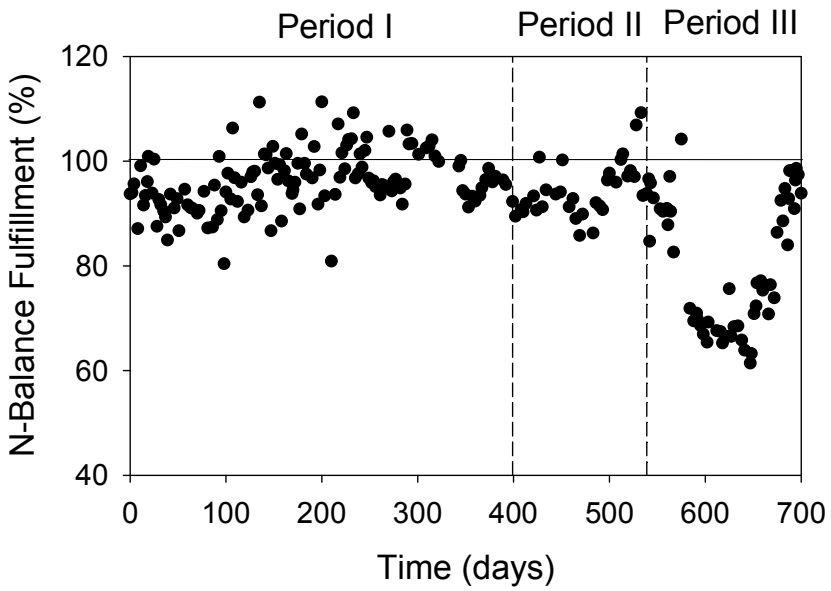

Fig. 7.4. Nitrogen balance fulfilment during the operation of the lab-scale airlift reactor treating a synthetic low-strength influent.

Overall, nitrogen balance was fulfilled during period I of operation, with an average value of 96 ± 6% and it was slightly unfulfilled in periods II, with an average value of 93 ± 9%. But the nitrogen balance was clearly unfulfilled during the operation on period III, more specifically between days 584–673 when the average value was 69 ± 4%. Therefore, the presence of anammox bacteria in the granular sludge of the anammox reactor was expected.

A batch test with anammox substrates (ammonium and nitrite) in the bulk liquid devoid of oxygen was performed on day 603 to evaluate the presence of anammox bacteria in the granular sludge airlift reactor (see details in Section 7.2.3). Fig. 7.5 shows the evolution of ammonium, nitrite and nitrate during the essay. On minute 98, the influent pump was stopped and a pulse of anammox substrates was done in the bulk liquid previously flushed with N_2 as explained in Section 7.2.3. The high consumption of ammonium and nitrite was not explained by the activity of AOB and NOB microorganisms since DO concentration was maintained below 0.3 mg O_2 L^{-1}. Furthermore, the nitrite to ammonium consumption and nitrate production to ammonium consumption ratios obtained (1.03 ± 0.07 and 0.22 ± 0.07, respectively) were close to the anammox stoichiometry (Lotti et al., 2014c; Strous et al., 1998). Hence, anammox bacteria developed in the granular sludge of the airlift reactor. This fact could be expected because: (i) DO concentration was considerably low during period II (Fig. 7.2) and, thus, the gradient of oxygen inside the granules could lead to an anoxic zone in the granule where the anammox bacteria developed, such as in the CANON process; (ii) the increase of temperature to 30 °C favoured anammox growth since optimal temperatures for anammox were reported to be between 30–40 °C (Dosta et al., 2008), and (iii) SRT was high enough to allow anammox growth.

Nitrogen balance was already slightly unfulfilled in period II when DO concentration in the bulk liquid was low, but temperature was not optimal for anammox microorganisms (20 °C). So it could be hypothesised that anammox were not the responsible of such unfulfillment in nitrogen balance. However, SRT was extremely high in period II and it was reported that anammox bacteria need a minimum SRT of 85 days at 15 °C (Morales et al., 2015), which is lower than the SRT in the airlift reactor and, furthermore, temperature was higher than 15 °C, which is expected to decrease such minimum SRT value. Therefore, the appearance of anammox could be expected already during period II.

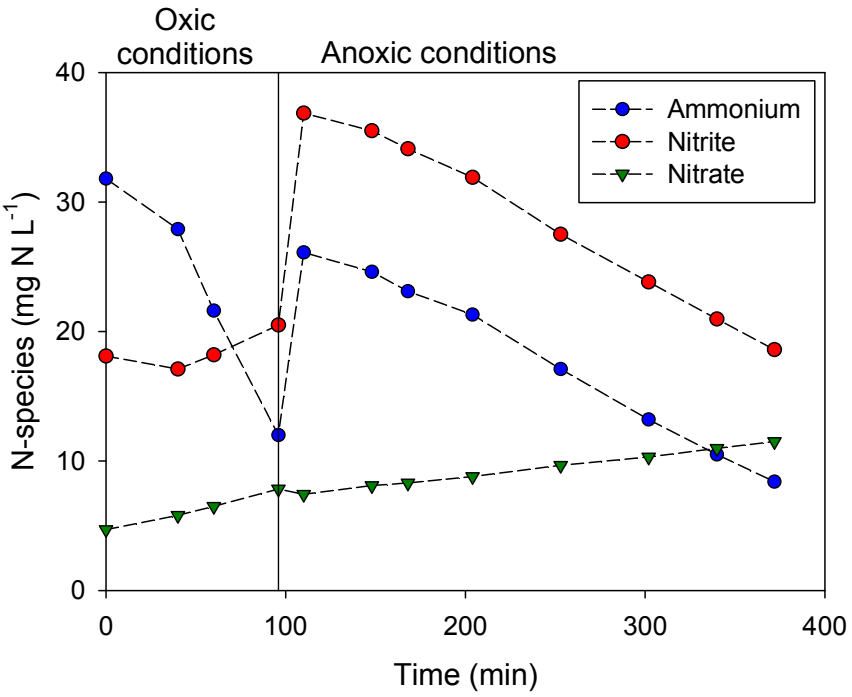

Fig. 7.5. Batch test performed on day 603 (period IV) for evaluating the anammox activity in the lab-scale airlift reactor. DO concentration < 0.3 mg O_2 L^{-1} during the anoxic conditions; pH = 8 ± 0.3.

Anammox bacteria use ammonium as electron donor and nitrite as electron acceptor to remove nitrogen as N_2 gas (i.e. anammox process consists of an autotrophic denitrification). However, part of the nitrite is oxidized to nitrate in the anammox reaction. Thus, anammox could contribute to the nitrate production in the bulk liquid of the granular airlift reactor. Nevertheless, the nitrate theoretically produced if the disbalance obtained in period II was due to the production of N_2 by anammox bacteria was calculated (see details in Section 7.2.5) and it resulted much lower than the nitrate actually produced in the reactor. Therefore, anammox bacteria could be present in the granular sludge but still its activity was dismissible compared to NOB activity. When temperature was lowered to 10 ºC at the end of period III (day 673) the nitrogen balance was recovered with an average value of 93 ± 4%. The wash out of anammox bacteria was expected due to the combination of the following facts: low temperature (10 ºC), high DO concentration (2.5 ± 0.6 mg O_2 L^{-1}) and low SRT (12 ± 12 days).

The growth of anammox in the lab-scale airlift reactor would be beneficial if the aim of the study was operating a one-stage system where both AOB and anammox bacteria were responsible of the autotrophic nitrogen removal in a single reactor. However, the present

study aimed for a two-stage system and more precisely for the development of a stable partial nitritation process in one single reactor, so the presence of anammox bacteria was not desirable. In any case, the temperature decrease had a negatively effect on the anammox population and anammox activity faded in the system. Once anammox activity did not take place in the reactor the nitritation activity increased. It was surmised that anammox bacteria could not compete with NOB for the nitrite anymore, and thus nitratation activity caused by NOB increased at the end of the operation.

7.3.3. Microbial characterization

The lab-scale airlift reactor was operated performing stable partial nitritation at 10 °C for 400 days and afterwards, the nitratation activity developed and fluctuated for 300 days more at different temperatures of operation (Fig. 7.1). The analysis of operational data suggested that the increase of nitratation activity could be due to the development of NOB in the granular sludge and also suggested that anammox bacteria developed in the granules. Therefore, an exhaustive microbial characterization of the granular biomass of the lab-scale airlift reactor was performed to confirm the hypothesis presented before about the appearance of different microorganisms (NOB and anammox bacteria). The microbial characterization was performed by using FISH-CLSM technique.

Samples of granular biomass from days 98, 233, 421, 498, 596, and 682 were analysed to evaluate the AOB enrichment, NOB repression and presence of anammox bacteria during the different periods of operation. Fig. 7.6 shows the results of FISH characterization together with the operation of the granular airlift reactor, more specifically together with the nitrite and nitrate concentrations in the bulk liquid.

A high enrichment in AOB was detected during the entire operation of the granular airlift reactor. In fact, even in period II, when the nitratation activity increased in the reactor, the abundance of AOB in the granular sludge was higher than 90% of the total population. However, the AOB relative abundance decreased until 71 ± 3% of the total population at the end of the operation (day 682), when the partial nitritation was totally destabilized, but still then, AOB were the predominant microorganisms in the granular sludge of the granular lab-scale airlift reactor.

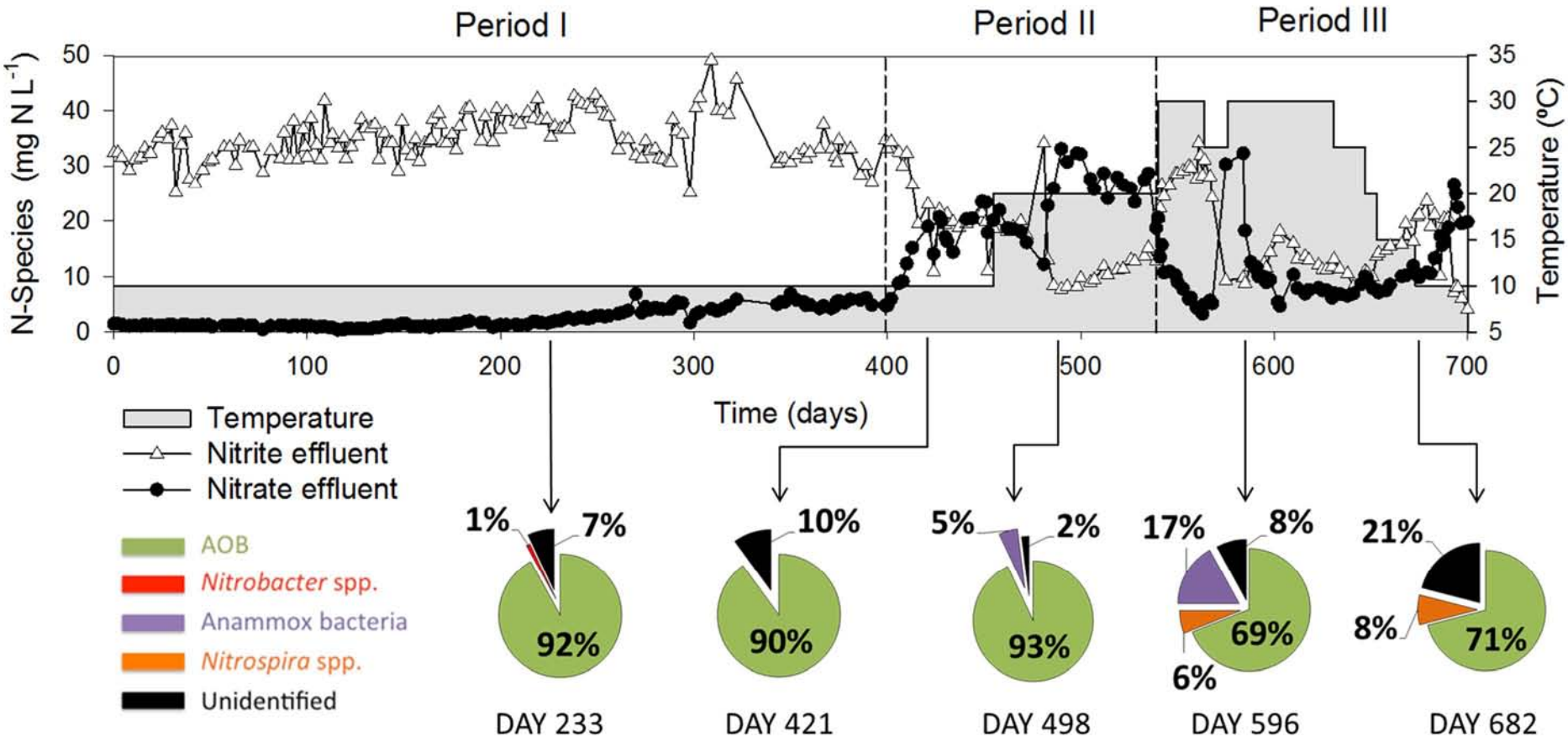

Fig. 7.6. FISH-CLSM results obtained through the long-term operation of the lab-scale airlift reactor treating a low strength synthetic influent. Nitrite and nitrate concentrations together with temperature were plotted to facilitate the understanding of the developed microorganisms.

Regarding anammox bacteria, FISH analysis detected the appearance of anammox microorganisms in the granular sludge at the end of period II (day 498) with a relative abundance of 5 ± 2% of the total population. This fact confirmed the hypothesis suggested in Section 7.3.2.2 where the presence of anammox bacteria in the granules was surmised according to the slight nitrogen disbalance observed. Furthermore, a high presence of anammox bacteria was detected in period III (day 596) with a relative abundance of 17 ± 2%, which agreed with the results of the anammox activity batch test performed on day 603 in the airlift reactor and with the nitrogen disbalance at the beginning of such period. Hence, anammox bacteria appeared in the granular sludge in period II when temperature increased to 20 ºC and continued growing in granules during period III. The presence of anammox bacteria was not analysed by FISH at the end of the operation (on day 682) since temperature was low (10 ºC) and nitrogen balance was fulfilled, however the relative high percentage of microorganisms without identification by FISH on the sample analysed (21%) could suggest that anammox bacteria still remained in the granules, which was expected since the wash out of microorganisms from the inner part of the granule should be gradual due to the slow detachment. In any case, FISH results regarding anammox bacteria agreed the operational results described in Section 7.3.2.2, by confirming the presence of anammox bacteria in the granular sludge of the airlift reactor in periods II and III.

FISH technique was also used to evaluate the presence of NOB species during the long-term operation of the granular sludge airlift reactor. NOB species were expected to be present in the granular sludge when nitratation activity was high, however FISH results were controversial. On the one hand, FISH-CLSM analysis confirmed the efficient NOB repression achieved in period I, since only 1 ± 1% of the total population was identified as NOB (specifically *Nitrobacter* spp.) on sample of day 233. *Nitrobacter* spp. are r-strategist microorganisms and, consequently, are favoured to grow at high TNN concentrations, such as those in the airlift reactor of this study. Hence, FISH confirmed the NOB repression observed during period I, and the presence of a NOB species expected to grow at the conditions of period I. However, at the beginning of period II (day 421) any NOB (nor anammox) species were detected in granules, despite the increase of nitratation activity, so FISH results could not explain the high production of nitrate in the bulk liquid of the reactor. Furthermore, FISH analysis did not show the presence of NOB either at the end of period II (day 498), when the highest nitratation activity was observed in the airlift reactor. Hence, FISH analysis could not confirm that the high nitrate production observed in the bulk liquid of the reactor was due to

the growth of NOB in the granular sludge of the lab-scale airlift reactor. Surprisingly, FISH analysis did identified the NOB species *Nitrospira* spp. for the first time during period III of operation, with relative abundances of 6 ± 4% and 8 ± 2% on days 596 and 682, respectively. *Nitrospira* spp. are k-strategist microorganisms which are favoured to grow at low TNN concentrations. During period I of operation, a residual TNN concentration was maintained in the bulk liquid (35 ± 4 mg $N\text{-}NO_2^-$ L^{-1}) and thus, *Nitrospira* spp. were not expected in the reactor. However, from period II onwards, TNN concentration decreased to 17 ± 8 mg N L^{-1}, which could favoured the appearance of the *Nitrospira* population.

Hence, according to FISH analysis, NOB population was barely detected during the period I of operation (specifically *Nitrobacter* spp.) but was clearly detected during the period III (specifically *Nitrospira* spp.); nevertheless, NOB population was never detected between those two periods, which corresponded to the moment with the highest nitratation activity of the operation. The reason why FISH could not identify any NOB species during period II and the reason why NOB population changed from *Nitrobacter* spp. to *Nitrospira* spp. remained unclear.

7.3.4. Development of an enriched NOB biofilm in the riser of the airlift reactor

The long-term operation of the lab-scale granular sludge airlift reactor treating a low-strength synthetic influent comprised a period of 400 days achieving a stable partial nitritation at 10 ºC, and afterwards the process destabilized and different periods of stable and unstable operation were defined for 300 days more. A high SRT and anammox growth were considered for trying to explain the destabilization of partial nitritation despite of using a low DO/TAN ratio strategy (reported to achieve a successful NOB repression); however, even when SRT was lowered, anammox activity decreased and an adequate DO/TAN concentrations ratio was applied in the reactor, *Nitrospira* spp. appeared and triggered to a high nitrate production and stable partial nitritation was never recovered in the system. Thus, on day 700 of operation the reactor was shut down.

When the reactor was emptied, a huge biofilm was observed all along the internal part of the riser of the airlift reactor (Fig. 7.7). A sample of this biofilm was analysed by FISH-CLSM to evaluate the presence of NOB on it, since such presence could explain the high content of nitrate in the period II of operation when NOB were not detected in granules. On the one hand, a 40 ± 4% of the total population was identified as AOB and, furthermore, the presence of NOB was detected with a 20 ± 2% of the total population, and more specifically,

it belonged to *Nitrospira* spp. On the other hand, *Nitrobacter* spp. were not detected in the riser biofilm and anammox bacteria were not analysed.

Therefore, the low DO/TAN concentrations ratio applied in the bulk liquid of the granular airlift reactor guaranteed a stable long-term operation for 400 days, however the development of a biofilm of *Nitrospira* spp. in the riser of the reactor triggered the nitrate production and destabilization of partial nitritation. The reason why the biofilm was formed was not clear, since it could be due to different factors which could contribute to enhance such formation: a high EPS formation at 10 ºC (see 5.3.3 of Chapter 5), a tendency of the biomass to form biofilms, the long SRT applied, etc; but the effect of these factors on the biofilm formation was not demonstrated in this study.

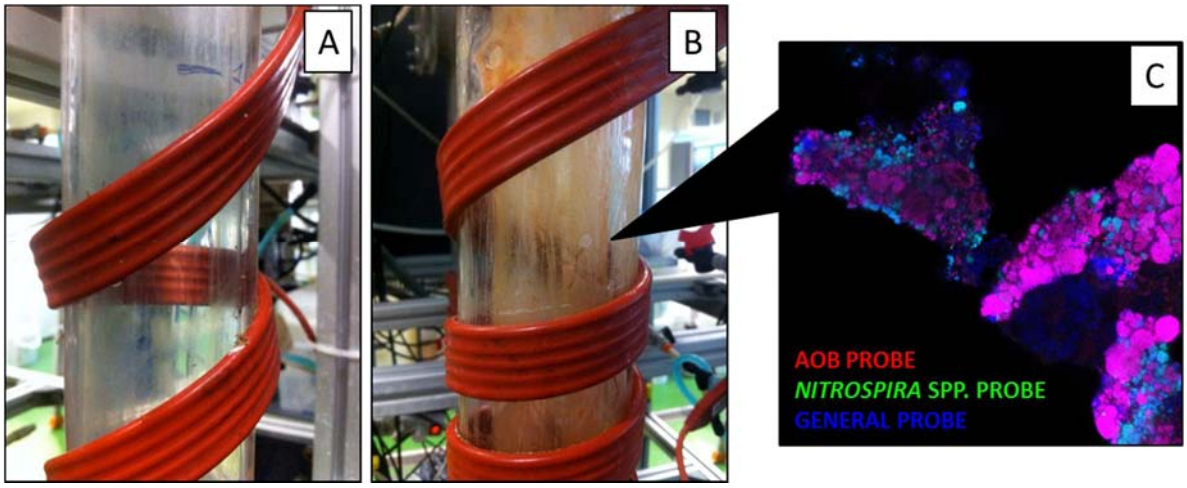

Fig. 7.7. A: Image of the clean riser of the airlift reactor; B: image of the riser of the airlift reactor when the biofilm developed; and C: overlapped FISH image of a sample from the biofilm developed in the riser of the airlift reactor, where AOB can be observed in pink and *Nitrospira* spp. in turquoise (mixture of dark blue and light green) (C).

In any case, NOB were not present in the granules until the end of the operation, despite of the formation of a biofilm full of NOB in the riser. This could be explained due to that the high TNN concentration in the bulk liquid of the reactor enhanced the selection of *Nitrobacter* spp. instead of *Nitrospira* spp. in the granules and thus, the DO/TAN strategy applied guaranteed strong oxygen limiting conditions in the granules, which allowed the NOB repression. The appearance of NOB in the granules from day 596 onwards was probably due to the detachment of NOB from the biofilm and, actually, the slight increase of NOB on day 682 was probably due to a more detachment from the biofilm rather than NOB growth in

granules, since DO/TAN strategy was demonstrated to achieve an efficient NOB repression in this system (period I) and in other similar systems (Bartrolí et al., 2010; Isanta, 2015a; Jemaat et al., 2013). Besides, the NOB present in samples from the end of the operation belonged to *Nitrospira* spp. as the NOB present in the riser biofilm, which supported the detachment hypothesis.

In any case, the formation of biofilm in the riser was unexpected and unstudied in the present work, and further research should be done to establish the causes of its appearance and the development of *Nitrospira* species on it. The reasons why *Nitrospira spp*. (k-strategists) appeared on the riser biofilm while *Nitrobacter* spp. did not appear on it remained unclear and further research should be needed. It could be hypothesised that *Nitrospira* spp. developed in the biofilm because it was the only available niche to compete with AOB since SRT was infinite and *Nitrospira* spp. were reported to have more affinity for oxygen than AOB (Regmi et al., 2014).

7.4. PRACTICAL IMPLICATIONS

The application of the DO/TAN strategy guaranteed the formation of aerobic granules enriched in AOB where NOB (specifically *Nitrobacter* spp.) were almost washed out from the system, guaranteeing a stable partial nitritation process at mainstream conditions. The operation was maintained stable for 400 days operating at 10 ºC, which demonstrated the strong effectiveness of the proposed system, which would be adequate for a further implementation at pilot-scale.

However, the operation was destabilized for reasons unknown but highly discussed above, which maybe could be avoided if more attention was previously paid to some operational issues:

- SRT should not be overlooked in any biological system although there was a different strategy applied to control competitions between microorganisms.
- The reactor walls should be periodically cleaned to avoid biofilm formation. The high presence of NOB were only detected in the biofilm of the riser, but never detected in granules, demonstrating the strong effectiveness of the DO/TAN strategy. Thus, the biofilm formation should be totally avoided. With a real-scale perspective, a periodical

cleaning of the reactor walls could be not feasible, so the use of anti-adherent materials for building the reactor could be an option.

7.5. CONCLUSIONS

The lab-scale granular sludge airlift reactor of the present study achieved a stable operation in the long-term at mainstream conditions.

Furthermore, the DO/TAN strategy was demonstrated as an efficient strategy to guarantee NOB repression, since NOB was strongly repressed in the granules even operating at an extremely high SRT.

The system destabilized most probably due to the appearance of a biofilm in the riser, which had a high content of *Nitrospira* spp.

Chapter 8

LOW-STRENGTH WASTEWATER TREATMENT IN AN ANAMMOX UASB REACTOR: EFFECT OF THE LIQUID UPFLOW VELOCITY

A modified version of this chapter is being prepared for publishing as:

Reino C. and Carrera J. 2016. Low-strength wastewater treatment in an anammox UASB reactor: effect of the liquid upflow velocity. *In preparation*.

Abstract

Two-stage systems have been proposed to overcome the drawbacks associated to the implementation of the autotrophic biological nitrogen removal process in the mainstream of urban wastewater treatment plants. In this study, an upflow anammox sludge blanket (UAnSB) reactor was successfully operated for 325 days treating a low-strength synthetic influent mimicking mainstream conditions. A nitrogen loading rate of up to 1.8 ± 0.2 g N L^{-1} d^{-1} was achieved and the nitrogen removal rate obtained (1.7 ± 0.1 g N L^{-1} d^{-1}) resulted considerably higher than most of the previously reported values for systems treating low-strength wastewater. FISH analysis showed a high enrichment in the anammox specie *Candidatus Brocadia anammoxidans* during the whole operation. The evolution of the granule diameter was followed throughout the operation of the UAnSB reactor and a direct correlation of the average granule diameter with the liquid upflow velocity (V_{up}) was established, being the higher the V_{up}, the bigger the granules. A stable granule diameter of 790 ± 40 μm was achieved by maintaining a V_{up} of 1.0 ± 0.1 m h^{-1}. The low V_{upS} applied avoid the use of effluent recirculation which would present a huge inconvenient to implement UAnSB reactors at real scale, however these low V_{upS} led to external mass transfer limitations in the reactor. In spite of the mass transfer limitations, not only a high specific anammox activity (0.26 ± 0.02 g N g^{-1} VS d^{-1}) was achieved in the UASB reactor but also a high nitrogen removal (80 ± 3%).

8.1. INTRODUCTION

The implementation of the autotrophic biological nitrogen removal (BNR) in the mainstream of urban wastewater treatment plants (WWTPs) has been proposed as the main alternative for achieving a neutral energy-consumer or even an energy-producer urban WWTP (Kartal et al., 2010; Siegrist et al., 2008). The neutral energy-consumer urban WWTP is based on the use of most of the entering organic matter to produce biogas (with the subsequent energy recovery) plus the reduction of aeration costs due to the lower oxygen requirements of the autotrophic BNR compared to the conventional BNR trough nitrification and heterotrophic denitrification. Hence, it consists of a first step of organic matter removal (A-stage) and a second step of autotrophic BNR (B-stage) where the A-stage effluent is treated through the nitritation and anaerobic ammonium oxidation (anammox) processes.

The B-stage can be implemented either in one single reactor (one-stage systems) or in two reactors (two-stage systems). Recently, many studies were focused on the implementation of autotrophic BNR treating low-strength wastewater by using one-stage systems (De Clippeleir et al., 2013; Gilbert et al., 2014; Hu et al., 2013; Lackner et al., 2015; Laureni et al., 2016), however these systems present some significant disadvantages: (i) low nitrogen removal rates achieved; (ii) destabilization of the partial nitritation in the long term; and (iii) competition for nitrite of nitrite oxidizing bacteria (NOB) and anammox bacteria with the subsequent destabilization of anammox process. Thus, the two-stage strategy has appeared as an alternative to overcome these problems, since allows a more stable performance and control of the partial nitritation and anammox processes (Isanta et al., 2015a; Ma et al., 2011a; Pérez et al., 2015; Reino et al., 2016).

The application of the anammox process at mainstream conditions (low-strength and low temperature) appears as a prerequisite for the implementation of a two-stage system for autotrophic BNR in urban WWTPs. Thus, many efforts have been made to achieve a successful start-up and operation of anammox reactors treating mainstream wastewater (Awata et al., 2015; Hendrickx et al., 2014; Laureni et al., 2015; Lotti et al., 2014b; Ma et al., 2013). The main disadvantage of anammox process is the very long doubling time (10–12 days) of the anammox bacteria (Dosta et al., 2008; Strous et al., 1999). This slow growth is even more troublesome at mainstream conditions because of (i) the low biomass growth rate due to the operation temperature under the optimum range and (ii) the low net biomass production due to the low nitrogen content of the stream (Morales et al., 2015). Therefore, a

high solids retention time (SRT) is needed to increase the biomass concentration in the system and guarantee the growth of anammox at mainstream conditions.

One efficient alternative to achieve a high SRT is the use of granular sludge (Fernández et al., 2008; Strous et al., 1998; van der Star et al., 2007). Different reactor configurations have been proposed for achieving granular sludge under anaerobic conditions: sequencing batch reactors (SBRs), filters, expanded bed and fluidized bed reactors, among others (Liu et al., 2003). However, the use of Upflow Anaerobic Sludge Bed (UASB) reactors appear as the most attractive alternative for the implementation of anammox process (Hulshoff Pol et al., 2004; Imajo et al., 2004; Tang et al., 2011). The main advantage over other reactors is that UASB reactors present a high biomass retention capacity which allows achieving extremely high loading rates, and furthermore, the requirements of area and reactor size are low. However, UASB reactors usually operate at upflow velocities as low as 0.5–1.5 m h^{-1} (Latif et al., 2011; van Haandel and van der Lubbe, 2012), which can trigger to external mass transfer limitations due to the lack of efficient mixing in the sludge bed.

Biomass granulation is a complex process that can be affected by different factors; either physical and chemical factors related to the process conditions applied, or even biological factors such as the cell-to-cell communication (quorum sensing) (Liu et al., 2003). The selection pressure imposed on the sludge is one of the factors affecting granulation. Thus, the selection pressure theory hypothesizes that granulation process strongly depends on the continuous selection of sludge particles that occurs in the reactors, in such a way that high selection pressure would wash-out light and dispersed sludge while heavier sludge could be retained in the system (Hulshoff Pol et al., 2004; Liu et al., 2003). The selection pressure may result from any environmental condition, such as temperature, pH, hydraulic retention time, upflow velocity (V_{up}), reactor configuration, etc. In the case of UASB reactors, selection pressure generally depends on the liquid V_{up} and gas production, which affect to the shear force imposed to biomass. Thus, high liquid V_{upS} lead to high hydrodynamic shear forces which enhance the granulation process (Arne Alphenaar et al., 1993; Liu and Tay, 2002).

Hence, to overcome the limitations associated to UASB reactors (specifically external mass transfer problems), a variant of UASB reactors, Expanded Granular Sludge Bed (EGSB) reactors, appeared as an alternative for implementing the anammox process. In this way, Lotti et al. (2014b) reported high nitrogen loading rates (NLRs) in an anammox EGSB reactor treating urban wastewater, even at 10 ºC. In EGSB reactors, liquid V_{up}s are higher than 4 m h^{-1} to cause the granular sludge bed to expand (Seghezzo et al., 1998). For the implementation

of the anammox process in EGSB such high V_{up}s are achieved by recycling part of the effluent, i.e. a liquid recirculation is used. Nevertheless, the need of a liquid recirculation is problematic for the real-scale implementation of the anammox process in the mainstream of urban WWTPs since too high recirculation flows would be needed and, consequently, operational costs would be unacceptable. By now, few studies have focused on the effect of V_{up} on the operation of anammox UASB (UAnSB) reactors and besides, to the best of the author's knowledge, all have focused on operations with high-strength wastewaters (Jin et al., 2012; Xing et al., 2014).

Therefore, this study aimed to (i) implement the anammox process in an UASB reactor treating a low-strength synthetic influent achieving high nitrogen removal rates and high effluent quality, and (ii) study in depth the effect of the liquid V_{up} on the UAnSB reactor operation, specifically by following the granulation through the operation of the reactor.

8.2. MATERIAL AND METHODS

8.2.1. Reactor, experimental set-up and operation

The anammox process was carried out in a lab-scale UASB reactor with a working volume of 2 L including the gas-liquid-solid separator. The detailed diagram of the reactor and set-up details are described in Section 4.1.2, Chapter 4. The pH was not controlled but measured offline and its value was 7.9 ± 0.2 during the whole operation, as the pH of the influent was set at 7.5 ± 0.2. Influent was devoid of oxygen and the dissolved oxygen concentration in the bulk liquid of the reactor was always 0 mg L^{-1}. The temperature was measured and controlled by means of an electric heater connected to a temperature controller. The cooling system detailed in Section 4.1.2, Chapter 4 was not used in the present study.

The reactor operation was divided in four different periods, each one corresponding to a different NLR applied. The period I (days 0–50) corresponded to the start-up period when the NLR was gradually increased until a stable value was achieved. During period I, the temperature was controlled at 32 ºC. Period II (days 50–200) corresponded to the stable operation at a fixed NLR. Period III (days 200–250) was a period of transition when NLR was gradually increased again, until achieving a stable value in Period IV (days 250–325). During periods II to IV, the temperature was controlled at 26 ºC. Since substrate concentrations were not changed during the operation, NLR was changed by varying the inflow. Thus, changes in

NLR lead to changes in liquid V_{up} in the UAnSB reactor since the liquid V_{up} was only linked to inflow (no recirculation was used), so the periods of operation could also be related to the different V_{up}s.

8.2.2. Inoculum and synthetic wastewater

The UAnSB reactor was inoculated with 500 ml (13 g L^{-1}) of settled anammox granular biomass from an anammox SBR working at 35 ºC under stable conditions for more than one year (Isanta et al., 2015b). The operational characteristics of the anammox SBR are shown in Table 8.1. The inoculum was enriched in anammox bacteria, more specifically in *Candidatus Brocadia anammoxidans*, with an abundance of 86 ± 3% as analysed by fluorescence *in situ* hybridization (FISH). *Candidatus Kuenenia stuttgartiensis* was also analysed by FISH but not detected in the sample. On day 27 more biomass from the inoculum was added to the reactor to increase a 25% the sludge bed volume.

Table 8.1. Operational characteristics of the SBR which provided the inoculum of the UASB anammox reactor. (NLR = Nitrogen Loading Rate; NRR = Nitrogen Removal Rate)

Parameter	Average value	Units
NLR	0.45 ± 0.09	g N L^{-1} d^{-1}
NRR	0.36 ± 0.09	g N L^{-1} d^{-1}
$[N\text{-}NH_4^+]_{influent}$	180 ± 30	mg N L^{-1}
$[N\text{-}NO_2^-]_{influent}$	190 ± 30	mg N L^{-1}
Average granule diameter	920 ± 90	μm

The UAnSB reactor was fed with a synthetic influent mimicking the effluent of a previous partial nitritation reactor treating a municipal low-strength wastewater as the one described in Chapter 5. During periods I, II and III the synthetic influent contained 35 mg N-NH_4^+ L^{-1} in the form of $(NH_4)_2SO_4$ and 35 mg N-NO_2^- L^{-1} in the form of $NaNO_2$, which meant a nitrite to ammonium concentrations ratio of 1. However, from day 244 onwards the nitrite to ammonium concentrations ratio in the influent was increased until 1.20 ± 0.06 to adjust the substrate concentrations to the reported anammox stoichiometry (Strous et al., 1998),

resulting in average concentrations of 33 mg $N\text{-}NH_4^+$ L^{-1} and 38 mg $N\text{-}NO_2^-$ L^{-1}. In addition, 1000 mg $KHCO_3$ L^{-1}, 50 mg NaH_2PO_4 L^{-1}, 100 mg $CaCl_2 \cdot 2H_2O$ L^{-1}, 200 mg $MgSO_4.2H_2O$ L^{-1}, 6.3 mg $FeSO_4$ L^{-1}, 6.3 mg EDTA L^{-1} and 1.25 mL L^{-1} of a trace elements solution with 15 g EDTA L^{-1}, 0.43 g $ZnSO_4.7H_2O$ L^{-1}, 0.24 g $CoCl_2.6H_2O$ L^{-1}, 0.99 g $MnCl_2.4H_2O$ L^{-1}, 0.25 g $CuSO_4.5H_2O$ L^{-1}, 0.20 g $NiCl_2.6H_2O$ L^{-1}, 0.20 g $NaSeO_4.10H_2O$ L^{-1}, 0.014 g H_3BO_3 L^{-1} and 0.22 g $NaMoO_4.2H_2O$ L^{-1} were supplied to the synthetic influent.

8.2.3. Calculations

The nitrogen removal rate (NRR) was calculated as the removal of substrates (ammonium and nitrite) without considering the nitrate produced in the anammox reaction. Conversely, nitrate produced was considered for the calculation of the nitrogen removal efficiency (NRE), since this parameter is more accurate to talk about N-removal from a real implementation point of view. Liquid V_{up} (in m h^{-1}) was determined with the inflow rate (Q, in m^3 h^{-1}) and the cross-sectional area of the reactor (A, in m^2), as follows:

$$V_{up} = \frac{Q}{A} \quad \text{(Eq. 8.1)}$$

ccording to film theory model, mass transfer between the bulk liquid and the granule (or external mass transfer) is driven by a concentration gradient across an external boundary layer (L_L, in m). The mass flow of a component in the L_L is proportional to the difference between the component concentration in the bulk liquid and the granule surface, with the proportionality constant being the external mass transfer coefficient (k_c, in m d^{-1}) (Prehn et al., 2012; Wanner et al., 2006). Then, the mass transfer coefficient is:

$$k_c = {}^{D_F}/_{L_L} \quad \text{(Eq. 8.2)}$$

where D_F is the diffusion coefficient for substrate in water (in m^2 h^{-1}).

For slow flow velocities or low turbulence, the value of L_L is large (high external mass transfer limitations) while for fast flow or high turbulence, L_L is small (low external mass transfer limitations). Both parameters (k_c and L_L) are usually calculated from experimental correlations (Wanner et al., 2006). For example, for laminar hydraulic flows around spherical particles, the following correlation can be used:

$$Sh = 2 + 0.6 * Re^{1/2} * Sc^{1/3} \quad \text{(Eq. 8.3)}$$

where Sh is the non-dimensional Sherwood number, Re is the non-dimensional Reynolds number and Sc is the non-dimensional Schmidt number.

Sherwood, Reynolds and Schmidt numbers are defined as:

$$Sh = \frac{k_c * d_g}{D_F} \quad \text{(Eq. 8.4)}$$

$$Re = \frac{d_g * \rho_w * V_{up}}{\mu_w} \quad \text{(Eq. 8.5)}$$

$$Sc = \frac{\mu_w}{D_F * \rho_w} \quad \text{(Eq. 8.6)}$$

where μ_w is the water viscosity (in kg m^{-1} h^{-1}), ρ_w is the water density (in kg m^{-3}), d_g is the average granule diameter (in m) and V_{up} is the upflow liquid velocity (in m h^{-1}).

8.2.4. Fluorescence *in situ* hybridization (FISH)

Relative abundances of anammox bacteria were analysed by FISH technique coupled with confocal laser scanning microscopy (CLSM) as described in Section 4.3.1 of Chapter 4. Specific probes for *Candidatus Brocadia anammoxidans* and *Candidatus Kuenenia stuttgartiensis* were 5'-TxRed-labeled. Hybridization protocol and probes are fully described in Section 4.3.1 of Chapter 4.

8.2.5. Specific analytical methods

Liquid samples from influent and effluent of the UAnSB reactor were withdrawn to determine ammonium, nitrite and nitrate concentrations three days per week, according to Section 4.2.1, Chapter 4. Average particle size and particle size distribution were periodically measured by a laser particle size analysis system (Malvern Mastersizer Series 2600, Malvern instruments Ltd., UK). Sampling for size analysis was always performed at 145 mm of height of the UAnSB reactor, except in the case of the stratification studies when the sampling point was specified in the corresponding section of the results. Maximum specific anammox activity (SAA) was determined by measuring the overpressure generated by the anammox sludge in closed bottles according to the methodology described by Dapena-Mora et al. (2007). During this protocol for determine maximum SAA, bottles were maintained in a shaker at 150 rpm to favour mixing and reduce external mass transfer limitations.

8.3. RESULTS AND DISCUSSION

8.3.1. Operation of the UAnSB reactor

The lab-scale UAnSB reactor was inoculated (day 0) with anammox biomass from a SBR which was operating at 35 ºC with an average SAA of 0.25 ± 0.02 g N g^{-1} VS d^{-1} (Isanta et al., 2015b). During the start-up period (days 0–50) NLR was gradually increased from 0.09 to 0.78 g N L^{-1} d^{-1} and NRE fluctuated between 73% and 84% until stable operation was achieved from day 50 onwards with an average NRE of 76 ± 3% on period II. Table 8.2 reports the operational parameters corresponding to the different periods of operation of the UAnSB reactor. During period II the reactor was operated for 150 days at an average NLR of 0.8 ± 0.2 g N L^{-1} d^{-1} and an average NRE of 76 ± 3%. Furthermore, in period IV the reactor achieved a stable operation for more than 2 months with an average NLR of 1.8 ± 0.2 g N L^{-1} d^{-1} and a better NRE (80 ± 3%).

The nitrite to ammonium consumption ratio and the nitrate produced to ammonium consumed ratio increased during operation until period IV, achieving average values of 1.20 ± 0.08 and 0.26 ± 0.03, respectively. These average values are close to the previously reported for anammox cultures (Lotti et al., 2014c; Strous et al., 1998). At the end of period III (on day 244) the nitrite to ammonium concentrations ratio in the influent was increased from 1.0 to 1.2 and an improvement of the ammonium removal efficiency was observed in the UAnSB reactor. In view of that, although nitrite was always observed in the effluent (3.8 ± 0.9 g N-NO_2^- L^{-1}), the reactor was limited by nitrite probably due to external mass transfer problems in the sludge bed.

The achieved NRR value in the UAnSB reactor (1.7 ± 0.1 g N L^{-1} d^{-1} in period IV) was considerably higher than most of the previously reported values for systems treating low-strength wastewater. Table 8.3 shows the NRR achieved in both, one and two-stage systems. On the one hand, NRR achieved in this study was much higher than any NRR reported in one-stage systems. On the other hand, regarding two-stage systems, the NRR achieved in this study was comparable to the one obtained by Lotti et al. (2014b) in an anammox EGSB reactor (1.40–1.85 g N L^{-1} d^{-1} at 20 ºC) and only lower than the reported by Ma et al. (2013) (5.7 g N L^{-1} d^{-1} at 30 ºC) which was achieved in a UAnSB reactor operating at a V_{up} as high as 11 m h^{-1}. Probably, the higher NRR achieved by Ma et al. (2013) was strongly correlated to the high V_{up} applied in its reactor, which favoured mixing and thus, overcame external mass transfer limitations in the sludge bed.

Table 8.2. Periods of operation and operational parameters of the UASB anammox reactor. Since in period I and III nitrogen loading rates were being increased and continuously changing, a range of NLR and NRR values was presented. (NLR = nitrogen loading rate; NRR = nitrogen removal rate; $N_{Tot\ effluent}$ = total nitrogen concentration in the effluent; $\Delta N\text{-}NO_2^-/\Delta N\text{-}NH_4^+$ = nitrite to ammonium consumption ratio; $\Delta N\text{-}NO_3^-/\Delta N\text{-}NH_4^+$ = nitrate produced to ammonium consumed ratio).

Period	**Days**	**NLR** (g N L^{-1} d^{-1})	**NRR** (g N L^{-1} d^{-1})	$N_{Tot\ effluent}$ (mg N L^{-1})	$\frac{\Delta N\text{-}NO_2^-}{\Delta N\text{-}NH_4^+}$	$\frac{\Delta N\text{-}NO_3^-}{\Delta N\text{-}NH_4^+}$
I	0-50	0.09–0.78	0.08–0.69	15 ± 4	1.01 ± 0.12	0.21 ± 0.08
II	50-200	0.8 ± 0.2	0.7 ± 0.1	17 ± 2	1.05 ± 0.07	0.25 ± 0.02
III	200-250	1.0–1.6	0.9–1.5	17 ± 2	1.14 ± 0.08	0.25 ± 0.02
IV	250-325	1.8 ± 0.2	1.7 ± 0.1	15 ± 2	1.20 ± 0.08	0.26 ± 0.03

Table 8.3. Review of the NRR achieved in different reactors treating low-strength wastewater either in one or two-stage systems.

Type of System	Type of Reactor	Influent	T (ºC)	V_{up} (m h^{-1})	NRR (g N L^{-1} d^{-1})	Reference
One-stage systems	Rotating Biological Contactor	Synthetic	29	-	0.47	De Clippeleir et al. (2013)
	Rotating Biological Contactor	Synthetic	25	-	0.44	De Clippeleir et al. (2011)
	Gaslift Reactor	Semi-synthetic	25	-	0.26	Hendrickx et al. (2012)
	Moving Bed Biofilm Reactor	Synthetic	20	-	0.04	Gilbert et al. (2014)
	Airlift Reactor	Synthetic	20	-	0.44	Lotti et al. (2014a)
	Plug-Flow Granular Pilot-Scale Reactor	Urban wastewater	19	-	0.20	Lotti et al. (2015a)
Two-stage systems	UAnSB Reactor	Urban wastewater	30	11	5.72	Ma et al. (2013)
	UAnSB Reactor	Urban wastewater	27–30	1.2	0.40	Ma et al. (2011)
	Upflow Fixed Bed Biofilm Reactor	Synthetic	27	-	0.81	Gao et al. (2014)
	EGSB Reactor	Urban wastewater	20	20	1.40–1.85	Lotti et al. (2014b)
	UAnSB Reactor	Synthetic	26	1	1.7 ± 0.1	This study

Biomass concentration of the sludge bed in the UAnSB reactor was stable throughout the study, with an average value of 6.3 ± 0.4 g VS L^{-1}. The sludge had a high inorganic content since the VS/TS ratio was 0.48 ± 0.05. Biomass concentration in the effluent accounted for less than 9 mg VS L^{-1} resulting in a SRT of 45 ± 15 days. Regarding to the settling properties of the granules, a good settling ability was observed during the whole operation, with an increase of the settling velocity from 19 ± 5 m h^{-1} in period II to 31 ± 9 m h^{-1} in period IV. Settling velocities achieved were in the range of the corresponding to granular biomass (from 20 to 150 m h^{-1}; Cervantes, 2009).

Regarding the microbiological characterization of the sludge, FISH-CSLM was used to determine the enrichment in anammox bacteria during the whole operation of the UAnSB reactor. Thus, biomass samples on day 0 (inoculum), day 117 (period II) and day 314 (period IV) were analysed. On the one hand, *Candidatus Brocadia anammoxidans* was found to be the predominant microbial specie in the sludge bed, with percentages of the total population of 86 ± 3% (day 0), 92 ± 2% (day 117) and 93 ± 2% (day 314). Hence, a high enrichment in anammox bacteria was maintained in the granular sludge bed throughout the study. On the other hand, *Candidatus Kuenenia stuttgartiensis* appeared at the end of the operation of the UAnSB reactor (1 ± 1% on day 314) although it was not detected in the inoculum nor in the sample of day 117.

8.3.2. Effect of the liquid upflow velocity on the operation of the UAnSB reactor

8.3.2.1. Granulation

The evolution of the granule diameter was followed throughout the study (Fig. 8.1C). The inoculum had an average diameter of 920 ± 90 μm, however during period I (start-up) and the beginning of period II the granule size decreased gradually until reaching a minimum value of 350 ± 10 μm after 100 days of operation. The slight increase of diameter on day 46 was due to the addition of new biomass from the inoculum on day 27. After day 105, the granule diameter started to increase until reaching an average stable value of 790 ± 40 μm from day 220 onwards. Additionally, Fig. 8.1C shows the fraction of biomass considered as granule, i.e. particle diameter bigger than 200 μm, which resulted higher than 70% during the whole operation of the UAnSB reactor, even when the granulation was dropping. Moreover, Fig. 8.2 shows the granule size distribution at different reactor operation days. The unimodal shape was maintained despite of the first decrease and later increase of

the mean diameter, so there was mainly only one type of granule and the homogeneity of the sludge was maintained during the operation.

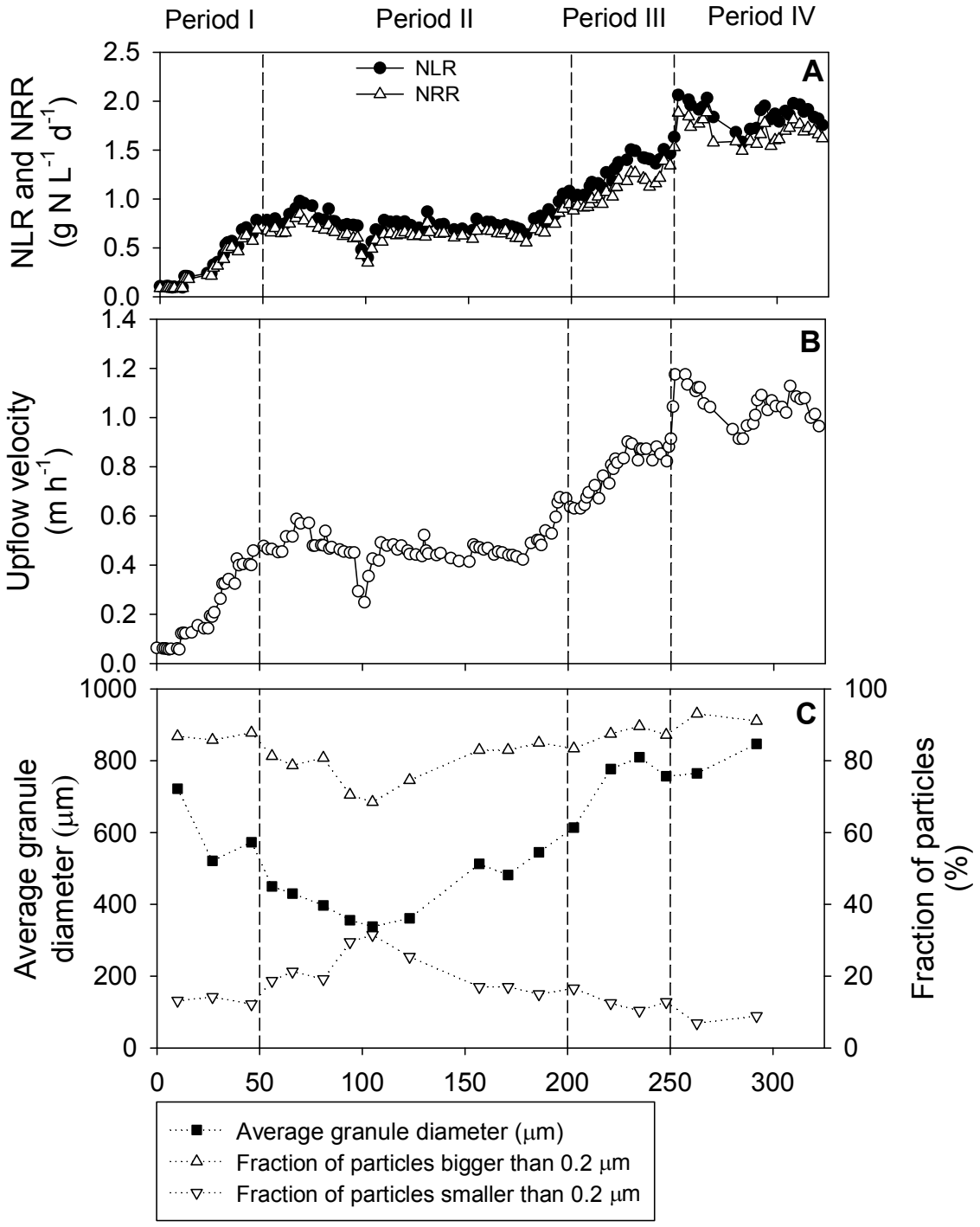

Fig. 8.1. Continuous operation of the UAnSB reactor treating low-strength synthetic wastewater. (A) Nitrogen loading rate (NLR) and nitrogen removal rate (NRR); (B) Upflow liquid velocity; (C) Granule size evolution.

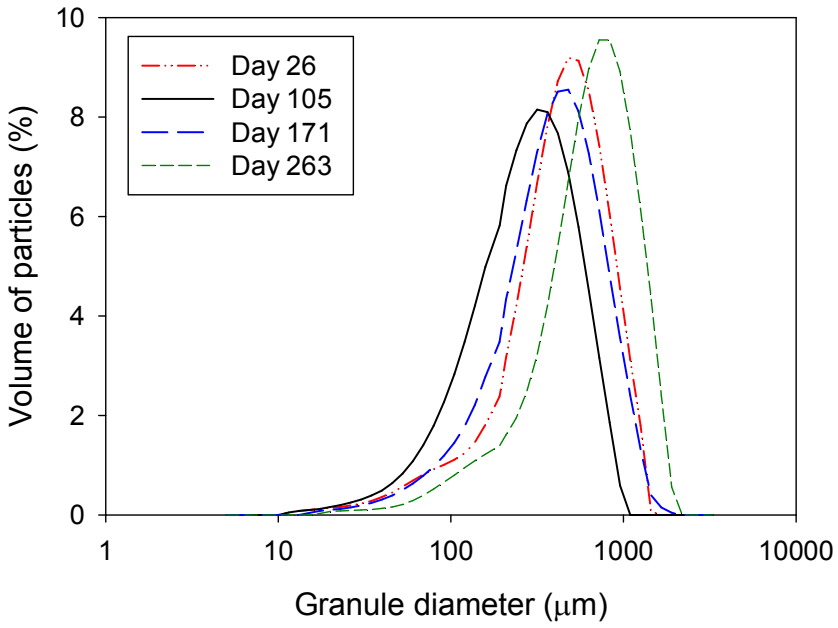

Fig. 8.2. Time course of the granule size distribution during the operation of the UAnSB reactor treating low-strength synthetic wastewater.

It is known that high V_{up}s favour granulation in UASB reactors (Arne Alphenaar et al., 1993; Liu and Tay, 2002) and, in fact, a correlation of the mean granule diameter with the liquid V_{up} was determined with data of periods II, III and IV when the granule size was increasing (Fig. 8.3). It can be observed that there was a direct correlation (R^2 = 0.86) between both parameters. Moreover, two statements can be established from Fig. 8.3: (i) in the range 0.4–0.6 m h^{-1}, higher upflow velocities enhanced granulation leading to higher granule diameters and (ii) there was a range of upflow velocities (from 0.8 m h^{-1} onwards) from which the granule diameter reached a maximum stable value. Nevertheless, despite of the liquid V_{up} was gradually increased during period I of operation, the average granule diameter decreased significantly (Fig. 8.1B and 8.1C), and thus, data from period I were not considered for the correlation of Fig. 7.3. The decreasing in granule size could be explained because the range of V_{up}s between 0–0.4 m h^{-1} was not high enough to maintain granulation, but once V_{up} was higher in period II (0.4–0.6 m h^{-1}) the drop in granule size was stopped and, after some time operating under this higher range, the average granule diameter started to increase. This meant that V_{up} was not a parameter with immediate effect on the granulation, i.e. some time was needed to notice its effect. In addition to the low V_{up} applied in period I, the change of the inoculum from an SBR to an UASB reactor could also be a reason to explain the dropping in granule size. The settling time imposed to the biomass is the driving force to favour granulation in SBRs, while in the case of UASB reactors the driving force is

the liquid V_{up} (Fig. 8.3). Hence, the change in the type of stress induced to the granules could contribute to the destabilization and decrease in granule diameter during start-up and the beginning of period II.

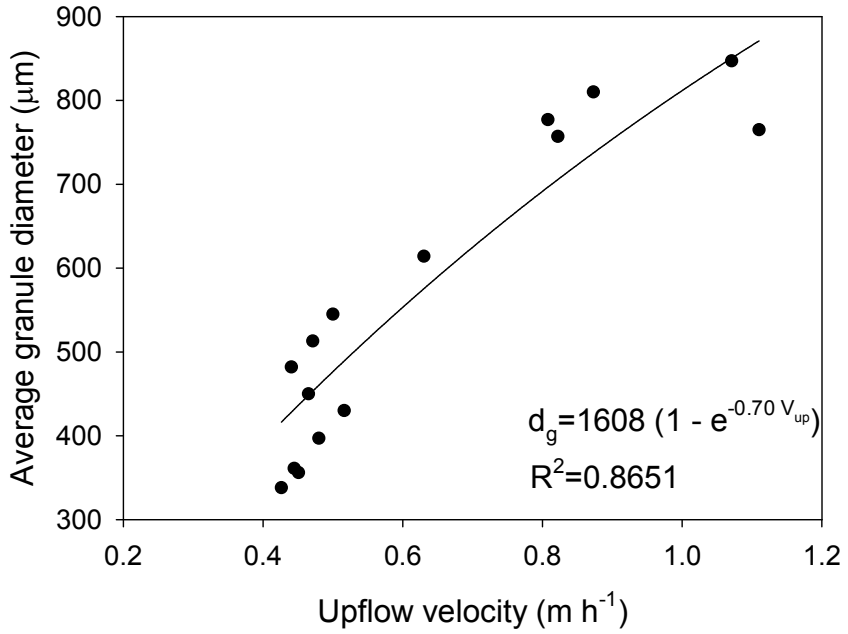

Fig. 8.3. Correlation of the diameter of the granules (d_g) with the upflow liquid velocity (V_{up}) applied in the UAnSB reactor. Correlation was obtained with data from periods II, III and IV of operation.

As previously mentioned, the effect of V_{up} on the granulation was evident but not immediate noticeable. Thus, during period III the V_{up} was sharply increased and as a result, the diameter of the granules increased until reaching a stable value in period IV (average value of 790 ± 40 μm at 1.0 ± 0.1 m h^{-1}) (Fig. 8.1). However, throughout these periods, a stratification of the granular sludge was visually observed along the longitude of the reactor; there were big granules at the bottom and smaller granules at the top. Therefore, sludge samples were taken and analysed at different heights of the UAnSB reactor (at 50, 145, 240, 345 and 450 mm of height) in order to confirm that the observed stratification was a fact indeed. Fig. 8.4 shows the average granule diameter at different heights of the UAnSB reactor during different operation days from the beginning of period III to the end of period IV (V_{up} of 0.6–0.9 m h^{-1} in period III and 0.9–1.2 m h^{-1} in period IV). On the one hand, a clear difference between granule diameters along the heights of the reactor was observed from day 203 to 235, with consecutive increases of diameter in consecutive decreases of height. This difference was more evident comparing the three lower heights (50, 145, 240 mm) versus the

two highest heights (345 and 450 mm). On the contrary, samples of days 263 and 292 showed similar average diameters along the reactor heights, demonstrating the homogenization of diameters through the sludge bed of the UAnSB reactor. Hence, in period IV not only the average granule diameter was stabilized (Fig. 8.1C) but also the stratification of the sludge bed was eradicated. Therefore, the increase in granule diameters caused by the operation of the UAnSB at high V_{upS} was progressive and stratified along the reactor which indicated that the granule stabilization was a long process where almost 100 days were needed to achieve a sludge bed stable in size and not stratified (Fig. 8.4).

The improvement of the granulation process at high liquid V_{up}s was reported before. Alphenaar et al. (1993) compared granulation in two UASB reactors performing anaerobic treatment of sulphate-containing wastewater and observed considerably higher granule diameters in the reactor with higher V_{up}s (0.5 mm with 0.05 m h^{-1} and 1.2 mm with 0.65 m h^{-1}). Regarding anammox reactors, Ma et al. (2013) operated an UAnSB reactor treating low-strength urban wastewater and observed the improvement of granulation when the V_{up} increased from 1.26 to 11 m h^{-1} at 30 ºC. Even higher V_{up}s (20 m h^{-1}) were used by Lotti et al. (2014b) in a lab-scale EGSB reactor treating also low-strength urban wastewater. However, high recirculation of the effluent was needed to achieve these elevated V_{up}s. Such a high recirculation ratios (a recirculation ratio of 39 was used by Lotti et al. (2014b)) would present a huge inconvenient to implement UAnSB reactors at real scale, since too high pumping costs would be associated. In this study, substantially lower V_{up}s (0.8 m h^{-1}) were needed to maintain granulation and stable operation of an UAnSB reactor treating a low-strength synthetic wastewater. This would imply that no recirculation is needed and thus, a more realistic implementation of the process in an urban WWTP would be possible.

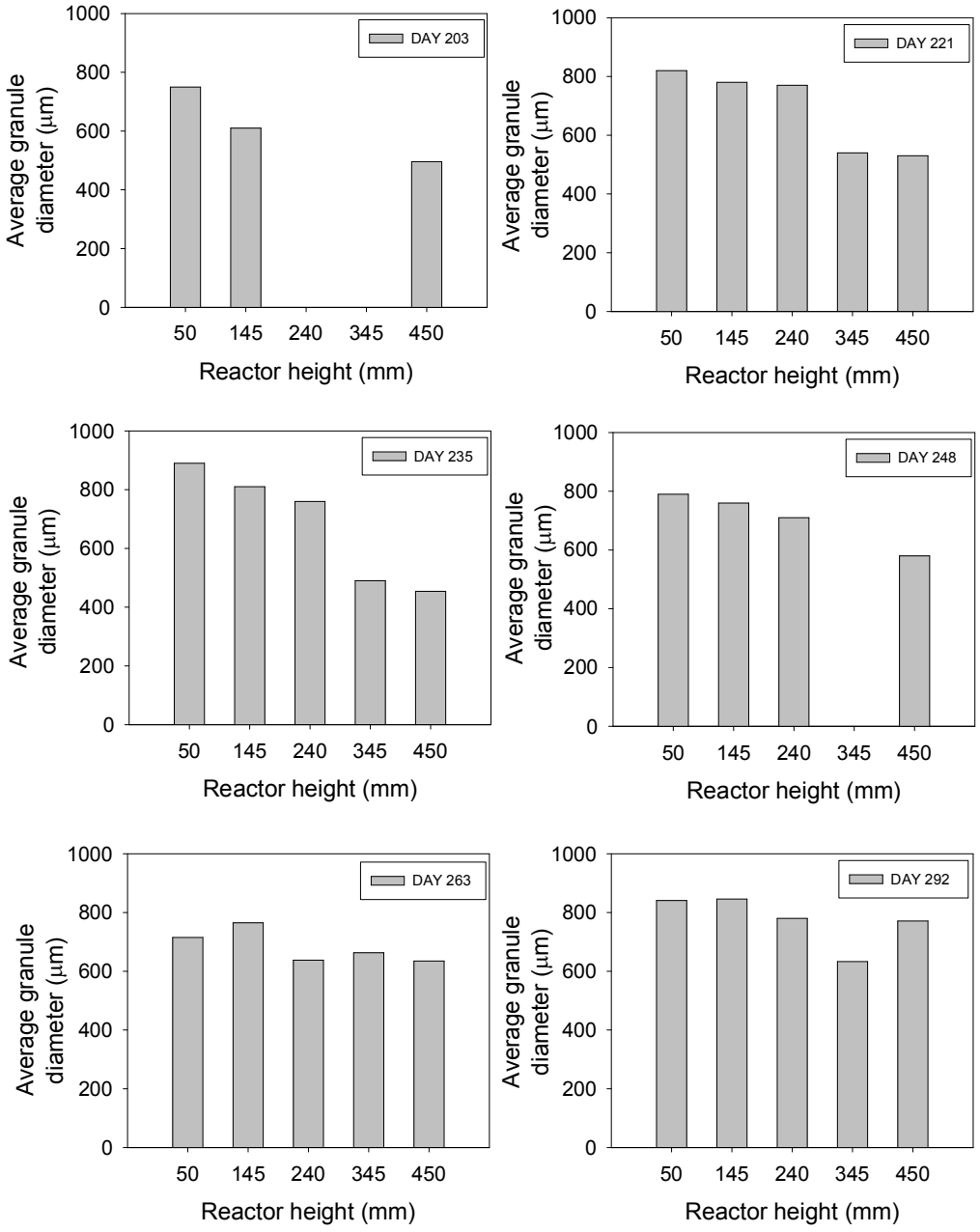

Fig. 8.4. Stratification of the granular sludge bed in the UASB anammox reactor treating low-strength synthetic wastewater. Missing values on day 203 and 248 were due to the lack of sampling at the corresponding heights.

8.3.2.2. External mass transfer limitations

In UASB reactors, liquid V_{up} not only affects to granulation process but also to the external mass transfer in the system. Low V_{up}s can lead to insufficient mixing which produce external mass transfer limitations and, hence, substrate accumulation in the effluent. But also too high V_{up}s can lead to the formation of preferential flows that contribute to external mass transfer limitations as well. In fact, Lotti et al. (2014b) reported external mass

transfer limitations in an EGSB reactor treating urban wastewater due to the formation of preferential flows, regardless of using high liquid V_{up}s (20 m h^{-1}). Thus, high substrate concentrations were accumulated in the EGSB reactor (14 ± 9 mg $N\text{-}NH_4^+$ L^{-1} and 13 ± 11 mg $N\text{-}NO_2^-$ L^{-1}).

Table 8.4 shows the values of Reynolds (Re) and Sherwood (Sh) numbers, boundary layer (L_L) and transfer coefficient (k_c) calculated for each operational period of the UAnSB reactor of this study. Before to discuss the calculations made to quantify the external mass transfer limitations, it is important to note that: (i) the nitrite concentration in the bulk liquid was never lower than 2.5 mg $N\text{-}NO_2^-$ L^{-1}, showing that the performance of the UAnSB was probably affected by mass transfer limitations and (ii) Re values indicate that the liquid flow in the UAnSB was clearly laminar for any V_{up} used in this study, and consequently, Sh number can be calculated with Eq. (8.3).

Regarding the calculations of the external mass transfer limitations, Table 8.4 shows that as V_{up} increased, Re and Sh numbers increased. This could result in an improvement of the mass transfer from the bulk liquid to the granule as reflected in the increase of the achieved NRR. However, this improvement in the external mass transfer was not reflected in the values of the mass transfer coefficient (k_c) and the boundary layer (L_L). In fact, the highest k_c, and consequently, the lowest L_L were achieved in period II with a V_{up} half than the used in period IV. Both values (k_c and L_L) not only depend on V_{up} but also depend on granule diameter (d_g). From Eqs. (8.2–8.6), it can be deduced that as d_g increases, k_c decreases and L_L increases, that is, external mass transfer limitations increase. Consequently, when V_{up} and d_g jointly increase, as it happened in periods II–IV, there exist two opposite effects over the external mass transfer. In this study, both opposite effects caused that L_L values were quite similar in periods II, III and IV. In any case, L_L values were always in the range 160–200 μm, indicating that the UAnSB was always affected by external mass limitations. In fact, substrate concentrations were always present in the effluent, with average values of 3 ± 1 mg $N\text{-}NH_4^+$ L^{-1} and 3.5 ± 0.8 mg $N\text{-}NO_2^-$ L^{-1}. Besides, SAA tests showed an average value of 0.35 ± 0.02 g N g^{-1} VS d^{-1} in period IV, while specific NRR in the UAnSB was of 0.26 ± 0.02 g N g^{-1} VS d^{-1} during the same period. SAA tests were performed in bottles under continuous agitation which enhanced mixing and reduced external mass transfer limitations. Hence, the difference between SAA and NRR also demonstrated that the UAnSB reactor was affected by external mass transfer limitations.

Table 8.4. Operational parameters of the UAnSB for each period and external mass transfer calculations. Granule diameter (d_g), upflow velocity (V_{up}), nitrogen removal rate (NRR) and nitrite concentration in the bulk liquid are the experimental average values for each period. Reynolds (Re) and Sherwood number (Sh) were calculated with Eqs. (8.5) and (8.3), respectively. External mass transfer coefficient (k_c) was calculated with Eq. (8.4) while external boundary layer was calculated with Eq. (8.2).

Period	d_g (mm)	V_{up} (m d^{-1})	Re	Sh	k_c (m h^{-1})	NRR (g N L^{-1} d^{-1})	L_L (µm)	$[N\text{-}NO_2^-]_{bulk\ liquid}$ (mg N L^{-1})
I	605 ± 105	0.21 ± 0.14	0.04	3.0	0.030	0.3 ± 0.2	204	3.6 ± 1.0
II	545 ± 74	0.48 ± 0.07	0.08	3.4	0.037	0.7 ± 0.1	162	4.0 ± 0.6
III	740 ± 87	0.79 ± 0.09	0.19	4.1	0.033	1.1 ± 0.3	182	3.7 ± 0.8
IV	806 ± 58	1.05 ± 0.07	0.27	4.5	0.034	1.7 ± 0.2	180	3.5 ± 1.0

Nevertheless, despite of the fact that the UAnSB reactor of the present study presented external mass transfer limitations, the resulting operation with a NRR of 1.7 ± 0.1 g N L^{-1} d^{-1}, a NRE of 80 ± 3% and a successful anammox granulation demonstrated that UASB reactors appear as a good alternative to implement the anammox process at mainstream conditions.

8.4. CONCLUSIONS

Stable anammox reaction was maintained in an UASB reactor treating a low-strength synthetic wastewater. Significantly high NLR, NRR and NRE were achieved compared to other similar systems. The anammox culture was highly enriched in *Candidatus Brocadia anammoxidans* during the whole operation of the reactor.

Liquid upflow velocity was demonstrated to be a key parameter to be aware of for the implementation of the anammox process in UASB reactors, since it affected to granulation and external mass transfer. On the one hand, liquid V_{up} presented a direct but not immediate effect on the anammox granulation, being the higher the V_{up} the bigger the granules. On the other hand, the low liquid V_{upS} applied led to external mass transfer limitations which in any case affected to the high nitrogen removal of the UAnSB.

Chapter 9

STABLE LONG-TERM OPERATION OF AN ANAMMOX UASB REACTOR AT MAINSTREAM CONDITIONS

A modified version of this chapter is being prepared for publishing as:

Reino, C., Suárez-Ojeda, M.E., Pérez, J., Carrera, J., 2016. Stable long-term operation of an anammox UASB rector at mainstream conditions. *In preparation.*

Abstract

Efforts of implementing the anammox process at mainstream conditions with high nitrogen removal rates have gained much attention in the race for achieving an energy-positive urban wastewater treatment plant. A successful stable long-term operation at mainstream conditions in an Upflow Anammox Sludge Bed (UAnSB) reactor treating a low-strength synthetic influent for 350 days and a real urban wastewater for 110 days was presented in this study. An exhaustive study of the effect of temperature on anammox activity was performed when the synthetic influent was treated at mainstream conditions, and an adaptation of anammox bacteria after long-term operation at low temperatures was observed. In fact, a nitrogen loading rate as high as 0.93 ± 0.05 g N L^{-1} d^{-1} with a 82 ± 4% of nitrogen removal were obtained at 11 ºC. Furthermore, the effect of treating a real urban wastewater at 11 °C at long-term in the UAnSB reactor was also evaluated, and a stable operation was achieved with an extremely high average nitrogen removal rate (0.59 ± 0.05 g N L^{-1}). Anammox enrichment was maintained during the whole operation with abundance higher than 70% according to fluorescence *in situ* hybridization, being *Candidatus Brocadia anammoxidans* the predominant microbial species. The presence of heterotrophs in the sludge bed was demonstrated through pyrosequencing technique and heterotrophic batch tests, but anammox activity was demonstrated to be higher than heterotrophic activity, even when the synthetic influent was replaced by the real urban wastewater. The feasibility of operating an enriched anammox reactor at high nitrogen removal rate in the long-term at mainstream conditions was demonstrated in this study.

9.1. INTRODUCTION

The anammox-based processes have been proposed as the most promising alternative to guarantee energy-producer urban wastewater treatment plants (WWTPs) (Kartal et al., 2010; Siegrist et al., 2008). Over the past decade, the anammox-based processes have been successfully implemented in the sidestream treatment of urban WWTPs (Lackner et al., 2014), however they has never been implemented in the mainstream. The main challenge of the mainstream application is to achieve high nitrogen removal rates that guarantee a good effluent quality. Thus, achieving a stable anammox operation in the long-term under mainstream conditions is of paramount importance.

One of the challenges of the mainstream implementation of anammox process is the need of dealing with a much lower range of wastewater temperatures compared to that of the sidestream process (>30 °C). In fact, gradient from 20 to 10 °C was proposed by Gilbert et al. (2015) to simulate the temperature gradient observed in urban WWTPs in moderate climates. Low temperatures have been reported to cause a considerable decrease of anammox activity (Dosta et al., 2008; Hu et al., 2013; Vázquez-Padín et al., 2011) despite that anammox bacteria were reported to grow in natural ecosystems at temperatures below 4 °C, such as the ones occurring on marine sediments (Rysgaard et al., 2004). Another challenge of the mainstream operation of anammox processes is the nitrogen concentrations of the influent, orders of magnitude lower than that of sidestream, which lead to decreasing net biomass production. This is inadvisable since anammox bacteria are microorganisms with an intrinsic very long doubling time (Strous et al., 1999). Thus, high solids retention time is needed to increase the biomass concentration in the system in order to guarantee the growth of anammox at mainstream conditions.

Recently, many studies were focused on the implementation of anammox process at mainstream conditions, either in one-stage systems such as CANON (Completely Autotrophic Nitrogen removal Over Nitrite) and OLAND (Oxygen-Limited Autotrophic Nitrification/Denitrification) technologies; or in two-stage systems with the separation of partial nitritation and the anammox process in two different reactors. On the one hand, a first approach to mainstream conditions was done using one-stage systems, commonly called partial nitritation/anammox (PN/A) systems. Lotti et al. (2014a) reported a stable operation treating a synthetic influent at 15 °C, but process destabilization occurred in the long-term operation at 10 °C. Similar results were obtained by Gilbert et al. (2015) which compared

different reactor configurations for PN/A systems treating a synthetic wastewater, but destabilization occurred at temperatures below 13 °C. In the case of PN/A systems treating real urban wastewater, perspectives were not better and Laureni et al. (2016) recently reported the suppression of anammox activity at 11 °C. On the other hand, few studies reported stable operation of single anammox reactors treating real urban wastewater at low temperatures. However, these studies reported either low nitrogen removal rates which would lead to very large bioreactor volumes (Hendrickx et al., 2014; Laureni et al., 2015) or high nitrogen removal rates but high effluent recirculation ratios which would present a huge inconvenient to implement the process at real scale (Lotti et al., 2014b; Ma et al., 2013).

The present study aimed at demonstrating the feasibility of achieving high nitrogen removal rates through the anammox process operating at mainstream conditions in an Upflow Anammox Sludge Blanket (UAnSB) reactor. Hence, an exhaustive study of the effect of low temperatures (13 and 11 °C) on the long-term anammox operation at mainstream conditions and, furthermore, the effect of treating a real urban wastewater on the anammox activity were performed.

9.2. MATERIALS AND METHODS

9.2.1. Reactor set-up and operation

A lab-scale UASB reactor of 2 L of working volume including the gas-liquid-solid separator was used. The experimental set-up details and a diagram of the reactor were described in Section 4.1.2. of Chapter 4. The pH was not controlled, but measured off-line and its value was of 8.1 ± 0.3 during the whole operation. Dissolved oxygen (DO) concentration measured in the bulk liquid was always 0.0 mg L^{-1}. The temperature was measured and controlled by means of a cooling system and an electric heater (HBSI 0.8m, HORST, Germany) connected to a temperature controller (BS-2400, Desin Instruments, Spain). Three different temperatures of operation were tested: 22, 13 and 11 °C.

The operation of the UAnSB reactor was divided in two main parts regarding the type of influent treated. Between days 0–350 a synthetic influent was treated at 22, 13 and 11 °C, and between days 351–460 a real urban wastewater was treated at 11 °C.

9.2.2. Inoculum and wastewater characteristics

The UAnSB reactor was previously operated at high temperature (30–26 °C) treating a synthetic influent for 325 days (details of the operation can be found in Chapter 8). Hence, at the start of the current operation, the sludge bed was highly enriched in anammox bacteria, with an abundance of 93 ± 2% of *Candidatus Brocadia anammoxidans* and 1 ± 1% of *Candidatus Kuenenia stuttgartiensis* as identified by fluorescence *in situ* hybridization (FISH).

From day 0 to 350 the UAnSB reactor treated a synthetic influent mimicking the effluent of a previous partial nitritation reactor treating a municipal wastewater as the one described in Chapter 5. The low-strength synthetic influent contained: 33 mg $N\text{-}NH_4^+$ L^{-1}, 38 mg $N\text{-}NO_2^-$ L^{-1}, 1000 mg $KHCO_3$ L^{-1}, 50 mg NaH_2PO_4 L^{-1}, 100 mg $CaCl_2 \cdot 2H_2O$ L^{-1}, 200 mg $MgSO_4.2H_2O$ L^{-1}, 6.3 mg $FeSO_4$ L^{-1}, 6.3 mg EDTA L^{-1} and 1.25 mL L^{-1} of a trace elements solution (van de Graaf et al., 1996).

From day 351 to 460 the UAnSB reactor treated a real urban wastewater influent. A real effluent from a partial nitritation reactor treating urban wastewater was not available at the time the experiments were performed and, thus, a nitrite-amended secondary clarifier effluent from an urban WWTP without nitrogen removal treatment located in an industrial area of Catalonia (north-east of Spain) was used as influent. Nitrite was added as $NaNO_2$ to maintain a ratio between the nitrite and ammonium concentration of 1.3 ± 0.2. The tank with the influent was periodically flushed with dinitrogen gas (N_2) to guarantee a DO concentration lower than 0.3 mg L^{-1}. Table 9.1 shows the main characteristics of the real urban wastewater after the N_2 flushing and the nitrite addition.

9.2.3. Maximum specific heterotrophic activity

Heterotrophic activity of the biomass was evaluated with batch tests according to a methodology adapted from Dapena-Mora et al. (2007) by measuring the overpressure generated by the anammox enriched sludge in closed bottles. The overpressure generated in the bottles was measured during the first 8 hours of the experiment to determine the maximum activity of the biomass; however, the total duration of the experiments was about 24 hours in order to measure the final concentration of substrates and to confirm its removal. Temperature was set at 30 °C and agitation was maintained at 150 rpm. Tests were performed in triplicates. Two sets of tests were performed: (i) with biomass treating a synthetic influent at 13 °C (day 274) and (ii) with biomass treating a real urban wastewater at 11 °C (day 420).

Table 9.2 shows the substrates used in each batch test and the corresponding concentration. The ratio between carbon and nitrogen concentrations (C/N) was chosen to ensure that organic carbon was present in excess (theoretical C/N was reported to be about 0.64 mg C mg^{-1} N for denitrification via nitrite (Henze et al., 2008) and 0.88 mg C mg^{-1} N for denitrification via nitrate (Liu et al., 2007)).

Table 9.1. Characterization of the nitrite-amended secondary clarifier effluent used as the real influent of the UAnSB reactor. COD is the chemical oxygen demand, and COD_{Tot} and COD_{Sol} are the average concentrations of the total and soluble COD, respectively. DO is the dissolved oxygen concentration.

Parameter	Value	Units
$[N\text{-}NH_4^+]$	30 ± 7	mg N L^{-1}
$[N\text{-}NO_2^-]$	40 ± 7	mg N L^{-1}
$[N\text{-}NO_3^-]$	1 ± 1	mg N L^{-1}
COD_{Tot}	90 ± 20	mg O_2 L^{-1}
COD_{Sol}/COD_{Tot}	0.9 – 1	-
Total solids	10 ± 3	mg TS L^{-1}
pH	8.4 ± 0.3	-
Conductivity	1.9 ± 0.3	mS cm^{-1}
DO	0.2 ± 0.1	mg L^{-1}

9.2.4. Inorganic elements analysis

A semi-quantitative analysis of the inorganic elements present in the sludge bed was performed on days 0 and 423. Samples were pre-treated as follows: 0.1 grams of lyophilised biomass were digested in a microwave digester (Mars, CEM) with concentrated HNO_3 and HCl. The analysis and quantification was done by inductively coupled plasma mass spectrometry (ICP-MS, 7500ce, Agilent Technologies). The totality of this analysis was outsourced to the Servei d'Analisis of the Universitat Autònoma de Barcelona.

Table 9.2. Substrates used for the heterotrophic denitrification essays performed with biomass from the UAnSB reactor when a synthetic influent and a real urban wastewater were treated. Ratio C/N is the ratio between the concentrations of total carbon and nitrogen.

Biomass	**Test**	**[N-NO_2^-]** (mg N L^{-1})	**[N-NO_3^-]** (mg N L^{-1})	**[C-Acetate]** (mg C L^{-1})	**Ratio C/N** (mg C mg^{-1} N)
Biomass treating synthetic wastewater	Only nitrite	25 ± 5	-	-	-
	Nitrite + acetate	21 ± 8	-	35 ± 6	2 ± 1
	Only nitrate	-	16.7 ± 0.8	-	-
	Nitrate + acetate	-	20 ± 1	31 ± 9	1.5 ± 0.4
Biomass treating real urban wastewater	Only nitrite	29 ± 2	-	-	-
	Nitrite + acetate	29 ± 1	-	57 ± 3	1.9 ± 0.2
	Only nitrate	-	33.5 ± 0.5	-	-
	Nitrate + acetate	-	34 ± 1	64 ± 4	1.9 ± 0.1

9.2.5. Calculations

Nitrogen removal rate (NRR) was calculated as the removal of both substrates (ammonium and nitrite) without considering the nitrate produced in the anammox reaction. Conversely, nitrate produced was considered for the calculation of the nitrogen removal efficiency (NRE), since this parameter is more accurate when talking about N-removal from a point of view of real implementation. Specific nitrogen removal rate (sNRR) was calculated with Eq. 9.1.

$$sNRR\ (gN\ g^{-1}VS\ d^{-1}) = \frac{NRR}{VS} \qquad \text{(Eq. 9.1)}$$

where, NRR (g N L^{-1} d^{-1}) is the nitrogen removal rate obtained for each day of operation and VS (g VS L^{-1}) is the average volatile solids concentration during each period considered.

The application of Eq. 9.1 implied that all the working volume of the reactor (2 L) was considered to have the same biomass concentration than the sludge bed, which is where the samples for VS analysis were taken. However, the concentration of biomass was not uniform along the longitude of the reactor, and furthermore, it was obvious than higher biomass concentration was present in the sludge bed than in the reactor separator. Thus, Eq. 9.1 intrinsically led to an underestimation of the real value of sNRR.

9.2.6. Fluorescence *in situ* hybridization (FISH)

Relative abundances of anammox bacteria were analysed by FISH coupled to confocal laser scanning microscopy (CLSM) as described in Section 4.3.1. of Chapter 4. Specific probes for *Candidatus Brocadia Anammoxidans* and *Candidatus Kuenenia stuttgartiensis* were 5´-TxRed-labeled and specific probe for *Candidatus Brocadia Fulgida* was 5´-ALEXA594-labeled. In addition, a general probe for all anammox microorganisms was 5´-ALEXA488-labeled. Hybridization protocol and probes are fully described in Section 4.3.1 of Chapter 4.

9.2.7. Pyrosequencing

Identification of the microbial population was performed using next-generation sequencing at samples from days 240, 347 and 448 of the reactor operation. DNA extraction, pyrosequencing settings and bioinformatics applied are described in Chapter 4, Section 4.3.2.

Bacterial 16S rRNA variable regions V2-V4 were targeted using the primer pair 515F-909R. For bacteria biodiversity analysis and phylogenetic classification reads shorter than 100 bps and larger than 394 bps were trimmed, and the followed methodology is explained in detail in Section 4.3.2 of Chapter 4. Relative abundances of reads were determined by taxonomic level. Indices of biological diversity (Shannon), richness (Chao), and rarefaction curves were calculated for all libraries at 97, 95 and 90% of similitude. Table AI.2.1. and Figs. AI.2.1, AI.2.2 and AI.2.3 in the Annex I – Section II show the indices of biological diversity and rarefaction curves, respectively. All these results indicate the libraries were comparable in terms of abundance percentages and that good coverage of diversity was reached.

9.2.8. Specific analytical methods

Liquid samples from influent and effluent of the UAnSB reactor were withdrawn to determine ammonium, nitrite and nitrate concentrations three days per week, according to Section 4.2.1, Chapter 4. Average particle size and particle size distribution were periodically measured by a laser particle size analysis system (Malvern Mastersizer Series 2600, Malvern instruments Ltd., UK). Sampling for size analysis was always performed at 145 mm of height of the UAnSB reactor. Maximum specific anammox activity (SAA) was determined by measuring the overpressure generated by the anammox sludge in closed bottles according to the methodology described by Dapena-Mora et al. (2007).

9.3. RESULTS

9.3.1. Operation of the UAnSB reactor at low temperatures

The lab-scale UAnSB reactor was previously operated at high temperature (26–32 °C) for 325 days treating a low-strength synthetic influent (details of the operation can be found in Chapter 8). A stable nitrogen loading rate (NLR) of 1.8 ± 0.2 g N L^{-1} d^{-1} and nitrogen removal rate (NRR) of 1.7 ± 0.1 g N L^{-1} d^{-1} were maintained for 2 months at 26 °C before the temperature was directly lowered to 22 °C (day 0 of the present study).

Fig. 9.1.A,B shows the stable long-term operation of the UAnSB reactor when a low-strength synthetic influent was treated for 350 days. During this period, temperature was lowered to evaluate the effect of low temperature on the anammox process at mainstream conditions in the long-term. Firstly, after one month of stable operation at 22 °C, the

temperature was gradually lowered until achieving 13 °C on day 35. The day after lowering the temperature to 13 °C the nitrogen removal rate (NRE) resulted as low as 71% with a clear decrease in NRR until 1.32 g N L^{-1} d^{-1}. Nevertheless, despite the initial decrease of anammox activity, the operation was maintained stable for more than 8 months at 13 °C with an average NLR of 1.1 ± 0.2 g N L^{-1} d^{-1} and NRE of 82 ± 3%. Moreover, on day 280 the temperature was directly lowered to 11 °C and further maintained until day 350. Despite of operating at such a low temperature, the anammox activity was not considerably decreased compared to the operation at 13 °C (Fig. 9.1A) and average values of 0.93 ± 0.05 g N L^{-1} d^{-1} and 82 ± 4% of NLR and NRE were obtained. Furthermore, a specific nitrogen removal rate (sNRR) of 0.09 ± 0.01 g N g^{-1} VS d^{-1} was achieved in the UAnSB reactor operating at 11 °C.

The nitrite to ammonium consumption and the nitrate produced to ammonium consumed ratios were 1.3 ± 0.1 and 0.24 ± 0.08, respectively, during the first 350 days of the UAnSB reactor operation when synthetic influent was treated. In addition to the stability of the anammox process, a good effluent quality was achieved with an average effluent nitrogen concentration of 13 ± 4 mg N L^{-1} during the operation at 11 °C (Fig. 9.1B).

After achieving a stable performance of the anammox process treating the synthetic influent at a temperature at 11 °C, a real urban wastewater was treated to study the effect of real wastewater matrix on the system. The operation of the UAnSB reactor treating real urban wastewater at 11 ºC is depicted in Fig. 9.1C,D. Between days 350–380 NLR and NRR gradually decreased, but an abrupt increase was observed on day 382. This increase was not intentioned but due to a bad characterization of the urban wastewater during days 370–380. The anammox activity was maintained until day 400 despite of the sudden increase of NLR on day 382 but, afterwards, a decrease of NLR and NRR started to occur (Fig. 9.1C). Nevertheless, a stabilization of the rates was observed from day 420 onwards and average stable values of 0.59 ± 0.05 g N L^{-1} d^{-1} of NLR and 0.35 ± 0.06 g N L^{-1} d^{-1} of NRR were achieved during the last month of operation at 11 °C. Furthermore, the sNRR achieved was 0.021 ± 0.003 g N g^{-1} VS d^{-1}.

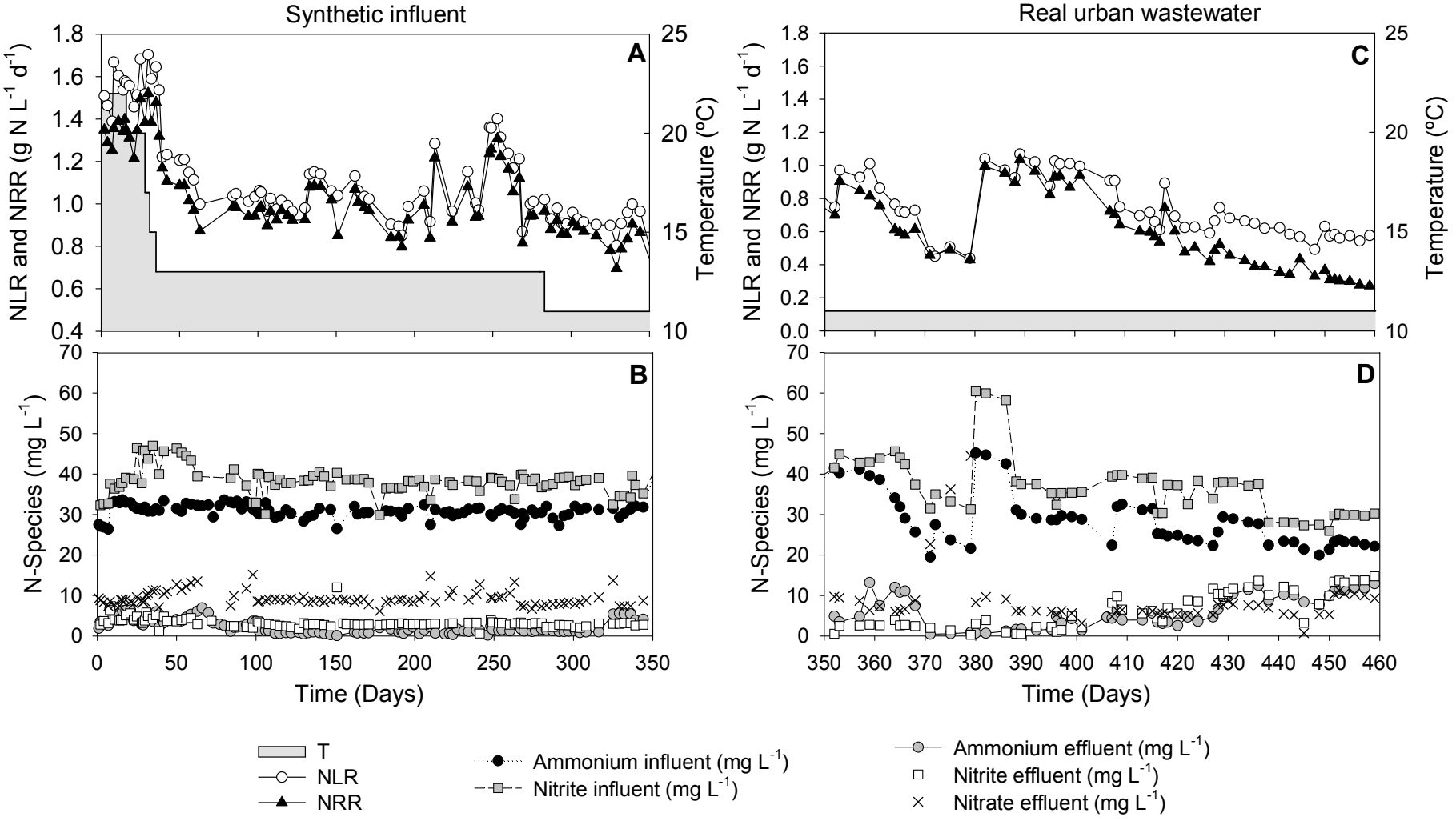

Fig. 9.1. Long-term operation of the UAnSB reactor at mainstream conditions. A,B: Synthetic influent treated at 22, 13 and 11 °C; C,D: real urban wastewater used as influent at 11 °C. NLR: Nitrogen Loading Rate; NRR: Nitrogen Removal Rate; T: Temperature.

When the UAnSB reactor was treating the real urban wastewater, the nitrite to ammonium consumption ratio increased until 1.4 ± 0.2, while the nitrate produced to ammonium consumed ratio decreased until 0.21 ± 0.05, comparing with the ratios obtained with the synthetic influent. Regarding the COD removal, an average value of 50 ± 10% (c.a. 45 mg O_2 L^{-1} removed) was achieved.

Fig. 9.2 shows the trend of the sNRR during the long-term operation of the UAnSB reactor. A gradually decrease of anammox activity was observed when temperature was lowered and, moreover, a sharply decrease of the anammox activity was produced when the synthetic influent was changed to the real urban wastewater. The effect of the temperature on the anammox process was evaluated by plotting the NRR to a conventional Arrhenius plot (Fig. 9.3) (R^2 = 0.98) and the activation energy (E_a) value obtained was 30 ± 3 kJ mol^{-1}. In addition, according to an Arrhenius-type equation (Eq. 9.2), a temperature coefficient of θ = 1.043 ± 0.004 was obtained. These calculations were performed by using the NRR values obtained from the long-term operation with synthetic influent at 22, 13 and 11 °C (data of the present study) and the NRR achieved at 26 °C in the previous study described in Chapter 8.

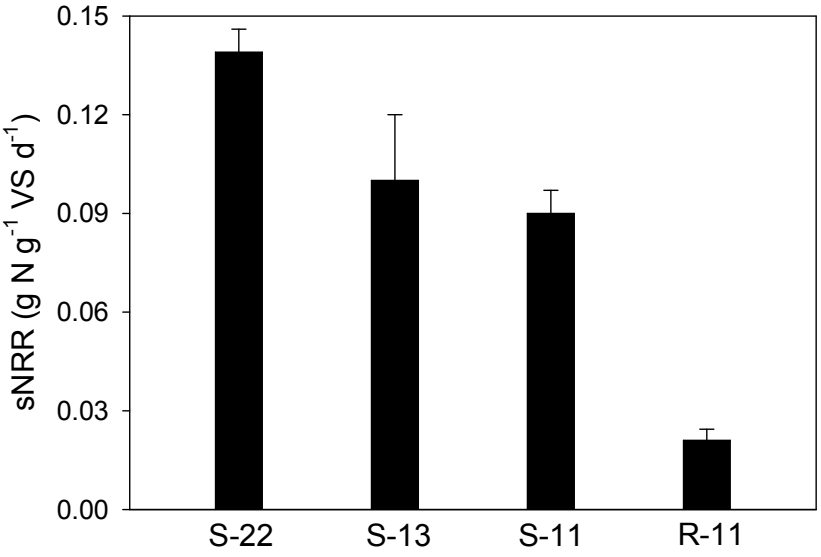

Fig. 9.2. Specific nitrogen removal rate (sNRR) during the operation of the UAnSB reactor at the different temperatures and influents tested. S-22: synthetic influent at 22 °C; S-13: synthetic influent at 13 °C; S-11: synthetic influent at 11 °C and R-11: real urban wastewater at 11 °C.

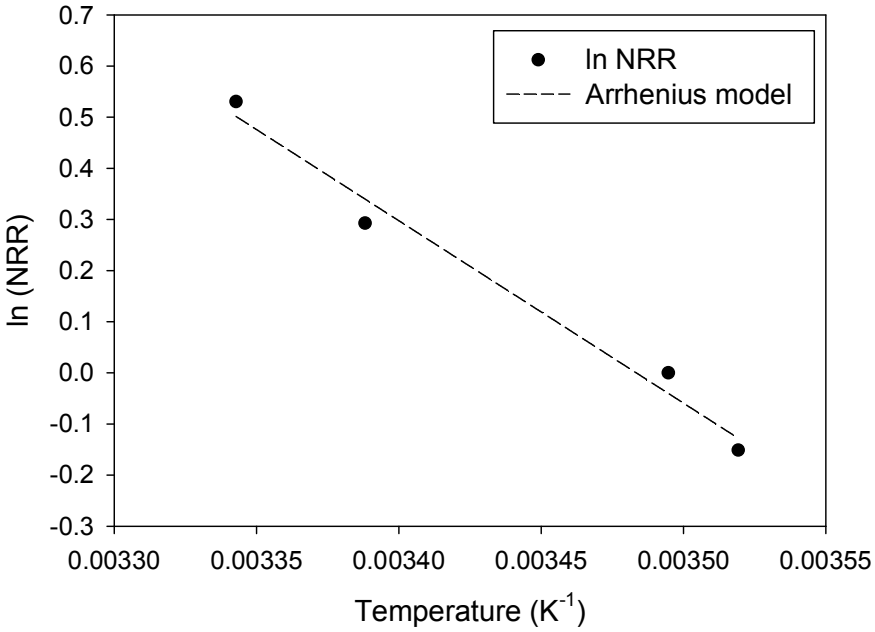

Fig. 9.3. Arrhenius plot for the anammox activity achieved in the UAnSB reactor in the long-term operation at low temperatures. NRR: nitrogen removal rate.

$$NRR_1 = NRR_{ref} \cdot \theta^{(T_1 - T_{ref})} \quad \text{(Eq. 9.2)}$$

where, NRR_1 (g N L^{-1} d^{-1}) is the nitrogen removal rate obtained in the long-term operation of the UAnSB reactor operating at 26, 22, 13 or 11 ºC; NRR_{ref} (g N L^{-1} d^{-1}): is the nitrogen removal rate obtained in the long-term operation of the UAnSB reactor operating at a temperature of reference; θ is the temperature coefficient; T_1 (ºC) is the temperature at the long-term operation (26, 22, 13 or 11 ºC); and T_{ref} (ºC) is the temperature chosen as reference.

One of the main differences between synthetic and real urban wastewater was the presence of organic matter, which could enhance the growth of heterotrophs in the sludge bed. The maximum heterotrophic activity was evaluated before and after the change from synthetic influent to real urban wastewater. Two types of tests were performed depending on the electron donor available for denitrification: (i) tests with an external organic matter addition (acetate as electron donor) and (ii) tests with the internal organic matter present in the sludge (i.e. decay products used as electron donors). Both nitrite and nitrate were added as electron acceptors. Fig. 9.4 shows both, the maximum specific heterotrophic activity and anammox activity achieved in batch tests performed with biomass from the UAnSB reactor of days 274 and 420, when synthetic and real influent were being treated, respectively.

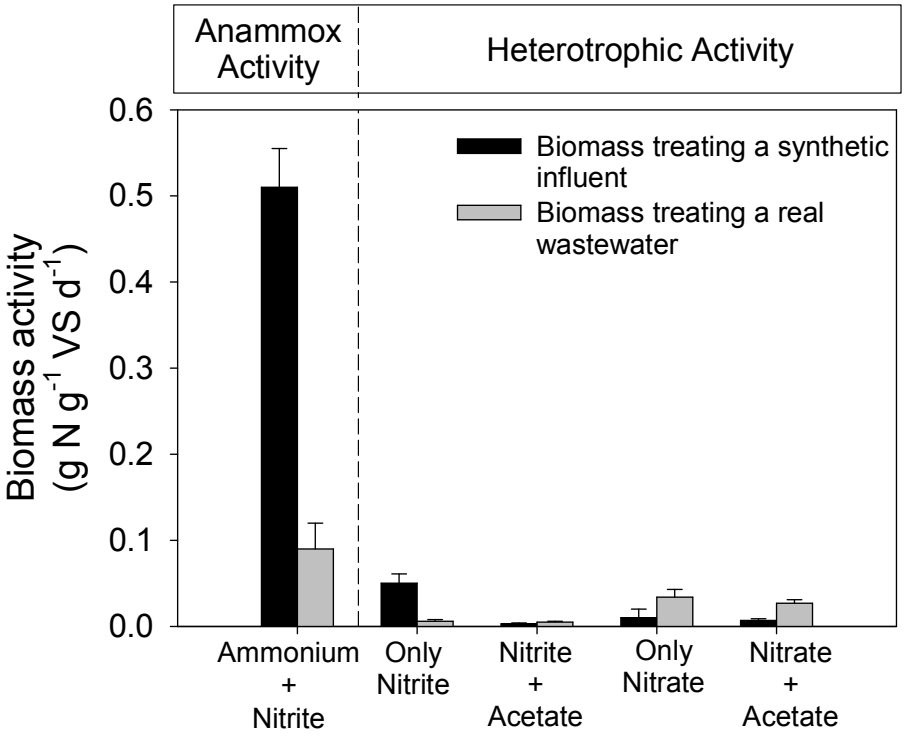

Fig. 9.4. Maximum denitrifying activity achieved when different substrates were used in batch tests performed in bottles maintained at 30 °C. Biomass used was either treating a synthetic influent at 13 °C (day 274) or treating real urban wastewater at 11 °C (day 420).

Overall, anammox activity was considerably higher than heterotrophic activity for both biomass samples. On the one hand, heterotrophic activity was barely observed in tests with biomass treating the synthetic wastewater, except for the test with nitrite and internal organic matter. On the other hand, heterotrophic activity was observed in tests with biomass treating the real urban wastewater when nitrate was used as electron acceptor. Hence, heterotrophic activity was 0.027 ± 0.004 g N L^{-1} d^{-1} when nitrate and acetate were used as substrates and 0.034 ± 0.009 g N L^{-1} d^{-1} when nitrate was used as the only substrate. Conversely, heterotrophic activity was barely detected when nitrite was used as a substrate with biomass treating the real wastewater. In any case, the batch tests demonstrated that maximum heterotrophic activity was 3-times lower than the maximum anammox activity for samples of biomass treating the real urban wastewater.

9.3.2. Physicochemical characterization of the sludge bed

Biomass concentration in the sludge bed of the UAnSB reactor was maintained stable during the whole operation treating the synthetic influent, with an average value of 10 ± 1 g VS L^{-1}. Total solids concentration was also stable with an average value of 13 ± 1 g TS L^{-1}.

Conversely, when the real influent was treated, both VS and TS increased until average values of 16.8 ± 0.5 g VS L^{-1} and 22.8 ± 0.9 g TS L^{-1}. The solids concentration in the effluent was maintained stable during the whole operation of the reactor (including synthetic and real influent) with an average value of (8 ± 6 mg TS L^{-1}) despite the real influent had a high content of solids (10 ± 3 mg TS L^{-1}) compared with the synthetic influent. Regarding the settling properties of the granules, the settling velocity and the sludge volumetric index at 5 min were maintained through the whole operation with average values of 41 ± 6 m h^{-1} and 30 ± 10 ml g^{-1} TS, respectively. A picture of the granules is depicted in Fig. 9.5.

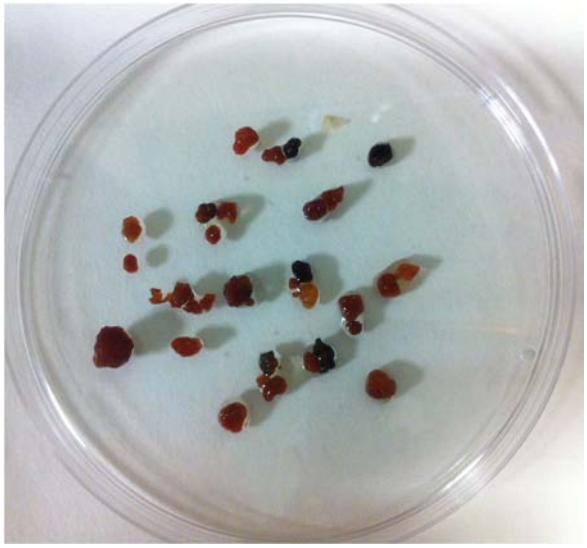

Fig. 9.5. Image of the granules of the UAnSB reactor over a petri dish.

The granule size was measured throughout the whole operation of the UAnSB reactor (Fig. 9.6B). During the first 300 days of operation the mean granule diameter was maintained in 820 ± 70 µm, however between days 300–400 a drop in granule size was observed until reaching a diameter as low as 368 µm on day 392. Then, the granule diameter started to increase until achieving a mean value of 800 ± 80 µm during the last month of the operation of the UAnSB reactor. In addition, Fig 9.7 shows the distribution of the granule diameter of samples from days 199, 373, 410 and 436, which corresponded with the period when the mean granule diameter presented more deviations as it is shown in Fig. 9.6B. On day 199 the unimodal shape confirmed that there was mainly one type of granule; then, on days 373 and 410 the granule size was low and two main sizes of granules were observed (two peaks in Fig. 9.7); finally on day 436 the granulation was recovered and the unimodal shape was obtained again.

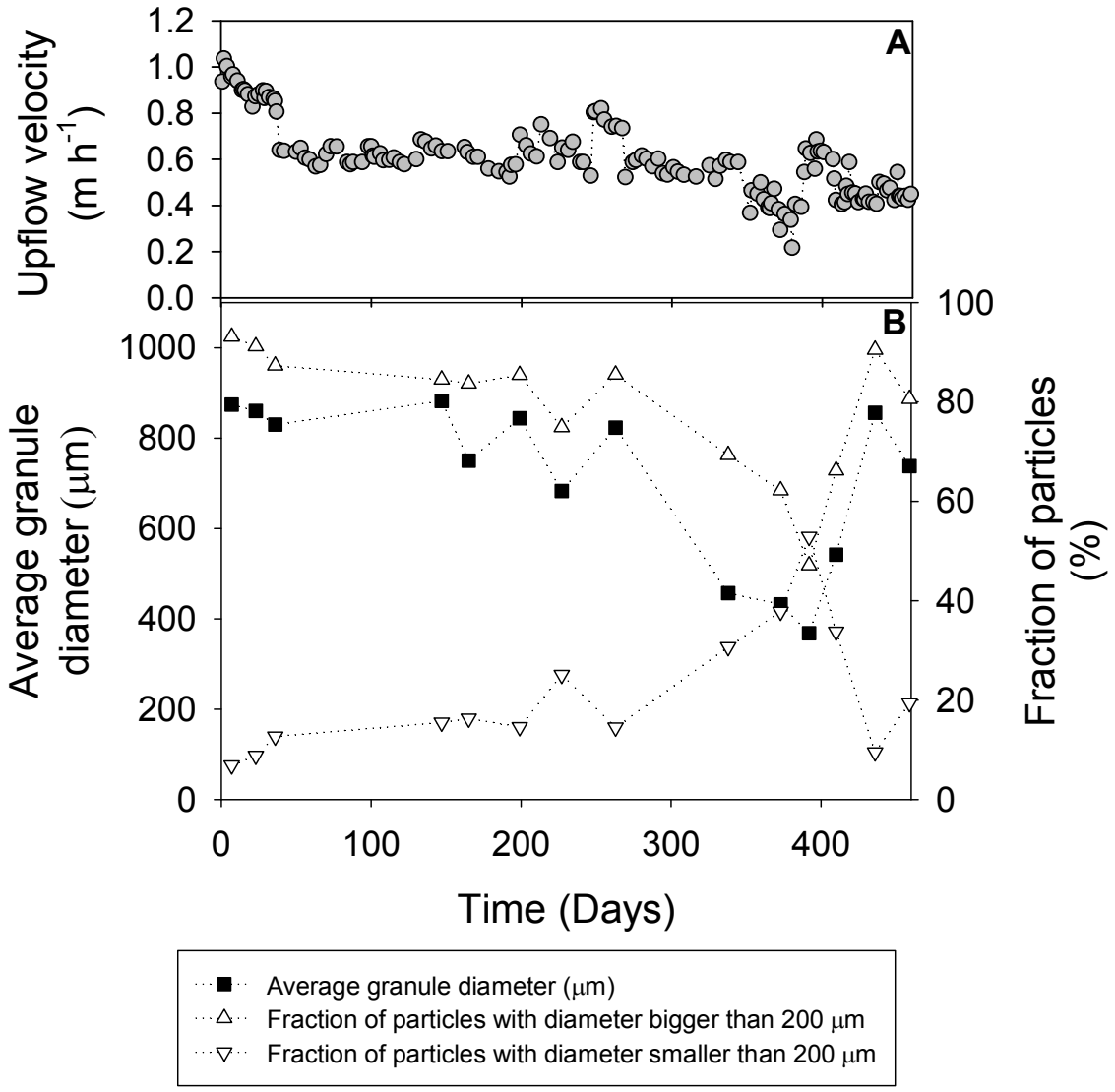

Fig. 9.6. Upflow velocity (A) and granule diameter (B) evolution during the long-term operation of the UAnSB reactor.

Since the real urban wastewater used as influent came from a WWTP of an industrial area, the presence of toxic or inhibitor compounds, which could be accumulated in the sludge of the UAnSB reactor, was a concern. Actually, the industrial activity of the area where the urban WWTP was located made the urban wastewater used as influent susceptible to present metals. Hence, a general screening of the most common elements present in sludge samples from days 0 and 423 was done (Table AII.1.1 of Annex II). Table 9.3 shows the most abundant metals found in the sludge.

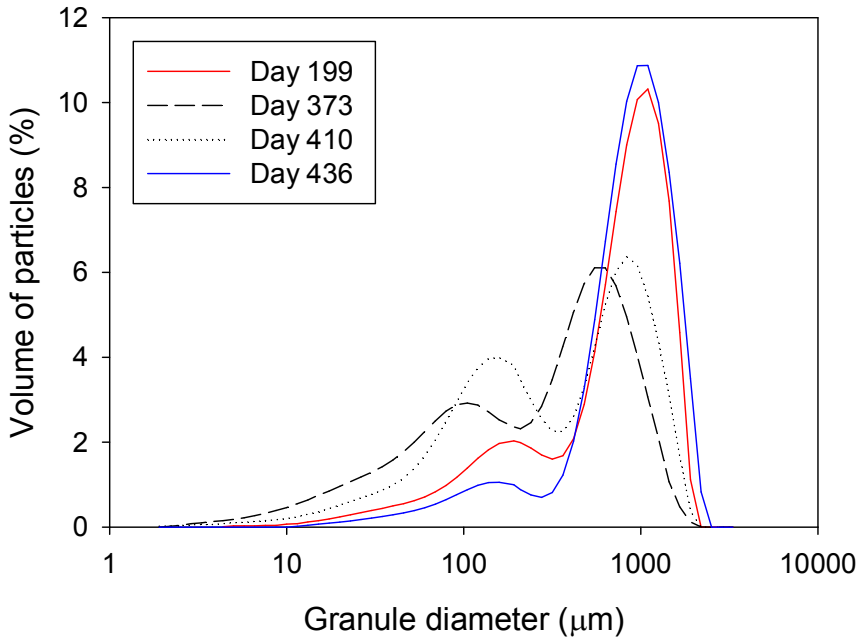

Fig. 9.7. Evolution of the granule diameter distribution during the operation of the UAnSB reactor at mainstream conditions.

Table 9.3. Most abundant metals found in samples of granular biomass from inoculum (day 0, synthetic influent) and from day 423 (real urban wastewater). The results are shown in micrograms of metal per gram of lyophilised biomass.

Element	**Day 0**	**Day 423**	**Units**
Zn	181	1882	$\mu g\ g^{-1}$
Sr	52	455	$\mu g\ g^{-1}$
Ba	28	266	$\mu g\ g^{-1}$
Cu	73	350	$\mu g\ g^{-1}$
Ni	68	114	$\mu g\ g^{-1}$
Sn	<5	266	$\mu g\ g^{-1}$
Pb	<5	37	$\mu g\ g^{-1}$
Co	5	45	$\mu g\ g^{-1}$

9.3.3. Microbial characterization of the sludge bed

Biomass samples from days 27 (22 °C, synthetic influent), 240 (13 °C, synthetic influent), 347 (11 °C, synthetic influent), 400 (11 °C, real wastewater) and 448 (11 °C, real wastewater) were analysed by using the FISH-CLSM technique to determine the enrichment in anammox bacteria during the long-term operation of the UAnSB reactor at mainstream conditions.

Regarding the FISH-CLSM analysis performed when the synthetic influent was used, *Candidatus Brocadia anammoxidans* was found to be the predominant microbial species identified in the sludge bed, although its abundance decreased when temperature decreased (Fig. 9.8). Thus, the inoculum (26 °C) contained a 93 ± 2% of *Candidatus Brocadia anammoxidans* and this percentage gradually decreased until 38 ± 5% on day 347 (11 °C). The anammox species *Candidatus Kuenenia stuttgartiensis* appeared in all the samples analysed with abundance always lower than 10%, and no change on this trend was observed when temperature decreased. The third anammox species identified was the *Candidatus Brocadia fulgida.* The higher abundance of this species (24 ± 3%) was found on day 240 (13 °C) and then, when temperature was lowered to 11 °C (day 347) its abundance decreased until 15 ± 2%. The presence of *Candidatus Brocadia fulgida* was not analysed neither in the inoculum nor on day 27 because the contributions of *Candidatus Brocadia anammoxidans* and *Candidatus Kuenenia stuttgartiensis* already covered almost the entire microbial population.

When the synthetic influent was replaced by the real urban wastewater but the UAnSB reactor continued operating at 11 °C, the abundance of the *Candidatus Brocadia anammoxidans* decreased from 38 ± 5% (day 347) to 27 ± 3% (day 400). However, the abundance of this species was maintained throughout the continuous operation of the UAnSB reactor treating real urban wastewater at 11 °C with an average value of 30 ± 6% of the total population (Fig 9.8). In the case of the *Candidatus Kuenenia stuttgartiensis,* the abundance was maintained as low as in the biomass samples from the synthetic influent. Regarding the *Candidatus Brocadia fulgida*, its abundance decreased during the long-term operation of the UAnSB reactor treating real urban wastewater at 11 °C, with a 3 ± 1% of abundance on day 448.

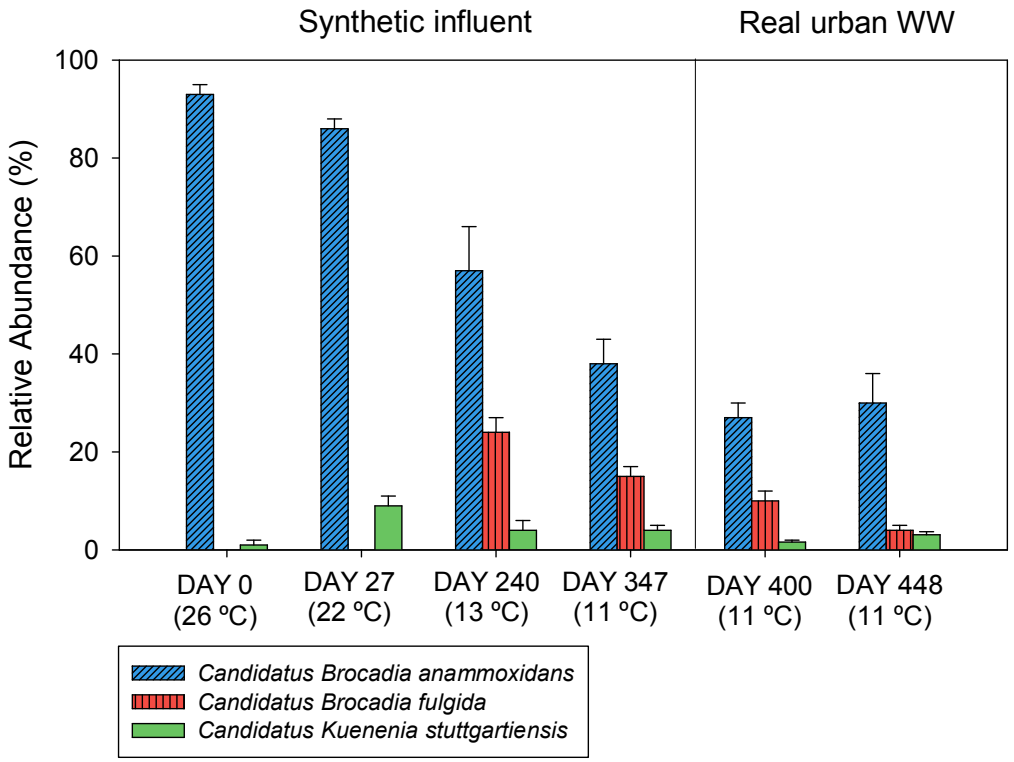

Fig. 9.8. Anammox species identified by the FISH-CLSM analysis performed on the granular sludge during the long-term operation of the UAnSB reactor. *Candidatus Brocadia fulgida* was not analysed in samples of days 0 (inoculum) and 27. Days 27, 240 and 347 corresponded to the treatment of the low-strength synthetic influent, while days 400 and 448 corresponded to the treatment of the real urban wastewater (WW).

FISH-CLSM was also used to quantify the abundance of all anammox bacteria in the biomass samples by using a general probe (AMX368; Table 4.1, Chapter 4) with specificity for all the anammox microorganisms. Hence, Fig. 9.9 shows the abundance of all anammox bacteria versus the sum of abundances of *Candidatus Brocadia anammoxidans, Candidatus Kuenenia stuttgartiensis* and *Candidatus Brocadia fulgida.* On days 0 and 27 the sum of species identified was close to 100% and, thus, the general anammox abundance with the general probe was not assessed. Fig. 9.9 shows that (i) the abundance of anammox bacteria was maintained higher than 80% in the sludge bed of the UAnSB reactor when the synthetic influent was treated (even at 11 °C) and it decreased until 72 ± 6% (day 448) in the long-term operation at 11 °C treating a real urban wastewater, and (ii) the sum of the different anammox species analysed compared to the total anammox bacteria identified with the general probe differed when temperature was lowered to 11 °C. This difference was maintained when the real urban wastewater was used as influent. This could mean that at least one anammox

species, which was not previously identified in the biomass samples (different from the analysed ones), appeared in the reactor when temperature decreased to 11 °C.

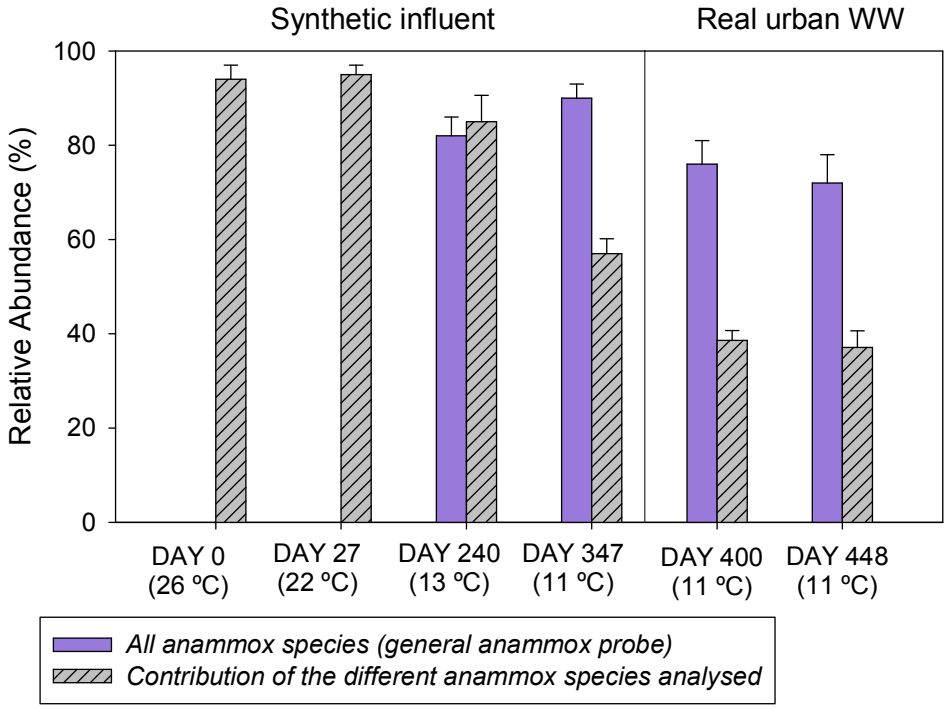

Fig. 9.9. Comparative of the abundance of anammox bacteria identified with the general probe by FISH-CLSM versus the sum of abundances of the species *Candidatus Brocadia anammoxidans, Candidatus Kuenenia stuttgartiensis* and *Candidatus Brocadia fulgida*. The general probe for all anammox was not used in sample of days 0 (inoculum) and 27. Days 27, 240 and 347 corresponded to the treatment of the low-strength synthetic influent, while days 400 and 448 corresponded to the treatment of the real urban wastewater (WW).

In addition to the FISH-CLSM analysis, pyrosequencing technique was used to examine the microbial community developed in the UAnSB reactor after the long-term operation at low temperatures treating the synthetic influent at days 240 (13 °C) and 347 (11 °C) and also the microbial community developed after the change of influent to a real wastewater at day 488 (11ºC).

Regarding the anammox population, *Candidatus Brocadia* was the most abundant genus with a relative abundance of 61% of the total reads on sample from day 240, which corresponded to the long-term operation treating the synthetic influent at 13 ºC (Fig. 9.10). However, its abundance decreased to 13% on sample from day 347 (operation at 11 °C) and resulted as low as 5% on sample from day 448, after the long-term operation treating a real urban wastewater at 11 °C. The genus *Candidatus Kuenenia* only appeared with an abundance

of 3% on sample from day 347. In addition, abundances of 15 and 7% on samples from days 240 and 347, respectively, were identified as *Planctomycetes* phylum, which is the phylum that anammox microorganisms belong to. Furthermore, on samples from days 347 and 448 there was a high abundance of reads which were unclassified at genus level (classified as bacteria at kingdom level). The centroid sequence from this unclassified selection (corresponding to abundances of 27% and 26% of total reads on samples of days 347 and 448, respectively) was run against BLAST and matched to the OTU B-3 found by Yamagishi et al. (2013) in the clone library analysis of a sample from a biofilm developed in a swine wastewater treatment facility with presence of anammox bacteria. Nonetheless, the reported study did not affiliate the OTU B-3 with any cultured bacteria.

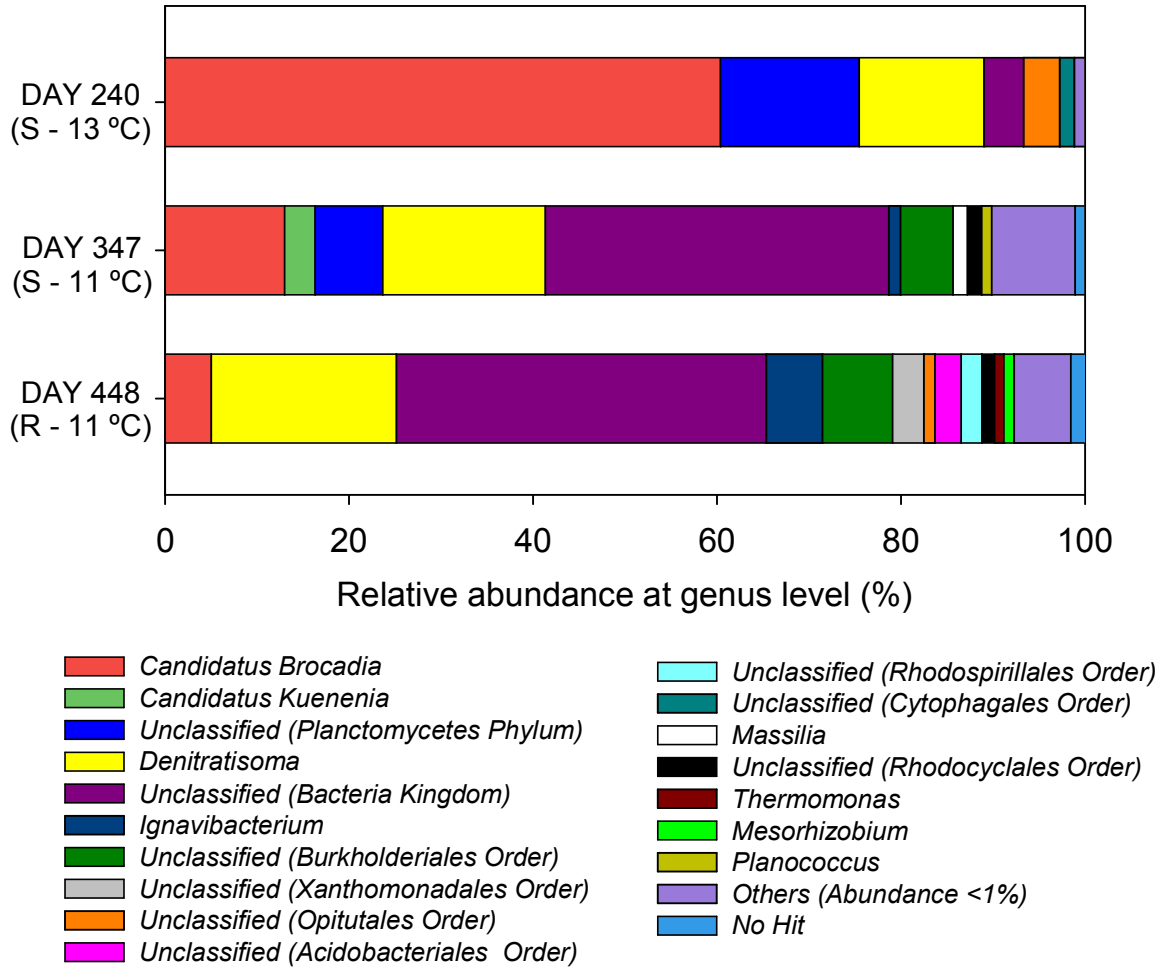

Fig. 9.10. Microbial diversity at genus level on days 240 (S–13°C: synthetic influent at 13°C), 347 (S–11°C: synthetic influent at 11°C) and 448 (R–11°C: real urban wastewater at 11°C). Relative abundance was calculated only considering those microorganisms in which the number of 16S copies was higher than 0.5% of the total copies.

Regarding other abundant genera present in biomass samples from the UAnSB reactor, *Denitratisoma* genus was found through the entire operation of the UAnSB reactor and, moreover, its abundance gradually increased during the operation, resulting in 14, 18 and 20% of the total reads on samples from days 240, 347 and 448, respectively. More specifically, the OTU identified corresponded to the *Denitratisoma oestradiolicum* species, which has been reported as heterotrophic denitrifying bacteria capable of using either nitrite or nitrate as electron acceptors (Fahrbach et al., 2006). In addition, the *Ignavibacterium* genus appeared on sample from day 347 when synthetic influent was treated with an abundance of 1% of total reads, which increased until 6% when the real urban wastewater was treated. *Ignavibacterium* genus was reported as a heterotrophic denitrifier capable of using nitrite but not nitrate as electron acceptor (Ramos et al., 2016).

9.4. DISCUSSION

9.4.1. Effect of low temperature on the anammox activity

The effect of the temperature decrease on the long-term operation of the UAnSB reactor was evaluated when a synthetic influent mimicking municipal wastewater was treated. A stable operation at high NLR and NRR with a good quality of effluent was achieved at any of the temperatures tested (Fig. 9.1A). Nevertheless, a considerable decrease of the specific anammox activity was observed when temperature decreased (Fig. 9.2). In fact, a decrease of 28% of anammox activity occurred when temperature decreased from 22 to 13 ºC. The unfavourable effect when temperature falls below 15 ºC was previously observed in anammox systems (Laureni et al., 2015) and, even more, many systems operating at temperatures below 15 °C triggered to the destabilization of anammox process either in one-stage (Laureni et al., 2016; Lotti et al., 2014a) or two-stage systems (Jin et al., 2013; Sánchez Guillén et al., 2016). When temperature was lowered to 11 °C, anammox activity only decreased a 10% compared to activity at 13 ºC, which could be explained by the adaptation of anammox biomass to low temperatures during the operation of the UAnSB reactor at 13 °C for more than 200 days. In fact, anammox biomass was reported to experiment adaptation to low temperatures (Dosta et al., 2008; Hu et al., 2013; Lotti et al., 2015c).

The effect of temperature on the anammox activity was successfully described according to the Arrhenius equation for the range of temperatures 26–11 ºC (Fig. 9.3). Thus,

the temperature dependency was described by one activation energy (E_a) and a single temperature coefficient (θ). This result differed from the reported by Lotti et al. (2015c) where different temperature dependencies were observed at different temperature intervals between 30–10 °C: the lower the temperature the higher the temperature dependency was. Moreover, the value of E_a (30 ± 3 kJ mol^{-1}) obtained was lower to that reported by Lotti et al. (2015c) for different anammox sludge and to that reported elsewhere, no matter if biomass adapted or not adapted to low temperature was used (Dapena-Mora et al., 2007; Hendrickx et al., 2012, 2014; Strous et al., 1999). The low value of E_a obtained implies that the anammox biomass of the present study had more resilience to temperature changes than those previously reported. This could be due to the fact that the reported studies performed short-term activity tests to determine the anammox activity; while in the present study anammox activity was measured after long-term operation at each temperature. Hence, it could be suggested that the anammox adaptation at each temperature helped to face the next temperature change, avoiding the temperature shock imposed to anammox activity when performing short-term tests. Furthermore, the low value of the temperature coefficient obtained (1.043 ± 0.004) demonstrated a low temperature dependency of the anammox biomass developed in the UAnSB reactor of the present study. In fact, the temperature coefficient value was in the range of the reported for heterotrophic denitrifiers (Carrera et al., 2003). These results suggest that anammox bacteria cannot be regarded anymore as intrinsically highly resilient to low temperatures. In the same way, Lotti et al., (2015b) recently published a study which demonstrated that anammox bacteria cannot be regarded anymore as an intrinsically slow growing microorganism, because the maximum growth rate can be increased when adequate cultivation conditions are imposed. Thus, possibilities for bioprocess design, such as the volume of the bioreactors, should take into consideration these recently results obtained in anammox cultures, especially for the implementation of anammox process in the mainstream of urban wastewater treatment plants.

In addition to the stable operation of the UAnSB reactor for more than 300 days at temperatures lower than 15 °C, high nitrogen removal rates were achieved in comparison to other similar systems. Hence, a NRR of 0.86 ± 0.07 g N L^{-1} d^{-1} was achieved at 11 °C, which was at least one order of magnitude higher than the reported for other anammox systems, either in one or two-stage systems operating at low temperature with synthetic wastewater (Gilbert et al., 2014; Sánchez Guillén et al., 2016). Furthermore, the nitrite to ammonium consumption ratio and the nitrate produced to ammonium consumed ratio obtained when the

synthetic influent was treated at low temperature were close to the previously reported for anammox cultures (Lotti et al., 2014c; Strous et al., 1998) and confirmed that anammox was the main process taking place in the UAnSB reactor (i.e. heterotrophic denitrification was negligible). In fact, heterotrophic denitrification was not expected to be significant in the system since no organic carbon was present in the influent and if any, it could be only expected by the use of the decay-products.

Additionally to the decrease in anammox activity, low temperatures affected the size of anammox granules: granule diameter sharply decreased when temperature decreased to 11 °C (Fig. 9.6B). It could be thought that the diameter decrease was due to a different parameter rather than temperature, such as the lost of activity or changes in the liquid upflow velocity (V_{up}). In fact, V_{up} was demonstrated to be a key parameter in the granulation and operation of UASB reactors (Arne Alphenaar et al., 1993; Liu and Tay, 2002). In this sense, an exhaustive study of the influence of V_{up} on the performance of the UAnSB reactor was presented in Chapter 8. It was previously demonstrated (Section 8.3.2.1, Chapter 8) that values of V_{up} lower than 0.4 m h^{-1} led to losing granulation when reactor operated at 26 °C while higher values enhanced granulation. For V_{upS} higher than 0.8 m h^{-1} granule diameter stabilized with no further increase. Thus, with the purpose of avoiding the deterioration of the granulation in the sludge bed of the UAnSB reactor of the present study, V_{up} was maintained at an average value of 0.48 ± 0.09 m h^{-1} (Fig 9.6A). Regarding the effect of activity, granule size increased at the end of the operation of the UAnSB reactor when the real influent was treated until recovering the original diameter (800 ± 80 μm). At this point, the anammox activity was the lower one of the whole operation, so the changes in granule size could not be associated to anammox activity but to other parameters, such as temperature. In contrast, Lotti et al. (2014b) reported an increase of granule diameter from 1.5 to 2.1 mm when temperature decreased from 20 to 10 °C.

Regarding the microbial characterization, FISH analysis demonstrated the preservation of a high anammox enrichment during the entire operation treating the synthetic influent at low temperatures, which could explain the high stability of the anammox process in the UAnSB reactor. The decrease of the most abundant species, *Candidatus Brocadia anammoxidans*, was observed in favour of the appearance of *Candidatus Brocadia fulgida* after the long-term operation at 13 °C (Fig. 9.8). *Candidatus Brocadia fulgida* was reported to be the dominant anammox species in anammox reactors operating at low temperature (Hendrickx et al., 2014; Laureni et al., 2015; Lotti et al., 2014b). Thus, the role of *Candidatus*

Brocadia fulgida under mainstream conditions was further supported by this study, which suggests that this species could have a competitive advantage at low temperatures. Actually, it also supports the fact that microbial ecology was determined by the influent and bulk liquid substrate concentrations, rather than the inoculum used (Park et al., 2010). When temperature decreased to 11 ºC, all the anammox species identified by FISH decreased (even the *Candidatus Brocadia fulgida,* Fig. 9.8) in agreement with the pyrosequencing analysis which showed a significant decrease of *Candidatus Brocadia* genus (Fig. 9.10). However, general anammox bacteria abundance was maintained according to the FISH results (Fig. 9.9), which could suggest that different anammox species with high adaptation to low temperature appeared in the sludge bed of the UAnSB reactor. In fact, a high abundance of unclassified bacteria were found by pyrosequencing on sample from day 347, and the anammox species not identified could be part of it. It could be hypothesised that for example *Candidatus Anammoxoglobus propionicus* appeared in the sludge bed, since Gonzalez-Martinez et al. (2016) reported the appearance of *Candidatus Anammoxoglobus propionicus* and the decreasing of *Candidatus Brocadia* species in a CANON reactor when temperature was lowered from 35 to 25 °C; they suggested that the former could have a higher tolerance to low temperatures than the later.

9.4.2. Effect of the real urban wastewater on the anammox activity

The effect of treating a real urban wastewater at 11 ºC at long-term in the UAnSB reactor was evaluated. The replacement of the synthetic influent by the real urban wastewater led to a considerable decrease of anammox activity (Fig. 9.2). However, a stable operation was achieved with an average NRR of 0.59 ± 0.05 g N L^{-1} d^{-1} during the last month of operation (Fig. 9.1C). The NRR achieved was considerably higher compared to other anammox systems treating a real influent at low temperatures. For instance, Laureni et al. (2015) reported a NRR of 0.046 g N L^{-1} d^{-1} in an anammox sequencing batch reactor (SBR) operating at 12.5 °C and Hendrickx et al. (2014) reported a NRR of 0.027 g N L^{-1} d^{-1} in a gas-lift anammox reactor operating at 10 ºC, which are one order of magnitude lower than the achieved in the present study. Besides, for a one-stage system Laureni et al. (2016) reported a maximum anammox activity of 0.1 g N L^{-1} d^{-1} in a PN/A SBR reactor operating at 15 ºC with an almost complete suppression of anammox activity at 11 ºC. The NRR achieved in the present study was only comparable to the achieved by Lotti et al. (2014b) in a lab-scale upflow fluidized granular sludge anammox reactor at 10 ºC, which resulted in a NRR of 0.43

g N L^{-1} d^{-1}. Furthermore, to the best of the author's knowledge, the average NRR obtained in this study (0.59 ± 0.05 g N L^{-1} d^{-1}) was the highest NRR reported hitherto at 11 °C treating a real urban wastewater.

The decrease of anammox activity in the UAnSB reactor when the synthetic influent was replaced by the real urban wastewater was not explained by the temperature effect, because temperature was already maintained at 11 °C for 70 days previous to the use of real wastewater. Hence, the lower anammox activity achieved when the real urban wastewater was treated should be associated to other factors rather than temperature such as: (i) the competition for nitrite of anammox bacteria with heterotrophs, (ii) the shift or decrease of anammox population and/or (iii) the presence of potential toxic or inhibitoring compounds for anammox bacteria in the real urban wastewater.

The presence of organic matter in the real urban wastewater (Table 9.1) could lead to the growth of heterotrophic bacteria which could use the nitrite and nitrate present in the bulk liquid as electron acceptors to perform heterotrophic denitrification in the UAnSB reactor (Henze et al., 2008; Liu et al., 2007). The destabilization of anammox process due to the competition for nitrite between anammox and heterotrophs was reported before. Chen et al. (2016) studied the effect of increasing the ratio between the concentrations of COD and total nitrogen (COD/N ratio) on anammox activity and observed the suppression of anammox activity when the ratio was higher than 1.6 in a lab-scale anaerobic buffer reactor at 30 °C. Similar results were reported by Lackner et al. (2008) with a modelling study of a PN/A biofilm system where anammox process could not be sustained at COD/N ratios higher than 2.

In the present study, heterotrophic activity tests and pyrosequencing analysis demonstrated the presence of heterotrophs in the sludge bed of the UAnSB reactor during the entire operation. The biomass treating synthetic wastewater showed the highest heterotrophic activity when nitrite was used as electron acceptor and the decay products were used as electron donors (Fig. 9.4). This was expected since biomass was used to grow on decay products since influent was devoid of carbon source. In fact, when an external organic matter addition was used, the heterotrophic activity was as low in the nitrite batch test as in the nitrate batch test. The reason why nitrite was preferred over nitrate was unexpected and unclear, since nitrate was always present in the bulk liquid while anammox bacteria were expected to won the competition for nitrite. Conversely, when biomass was treating the real urban wastewater, the heterotrophic activity tests showed that denitrification via nitrite was

not relevant compared to denitrification via nitrate (Fig. 9.4). It could be surmised, that heterotrophic bacteria present in the sludge bed were not used to consume nitrite as electron acceptor due to the fact that anammox won the competition by nitrite in the UAnSB reactor. However, nitrate was always available for denitrifiers and, thus, heterotrophic bacteria could be used to consume nitrate rather than nitrite. Likewise, when the real urban wastewater was treated, the nitrite to ammonium consumption ratio barely increased while the nitrate produced to ammonium consumed ratio considerably decreased comparing with the ratios obtained with the synthetic influent and the corresponding ratios observed for anammox bacteria (Lotti et al., 2014c; Strous et al., 1998). This meant that a slight more nitrite consumption and a considerably more nitrate consumption than the expected by anammox bacteria occurred in the reactor. Hence, heterotrophic bacteria denitrified nitrite and nitrate and competed with anammox bacteria, which was expected since a COD removal of 50 ± 10% (c.a. 45 mg O_2 L^{-1} removed) was achieved in the UAnSB reactor when the real urban wastewater was treated. In fact, pyrosequencing results confirmed the presence of two denitrifiers which are able to consume nitrite and/or nitrate: *Denitratisoma* and *Ignavibacterium* genus. In any case, the heterotrophic denitrification via nitrate was useful to remove the nitrate produced by anammox and, thus, helped to guarantee a good effluent quality. Hence, the presence of heterotrophs in the sludge bed of the UAnSB reactor is expected to be useful as long as they do not outcompete anammox bacteria. Nevertheless, activity tests showed that heterotrophic denitrification was considerably lower than the autotrophic denitrification via anammox (Fig. 9.4), so anammox were expected to win the competition for nitrite against heterotrophic bacteria.

The prevalence of anammox activity over heterotrophic denitrification was reported before in anammox reactors treating real urban wastewater. For example, Laureni et al. (2015) reported that anammox bacteria won competition against heterotrophs in the long-term in a SBR operating at 29 ºC where, despite the addition of different carbon sources, nitrite was only removed when ammonium was spiked. The same result was reported by Malovanyy et al. (2015) in an integrated fixed film activated sludge (IFAS) reactor operating at 25 ºC, where an influent with a COD/N concentrations ratio of 1.8 guaranteed that the anammox outcompeted heterotrophs. Hence, the high decrease in anammox activity when the real urban wastewater was treated in the UAnSB reactor at 11 °C was not explained by the competition with heterotrophic bacteria.

A decrease in anammox population could cause the loss of activity when the synthetic influent was replaced by the real urban wastewater. Pyrosequencing analysis showed a significant decrease in anammox population with only 5% of the total reads identified as *Candidatus Brocadia* genus (Fig. 9.10) when the real urban wastewater was treated. Conversely, FISH results showed a highly enrichment in anammox bacteria (72 ± 6%) in the sludge bed of the UAnSB reactor despite of the decrease of abundance compared with the synthetic influent treatment (Fig. 9.9). This difference may be explained considering that FISH technique points toward the abundance of rRNA in samples, while pyrosequencing points toward the abundance of DNA (Wittebolle et al., 2005). Thus, the microbial diversity present in the sludge bed when the real wastewater was treated could be detected by pyrosequencing although the activities of some of these microbes in the reactor were low or null, which would contribute to decrease the relative abundance of anammox bacteria. Hence, it could be hypothesised that a change in the anammox species instead of the decrease of anammox population could cause the activity decrease. Actually, the decrease in temperature to 11 °C led to the appearance of one single or more anammox species which were not identified by the molecular techniques used and were maintained during the entire operation with the real urban wastewater. The hypothesis of the presence of *Candidatus Anammoxoglobus propionicus* could be considered since it was reported to consume small organic acids such as acetate and propionate in the presence of ammonium (Kartal et al., 2007), which could give them an adaptive advantage when organic matter is present. Nevertheless, further molecular techniques should be applied to identify the anammox species that could have appeared in the sludge bed. In any case, it could be surmised that the anammox species that appeared in the sludge bed had a lower growth rate and activity than the previous species identified in this work, which could explain the decrease of activity in the UAnSB reactor.

The presence of toxic or inhibitory compounds for anammox bacteria in the real urban wastewater could also explain the decrease of activity in the reactor when influent changed from synthetic to real influent. Trace amounts of some metals are components of many enzymes or co-enzymes and play an important role in the microbial metabolism, however high concentrations can cause inhibition and can be even toxic (Yang et al., 2013). Sludge elemental analysis showed a high accumulation of metals in the granular sludge after treating the real wastewater compared with the inoculum (Table 9.3). The inhibitory effect of metals on anammox bacteria was previously demonstrated (Bi et al., 2014). Besides, Lotti et al.

(2012) reported a considerable decreased in anammox activity caused by the prolonged exposure to copper (Cu), and Li et al. (2015) reported inhibition after short-term exposure to Cu and zinc (Zn). Furthermore, Li et al. (2015) determined nickel (Ni) as moderately toxic while lead (Pb) was poorly toxic for anammox bacteria. The mentioned studies reported inhibition of anammox bacteria in presence of metals dissolved in the bulk liquid, however little has been published about the effect of the accumulation of heavy metals in the anammox granules. In this sense, it was reported that an accumulation of Cu in anammox granules caused disorders in metabolic pathways affecting to the energy metabolism and cell synthesis, which led to the suppression of anammox activity (Zhang et al., 2016a, 2015). Still, biomass adaptation to the presence of inhibitors could be hypothesised in the UAnSB reactor in the long-term, which could explain the anammox activity decrease during the first days of treating the real urban wastewater and the subsequent stabilization in the long-term operation. In fact, Zhang et al. (2016b) suggested that anammox biomass gain self-adaptation to Cu through acclimation (e.g. secreting more EPS for self-protection).

In addition to the accumulation of metals, there was an increase in the solids content of the sludge bed, which could not be explained just because of the growth of the biomass, even though considering the growth of heterotrophs, and it was probably due to the retention of solids of the influent in the sludge-bed. This retention of solids could have two opposite implications: (i) if low, it could help granulation and (ii) if high, it could lead to a high inorganic content in the granules leading to destabilization of granulation. As mentioned before, the granule size increased after some time treating the real urban wastewater despite of the temperature was maintained at 11 °C. Thus, it could be hypothesized that the use of urban wastewater with high solids content enhanced the granulation due to different factors such as the addition of inert nuclei for bacterial attachment and/or the probable excess of EPS produced by anammox bacteria due to the stress associated to the income of organic matter with the real influent (Liu et al., 2003). Nevertheless, the excessive increase of inorganic compounds could reduce the available space for biomass growth, could affect the biomass activity if such compounds were at the same time toxic or inhibitors as previously mentioned and could affect to the internal mass transfer of substrates.

The reason why the anammox activity decreased when the real urban wastewater was treated in the long-term at 11 °C was unclear, but it could be the combination of the above-mentioned hypothesis. Other similar studies tried to explain the decrease of anammox activity when a real urban wastewater was treated with different hypothesis. On the one hand, causes

for the anammox activity suppression at 11 °C in a PN/AMX SBR remained unclear in Laureni et al. (2016) and they suggested that further investigation would be needed for elucidating the mechanisms that limited anammox activity in such system. On the other hand, Laureni et al. (2015) performed an extensive study to elucidate the causes of anammox activity decrease and reported that neither the heterotrophic competition for nitrite nor the shift of anammox population explained the adverse effects observed in anammox activity in a SBR treating a pre-treated municipal wastewater, and suggested that anammox population needed acclimation to the real influent and/or the development of a side population beneficial to it.

9.5. CONCLUSIONS

A stable long-term operation of the UAnSB reactor was maintained at high nitrogen removal rates, both treating a synthetic low-strength influent and treating a real urban wastewater at 11 ºC.

The decrease of temperature caused a decrease in anammox activity, however the anammox bacteria showed an adaptation at each temperature tested, which helped to face the next temperature change.

The enrichment of the sludge bed in anammox bacteria was maintained during the long-term operation. However, the abundance of the most abundant species (*Candidatus Brocadia anammoxidans*) decreased when temperature decreased, and unidentified anammox species appeared in the microbial community.

Anammox bacteria were expected to win the competition for nitrite with the heterotrophs present in the sludge bed, since anammox activity was always considerably higher than heterotrophic activity.

The presence of inhibitors and/or toxic compounds and the high solids content in the real urban wastewater used as influent was suggested to adversely affect the anammox activity.

Chapter 10

GENERAL CONCLUSIONS

The main objective of the present thesis was accomplished, since the feasibility of operating a two-stage system for performing the autotrophic biological nitrogen removal at mainstream conditions was demonstrated through the partial nitritation and anammox reactors successfully running in the long-term.

Hence, two blocks of conclusions can be stated considering the partial nitritation and anammox processes separately:

- **Partial nitritation process at mainstream conditions**

  - A granular sludge airlift reactor was successfully operated at 10 ºC performing stable partial nitritation of a synthetic influent in the long-term.

  - High nitrogen removal rates and an adequate effluent for a subsequent anammox reactor were obtained.

  - NOB repression was effectively achieved, being the nitrate production barely detected in the bulk liquid of the airlift reactor.

  - Microbial characterization of the developed biomass demonstrated that the sludge was highly enriched in AOB, while NOB genera were hardly detected. Furthermore, the presence of a heterotrophic population was found in the sludge.

  - The nitrifier culture enriched in AOB presented high values of the kinetic parameters μ_{max} and $K_{S,TAN}$ compared to other studies, which could explain the high nitritation rates obtained in the reactor, which were advantageous for NOB repression.

  - Nitrous oxide emissions from the granular airlift reactor performing partial nitritation at mainstream conditions were low compared to the emissions from reactors treating high-strength influents.

  - A dependence of nitrous oxide production with temperature was observed, resulting the higher the temperature the higher the N_2O production.

- **Anammox process at mainstream conditions**

  - The implementation of anammox process in an UAnSB reactor was proposed since a stable operation was maintained treating an urban wastewater in the long-term.

  - Liquid upflow velocity was demonstrated to be a key parameter in the implementation of anammox process in UASB reactors, since it affected to granulation and external mass transfer. The higher the liquid upflow velocity was the bigger the granules and the lower the external mass transfer problems were.

  - High nitrogen removal rates and high nitrogen removal efficiencoes were obtained in the UAnSB reactor treating an urban wastewater at temperature as low as 11 °C.

  - The negative effect of the temperature decrease on anammox activity was ratified when temperature lowered from 22 to 11 °C, however an adaptation of the anammox bacteria was observed after the long-term operation at each temperature tested (22, 13, 11 °C).

  - Microbial characterization of the developed biomass demonstrated that the sludge was highly enriched in anammox bacteria during the whole operation of the UAnSB reactor, even at low temperature. Furthermore, *Candidatus Brocadia anammoxidans* was the most abundant anammox species in the sludge developed in the reactor, although its abundance decreased with the temperature decreased.

  - Microbial characterization of the developed biomass showed the presence of heterotrophic bacteria. However, anammox activity was always higher than heterotrophic activity in the UAnSB reactor.

Chapter 11

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ANNEX I

COMPLETE PYROSEQUENCING ANALYSIS RESULTS

Pyrosequencing technique was used to evaluate the diversity and relative abundance of the different microorganisms present in the granular sludge of the reactors of this thesis.

Hence, this annex compiles the results obtained from the pyrosequencing analysis performed in samples of the lab-scale airlift reactor during the operation described in Chapter 5 and the lab-scale upflow anammox sludge bed reactor during the operation described in Chapter 9.

Furthermore, the indices of biological diversity were calculated for the obtained libraries indicating that a good coverage of diversity was reached.

I. PYROSEQUENCING ANALYSIS RESULTS FOR THE SAMPLES ANALYSED DURING THE CHAPTER 5: Kinetic and microbiological characterization of aerobic granules performing partial nitritation of a low-strength wastewater at 10ºC.

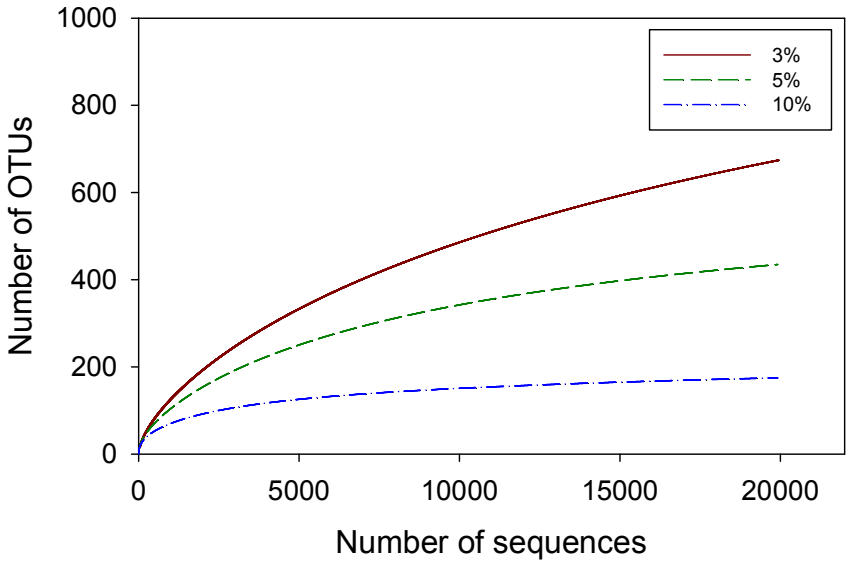

Figure AI.1.1. Rarefaction curves for the library of day 98. OTUs were defined at 3%, 5% and 10% distances, respectively.

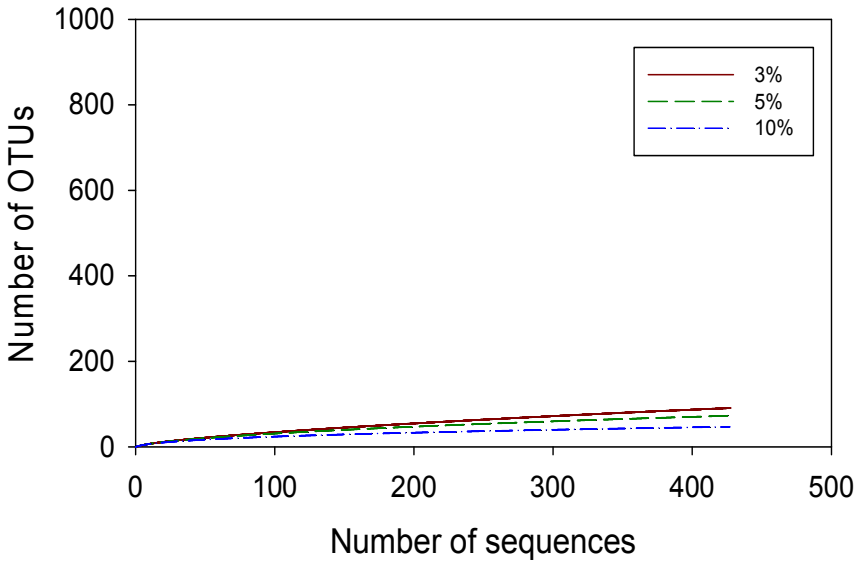

Figure AI.1.2. Rarefaction curves for the library of day 233. OTUs were defined at 3%, 5% and 10% distances, respectively.

Table AI.1.1. Indices of richness Chao1, diversity Shannon (H') and E of Eubacteria at 97, 95 and 90% of similitude for libraries d-98 and d-233 at which biomass samples were obtained.

Library	Chao1	Shannon (H')	E
97% Similitude			
d-98	1434	3.61	0.5358
d-233	267	3.20	0.7012
95% Similitude			
d-98	557	3.35	0.5513
d-233	147	3.02	0.7040
90% Similitude			
d-98	220	3.11	0.6013
d-233	70	2.71	0.7038

Table. AI.1.2. Pyrosequencing analysis results for the sample of day 98. Relative abundance was calculated considering only the microorganisms which the number of 16s copies was higher than 0.5% of the total copies.

Kingdom	Phylum	Class	Order	Family	Genus	Species	Relative Abundance (%)
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Nitrosomonadales*	*Nitrosomonadaceae*	*Nitrosomonas*	*Nitrosomonas sp*	40.8
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas sp*	7.9
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Nitrosomonadales*	*Nitrosomonadaceae*	*Nitrosospira*	*Nitrosospira sp*	7.2
Bacteria	*Actinobacteria*	*Actinobacteria (class)*	*Actinomycetales*	*Microbacteriaceae*	*Cryobacterium*	*Cryobacterium sp*	7.1
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingopyxis*	*Sphingopyxis macrogoltabida*	5.4
Bacteria	*Bacteroidetes*	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium sp*	4.7
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Comamonas*	*Comamonas nitrativorans*	4.5
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Dokdonella*	*Dokdonella sp*	4.4
Bacteria	*Bacteroidetes*	*Cytophagia*	*Cytophagales*	*Cytophagaceae*	*Flexibacter*	*Flexibacter sp*	4.0
No Hit	No Hit	No Hit	No Hit	No Hit	No Hit	No Hit	3.3
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Phyllobacteriaceae*	*Mesorhizobium*	*Mesorhizobium sp*	2.1
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Bradyrhizobiaceae*	*Nitrobacter*	*Nitrobacter sp*	1.5
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Brucellaceae*	*Mycoplana*	*Mycoplana sp*	1.3
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Rhodobacter*	*Rhodobacter sp*	0.9
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Devosia*	*Devosia insulae*	0.8
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Phyllobacteriaceae*	*Nitratireductor*	*Nitratireductor sp*	0.7
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Frateuria*	*Frateuria aurantia*	0.7
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Rhizobiaceae*	*Shinella*	*Shinella sp*	0.6
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas wittichii*	0.6
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Bradyrhizobiaceae*	*Afipia*	*Afipia sp*	0.6
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Rhizobiaceae*	*Sinorhizobium*	*Sinorhizobium sp*	0.5
Bacteria	*Bacteroidetes*	*Sphingobacteriia*	*Sphingobacteriales*	*Chitinophagaceae*	*Chitinophaga*	*Chitinophaga sp*	0.3

Table. AI.1.3. Pyrosequencing Analysis Results for the sample of day 233 after the bioinformatics treatment. Relative abundance was calculated considering only the microorganisms which the number of 16s copies was higher than 0.5% of the total copies.

Kingdom	Phylum	Class	Order	Family	Genus	Species	Relative Abundance (%)
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Nitrosomonadales*	*Nitrosomonadaceae*	*Nitrosomonas*	*Nitrosomonas europaea*	65.2
Bacteria	*Bacteroidetes*	*Cytophagia*	*Cytophagales*	*Unclassified*	*Unclassified*	*Unclassified*	14.6
Bacteria	*Bacteroidetes*	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	7.8
Bacteria	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Microbacteriaceae*	*Cryobacterium*	*Cryobacterium mesophilum*	2.1
Bacteria	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	1.8
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas sp*	1.6
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Comamonas*	*Comamonas nitrativorans*	1.5
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Acidovorax*	*Acidovorax sp*	1.1
Bacteria	*Bacteroidetes*	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium sp*	0.9
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Phyllobacteriaceae*	*Mesorhizobium*	*Mesorhizobium sp*	0.8
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonadales*	*Unclassified*	*Unclassified*	*Unclassified*	0.8
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*	*Brevundimonas*	*Brevundimonas sp*	0.7
Bacteria	*Chloroflexi*	*Anaerolineae*	*Anaerolineales*	*Unclassified*	*Unclassified*	*Unclassified*	0.6
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Devosia*	*Devosia sp*	0.6

II. PYROSEQUENCING ANALYSIS RESULTS FOR THE SAMPLES ANALYSED DURING THE CHAPTER 9: Stable long-term operation of an anammox UASB reactor at mainstream conditions

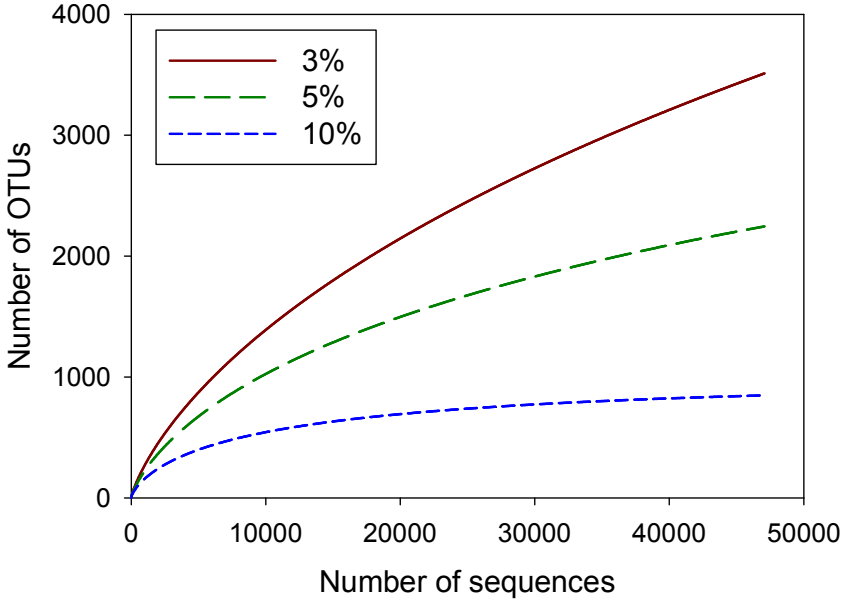

Figure AI.2.1. Rarefaction curves for the library of day 240. OTUs were defined at 3%, 5% and 10% distances, respectively.

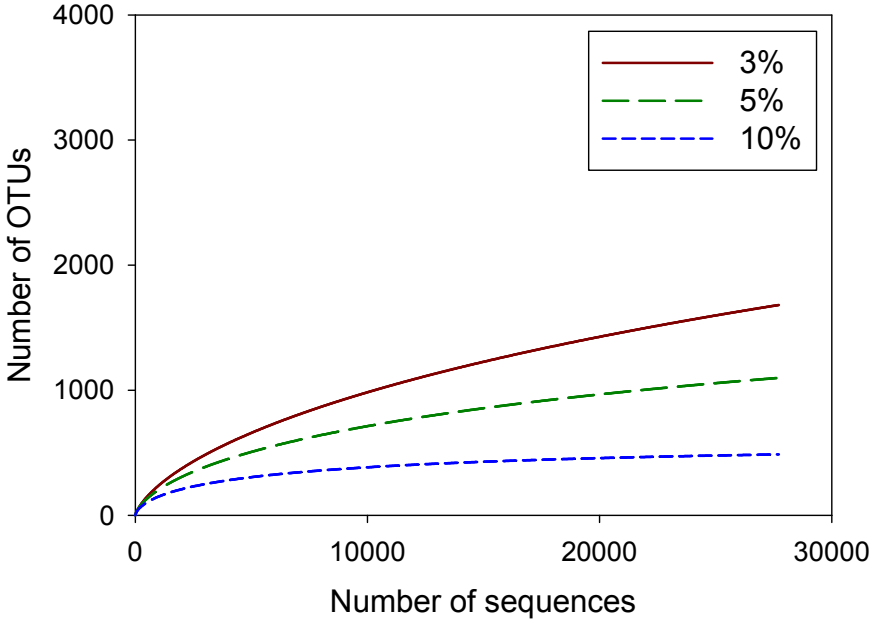

Figure AI.2.2. Rarefaction curves for the library of day 347. OTUs were defined at 3%, 5% and 10% distances, respectively.

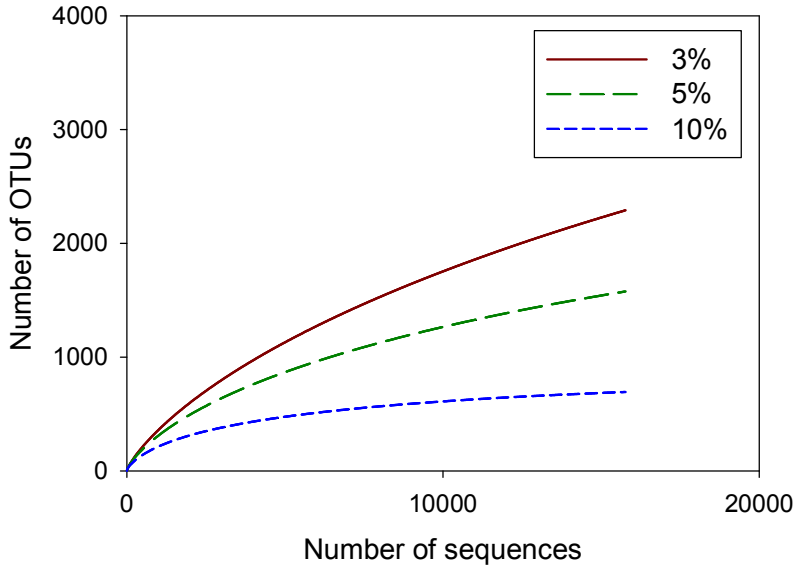

Figure AI.2.3. Rarefaction curves for the library of day 347. OTUs were defined at 3%, 5% and 10% distances, respectively.

Table AI.2.1. Indices of richness Chao1, diversity Shannon (H') and E of Eubacteria at 97, 95 and 90% of similitude for libraries d-240, d-374 and d-448 at which biomass samples were obtained.

Library	Chao1	Shannon (H')	E
97% Similitude			
d-240	9590	4.95	0.5897
d-374	3864	4.59	0.6022
d-448	6100	5.47	0.6903
95% Similitude			
d-240	4944	4.22	0.5295
d-374	2099	4.18	0.5784
d-448	3362	5.08	0.6710
90% Similitude			
d-240	1204	3.22	0.4636
d-374	801	3.75	0.5772
d-448	1034	4.39	0.6526

Table. AI.2.2. Pyrosequencing Analysis Results for the sample of day 240 after the bioinformatics treatment. Relative abundance was calculated considering only the microorganisms which the number of 16s copies was higher than 0.5% of the total copies.

Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Relative Abundance (%)**
Bacteria	*Planctomycetes*	*Planctomycetia*	*Candidatus Brocadiales*	*Candidatus Brocadiaceae*	*Candidatus Brocadia*	*Candidatus Brocadia sp*	54.6
Bacteria	*Planctomycetes*	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	15.1
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Denitratisoma*	*Denistratisoma oestradiolicum*	13.6
Bacteria	*Planctomycetes*	*Planctomycetia*	*Candidatus Brocadiales*	*Candidatus Brocadiaceae*	*Candidatus Brocadia*	*Unclassified*	5.8
Bacteria	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	4.3
Bacteria	*Verrucomicrobia*	*Optitutae*	*Optitutales*	*Unclassified*	*Unclassified*	*Unclassified*	3.9
Bacteria	*Bacteroidetes*	*Cytophagia*	*Cytophagales*	*Unclassified*	*Unclassified*	*Unclassified*	1.6
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Unclassified*	*Unclassified*	*Unclassified*	0.6
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	0.6

Table. AI.2.3. Pyrosequencing Analysis Results for the sample of day 347 after the bioinformatics treatment. Relative abundance was calculated considering only the microorganisms which the number of 16s copies was higher than 0.5% of the total copies.

Kingdom	Phylum	Class	Order	Family	Genus	Species	Relative Abundance (%)
Bacteria	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	37.3
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Denitratisoma*	*Denistratisoma oestradiolicum*	17.7
Bacteria	*Planctomycetes*	*Planctomycetia*	*Candidatus Brocadiales*	*Candidatus Brocadiaceae*	*Candidatus Brocadia*	*Candidatus Brocadia sp*	13.0
Bacteria	*Planctomycetes*	*Planctomycetia*	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	7.3
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Unclassified*	*Unclassified*	*Unclassified*	5.7
Bacteria	*Planctomycetes*	*Planctomycetia*	*Candidatus Brocadiales*	*Candidatus Brocadiaceae*	*Candidatus Kuenenia*	*Candidatus Kuenenia sp*	3.3
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Massilia*	*Naxibacter sp*	1.6
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclales*	*Unclassified*	*Unclassified*	*Unclassified*	1.5
Bacteria	*Ignavibacteriae*	*Ignavibacteria*	*Ignavibacteriales*	*Ignavibacteriaceae*	*Ignavibacterium*	*Unclassified*	1.3
Bacteria	*Firmicutes*	*Bacilli*	*Bacillales*	*Planococcaceae*	*Planococcus*	*Planococcus sp*	1.1
No hit	No hit	No hit	No hit	No hit	No hit	No hit	1.1
Bacteria	*Firmicutes*	*Bacilli*	*Bacillales*	*Unclassified*	*Unclassified*	*Unclassified*	0.95
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*	*Geobacteraceae*	*Unclassified*	*Unclassified*	0.93
Bacteria	*Bacteroidetes*	*Cytophagia*	*Cytophagales*	*Unclassified*	*Unclassified*	*Unclassified*	0.84
Bacteria	*Chloroflexi*	*Anaerolineae*	*Anaerolineales*	*Unclassified*	*Unclassified*	*Unclassified*	0.82
Bacteria	*Verrucomicrobia*	*Opitutae*	*Opitutales*	*Unclassified*	*Unclassified*	*Unclassified*	0.82
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Devosia*	*Devosia sp*	0.76
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Paracoccus*	*Paracoccus sp*	0.75
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Stenotrophomonas*	*Stenotrophomonas sp*	0.72
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp*	0.66
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	0.64
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*	*Brevundimonas*	*Brevundimonas sp*	0.59
Bacteria	*Acidobacteria*	*Acidobacteriia*	*Acidobacteriales*	*Unclassified*	*Unclassified*	*Unclassified*	0.58

Table. AI.2.4. Pyrosequencing Analysis Results for the sample of day 448 after the bioinformatics treatment. Relative abundance was calculated considering only the microorganisms which the number of 16s copies was higher than 0.5% of the total copies.

Kingdom	***Phylum***	***Class***	***Order***	***Family***	***Genus***	***Species***	**Relative Abundance (%)**
Bacteria	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	40.2
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Denitratisoma*	*Denistratisoma oestradiolicum*	20.1
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Unclassified*	*Unclassified*	*Unclassified*	6.5
Bacteria	*Ignavibacteriae*	*Ignavibacteria*	*Ignavibacteriales*	*Ignavibacteriaceae*	*Ignavibacterium*	*Unclassified*	6.1
Bacteria	*Planctomycetes*	*Planctomycetia*	*Candidatus Brocadiales*	*Candidatus Brocadiaceae*	*Candidatus Brocadia*	*Candidatus Brocadia sp*	5.0
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonadales*	*Unclassified*	*Unclassified*	*Unclassified*	3.4
Bacteria	*Acidobacteria*	*Acidobacteriia*	*Acidobacteriales*	*Unclassified*	*Unclassified*	*Unclassified*	2.8
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhodospirillales*	*Unclassified*	*Unclassified*	*Unclassified*	2.2
No hit	No hit	No hit	No hit	No hit	No hit	No hit	1.5
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclales*	*Unclassified*	*Unclassified*	*Unclassified*	1.4
Bacteria	*Verrucomicrobia*	*Opitutae*	*Opitutales*	*Unclassified*	*Unclassified*	*Unclassified*	1.2
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Unclassified*	*Unclassified*	1.1
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Phyllobacteriaceae*	*Mesorhizobium*	*Mesorhizobium sp*	1.1
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Thermomonas*	*Thermononas sp*	1.0
Bacteria	*Chloroflexi*	*Anaerolineae*	*Anaerolineales*	*Unclassified*	*Unclassified*	*Unclassified*	1.0
Bacteria	*Bacteroidetes*	*Cytophagia*	*Cytophagales*	*Unclassified*	*Unclassified*	*Unclassified*	0.9
Bacteria	*Acidobacteria*	*Solibacteres*	*Solibacterales*	*Solibacteraceae*	*Candidatus Solibacter*	*Candidatus Solibacter sp*	0.8
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclales*	*Unclassified*	*Unclassified*	*Unclassified*	0.8
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Acidovorax*	*Acidovorax sp*	0.7
Bacteria	*Bacteroidetes*	*Flavobateriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium sp*	0.7
Bacteria	*Planctomycetes*	*Planctomycetia*	*Unclassified*	*Unclassified*	*Unclassified*	*Unclassified*	0.6
Bacteriaa	*Gemmatimonadetes*	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadaceae*	*Gemmatimonas*	*Gemmatimonas sp*	0.6

ANNEX II

INORGANIC ELEMENTS ANALYSIS

This annex compiles the detailed results obtained from the semi-quantitative analysis performed to the sludge samples of the lab-scale upflow anammox sludge bed reactor during the operation described in Chapter 9. A general screening of the most common inorganic elements present in the sludge treating a real urban wastewater was performed.

Table AII.1.1. Inorganic elements detected in samples of granular biomass from inoculum (day 0, synthetic influent) and from day 423 (real urban wastewater). The results are presented in micrograms of metal per gram of lyophilised biomass.

Element	Inoculum	Day 423
Na	21	14
Mg	3	2
Al	< 0,25	0.7
P	20	24
S	13	< 10
K	3	1
Ca	31	37
Mn	0.2	0.2
Fe	7	11
Li	< 5	< 5
B	< 50	60
V	< 5	< 5
Cr	221	73
Co	5	45
Ni	68	114
Cu	73	350
Zn	181	1882
As	< 5	< 5
Se	31	20
Br	< 5	< 5
Rb	< 5	< 5
Sr	52	455
Mo	11	< 5
Ru	< 5	< 5
Rh	< 5	< 5
Pd	< 5	< 5
Ag	< 5	< 5
Cd	< 5	< 5
Sn	< 5	32
Sb	< 5	< 5
Te	< 5	< 5
I	< 5	< 5
Ba	28	266
La	< 5	< 5
Ce	< 5	< 5
Pr	< 5	< 5

Table AII.1.1. Continuation.

Element	Inoculum	Day 423
Nd	< 5	< 5
Sm	< 5	< 5
Eu	< 5	< 5
Gd	< 5	< 5
Dy	< 5	< 5
Ho	< 5	< 5
Er	< 5	< 5
Tm	< 5	< 5
Yb	< 5	< 5
Lu	< 5	< 5
Re	< 5	< 5
Os	< 5	< 5
Ir	< 5	< 5
Pt	< 5	< 5
Au	< 5	< 5
Hg	< 5	< 5
Pb	< 5	37
Bi	< 5	< 5
Th	< 5	< 5
U	< 5	< 5