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Evaluation of nitrogen elimination in anammox granular system for the treatment of industrial wastewater effluents

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Evaluation of nitrogen elimination in anammox granular system for the treatment of industrial wastewater effluents

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Outline of the thesis

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Summary (English)

The present doctoral thesis was focused on the application of the partial nitritation-anammox (PN-AMX) processes in a single unit with granular biomass for the removal of nitrogen from industrial wastewater effluents. Global population growth, water scarcity, climate change and more stringent effluent quality requirements (regarding the nitrogen, for example) are pushing the boundaries of the current state-of-the-art wastewater treatment for improved management of emissions before discharge.

The industrial effluents are complex liquors with high concentrations of nitrogen and organic matter. The removal of nitrogen was traditionally performed with the nitrification-denitrification biological processes, but the shortcut in the nitrogen cycle represented by the anammox bacteria provides as advantages lower energy (aeration) requirements, lower sludge production and no need for an external source of organic matter. Still, the effect of some characteristics of the industrial effluents needs further research in order to ensure good performance of the biological processes.

The application of the PN-AMX processes to the treatment of the supernatant from the anaerobic digestion (AD) of three hot-topic, challenging industrial effluents: fish-canning wastewater, pig slurry and the organic fraction of municipal solid waste (OFMSW) was studied in lab-scale reactors. For the OFMSW, the effect of an ammonia stripping pre-treatment before to the AD over the PN-AMX post-treatment processes was also assessed. Through the experimental results obtained, the applicability limits and potential destabilization of the PN-AMX processes were evaluated regarding the effect of high salinity and organic matter concentrations, as well as the evolution of the PN-AMX microbial populations and their potential competition or synergy with other biological processes, such as the heterotrophic denitrification. The gathered knowledge will be useful to address potential issues during the start-up of the process for its application at full-scale.

Resumen (Spanish)

La presente tesis doctoral se ha centrado en la aplicación del proceso combinado de nitritación parcial-anammox (PN-AMX) en una sola unidad con biomasa granular para la eliminación de nitrógeno de aguas residuales industriales. El crecimiento de la población mundial, la escasez de agua, el cambio climático y los requisitos de calidad del agua cada vez más estrictos (en relación al nitrógeno, por ejemplo) están ampliando las fronteras de los tratamientos de aguas residuales actuales para una gestión mejorada y vanguardista de las emisiones antes de la descarga final.

Las aguas residuales industriales son efluentes complejos con elevadas concentraciones de nitrógeno y materia orgánica. La eliminación de nitrógeno se ha realizado tradicionalmente mediante los procesos biológicos de nitrificación-desnitrificación; sin embargo, el atajo en el ciclo del nitrógeno que representan las bacterias anammox proporciona como ventajas un menor consumo de energía (aireación), una menor producción de lodos y la ausencia de necesidad por fuentes externas de materia orgánica. Aun así, el efecto de ciertas características de los efluentes industriales requiere de una mayor investigación para asegurar el correcto funcionamiento de los procesos biológicos.

La aplicación del proceso combinado PN-AMX al tratamiento del sobrenadante de la digestión anaerobia (AD) de tres tipos de aguas residuales industriales que suponen todo un desafío, como son el agua de conservera, los purines de cerdo y la fracción orgánica de los residuos sólidos urbanos (OFMSW) se ha estudiado mediante reactores a escala de laboratorio. En el caso de la OFMSW, también se ha estudiado el efecto adicional de un pre-tratamiento antes de la AD consistente en el stripping de amonio, sobre el post-tratamiento con el proceso PN-AMX. A partir de los datos experimentales obtenidos, los límites para la aplicación del proceso combinado PN-AMX, así como su potencial desestabilización, han sido evaluados teniendo en cuenta el efecto de altas concentraciones de materia orgánica y salinidad, así como la evolución de las poblaciones microbianas del proceso PN-AMX y su potencial competición o sinergia con otros procesos biológicos tales como la desnitrificación heterótrofa. El conocimiento adquirido será de utilidad para hacer frente a potenciales problemas durante el arranque de los procesos para su aplicación a escala real

Resumo (Galician)

A presente tese doutoral centrouse na aplicación do proceso combinado de nitritación parcial-anammox (PN-AMX) nunha soa unidade con biomasa granular para a eliminación de nitróxeno de augas residuais industriais. O crecemento da poboación mundial, a escaseza de auga, o cambio climático e os requisitos de calidade da auga cada vez más estritos (en relación ao nitróxeno, por exemplo) están ampliando as fronteiras dos tratamentos de augas residuais actuais para unha xestión mellorada e vangardista das emisións antes da descarga final.

As augas residuais industriais son efluentes complexos con elevadas concentracións de nitróxeno e materia orgánica. A eliminación de nitróxeno realizouse tradicionalmente mediante os procesos biolóxicos de nitrificación-desnitrificación; non obstante, o atallo no ciclo do nitróxeno que representan as bacterias anammox proporciona como vantaxes un menor consumo de enerxía (aireación), unha menor produción de lodos e a ausencia da necesidade por fontes externas de materia orgánica. Aínda así, o efecto de certas características dos efluentes industriais require dunha maior investigación para asegurar o correcto funcionamento dos procesos biolóxicos.

A aplicación do proceso combinado PN-AMX ao tratamento do sobrenadante da dixestión anaerobia (AD) de tres tipos de augas residuais industrias que supoñen todo un desafío, como son a auga de conserveira, os puriños de porco e a fracción orgánica dos residuos sólidos urbanos (OFMSW) estudiáronse mediante reactores a escala de laboratorio. No caso da OFMSW, estudiouse asemade o efecto adicional dun pre-tratamento previo á AD consistente no stripping de amonio, sobre o post-tratamento co proceso PN-AMX. A partires dos datos experimentais obtidos, os límites para a aplicación do proceso combinado PN-AMX, así como a súa potencial desestabilización, foron avaliados tendo en conta o efecto das altas concentracións de materia orgánica e salinidade, así coma a evolución das poboacións microbianas do proceso PN-AMX e a súa potencial competición ou sinerxía con outros procesos biolóxicos tales coma a desnitrificación heterótrofa. O coñecemento adquirido será de grande utilidade para facer fronte a potenciais problemas durante o arranque dos procesos biolóxicos para a súa aplicación a escala real.

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List of acronyms and symbols

A/O	Anoxic/Oxic (reactor)	
ABF	Anaerobic Biological Filtrated (reactor)	
AcS	Activated Sludge	
AD	Anaerobic Digestion	
AD-DN	Anaerobic Digestion - Denitrification (single-stage)	
AER1/2	aeration [batch assays including -]	
AerD	Aerobically Digested	
AF	Anaerobic Filter (reactor)	
AFB	Anaerobic Fluidized Bed (reactor)	
AFF	Anaerobic Fixed Filter (reactor)	
AGS	Aerobic Granular Sludge	
AHR	Anammox Hybrid Reactor	
AMX	Anammox	
ANARWIA	ANaerobic-Adding Raw Wastewater-Intermittent Aeration	
AnD	Anaerobic Degradation	
AnMBR	Anaerobic Membrane Bioreactor	
ANOVA	ANalysis Of VAriance	
AOA	Ammonia Oxidizing Archaea	
AOB	Ammonium Oxidizing Bacteria	
AOP	Advanced Oxidation Process	
AOR	Ammonium Oxidizing Rate	g N/(L·d)
ARB	Aged Refuse Bioreactor	
AS	Ammonia Stripping	
AS1/2/3/4	Activated Sludge [batch assays including -]	
AS-SBR	SBR treating OFMSW with AS pre-treatment	
AUSB	Aerated Upflow Sludge Bed (reactor)	
BMP	Biomethane Potential	
BNR	Biological Nitrogen Removal	
C	Carbon	
C/N	Carbon to Nitrogen ratio	g C/g N
CAB	Contact Aeration Basin	
CAD	Central Activity Digester	

CANON	Completely Autotrophic Nitrogen removal Over Nitrite	
CBR	Circulating Bed Reactor	
CF	Carbon Filter (reactor)	
CN	Carbon-Nitrogen (aerobic removal system)	
CNP	Carbon-Nitrogen-Phosphorus (aerobic removal system)	
COD	Chemical Oxygen Demand	g COD/L
COD/N	COD to Nitrogen ratio	g COD/g N
comammox	COMplete AMMonia Oxidiser	
CSTR	Continuously Stirred Tank Reactor	
db-RDA	distance-based Redundancy Analysis	
DEAMOX	DEnitrifying AMmonium OXidation	
DEMON	DEaMmONification (process)	
DIB	Deammonification in Interval-aerated Biofilm systems	
DN	Denitrification	
DNA	Deoxyribo-Nucleic Acid	
DO	Dissolved Oxygen	mg O_2/L
DSFF	Downflow Stationary Fixed Film (reactor)	
EDX	Energy Dispersive X-ray (spectroscopy)	
EGSB	Expanded Granular Sludge Bed (reactor)	
ELAN®	ELiminación Autótrofa de Nitrógeno	
EO	Electrochemical Oxidation	
EPS	Extracellular Polymeric Substances	
EU	European Union	
FA	Free Ammonia	g NH_3-N/L
FBBR	Fixed Bed Biofilm Reactor	
FBR	Fluidized Bed Reactor	
FFC	Fish and Food Cannery (wastewater)	
FGD	Flue Gas Desulphurisation	
FISH	Fluorescence *In Situ* Hybridization	
FNA	Free Nitrous Acid	g FNA/L
GAC	Granular Activated Carbon	
GSBAR	Granular Sequencing Batch Airlift Reactor	
HAR	Hybrid Anammox Reactor	
HB	Heterotrophic Bacteria	
HD	Heterotrophic Denitrification	
HDB	Heterotrophic Denitrifying Bacteria	
HRT	Hydraulic Retention Time	d
HSBR	Hybrid Sequencing Batch Reactor	
IASBR	Intermittently Aerated Sequencing Batch Reactor	

IC	Inorganic Carbon	g IC/L
ICAR	Internal Circulation Anammox Reactor	
ICAnR	Internal Circulation Anaerobic Reactor	
IC_{50}	half maximal Inhibitory Concentration	g/L
IFAS	Integrated Fixed-film and Activated Sludge reactor	
IFD	Integrated Fermentation-Denitritation	
k	conductivity	mS/cm
LFBR	LandFill BioReactor	
LFD	Liquid Fraction of Digestate	
LL	Landfill Leachate	
MABR	Membrane-Aerated Biofilm Reactor	
MAF	Mesophilic Anaerobic Filter	
MBR	Membrane Bioreactor	
MBBR	Moving Bed Biofilm Reactor	
MRWW	Municipal Reject Wastewater	
MSG	MonoSodium Glutamate (wastewater)	
N	Nitrogen	
N/IC	Nitrogen to Inorganic Carbon ratio	g N/g IC
NA	Not Applicable	
NAS	Nitrification Activated Sludge	
nAS-SBR	SBR treating OFMSW without AS pre-treatment	
ND	No Dilution	
NLR	Nitrogen Loading Rate	g N/(L·d)
N-ADN	Nitrification - Autotrophic Denitrification	
N-DN	Nitrification - Denitrification	
NOB	Nitrite Oxidizing Bacteria	
NOR	Nitrogen Oxidizing Rate	g N/(L·d)
NRBC	Non-woven Rotating Biological Contactor	
NRR	Nitrogen Removal Rate	g N/(L·d)
NRR_{max}	maximum NRR	g N/(L·d)
NS	Not Specified	
NT	Not Traceable	
OFMSW	Organic Fraction of Municipal Solid Waste	
OLAND	Oxygen Limited Autotrophic Nitrification Denitrification	
OMR	Organic Matter Removal	
OTU	Operational Taxonomic Unit	
P	Phosphorus	
PBS	Phosphate Buffer Solution	
PD-AMX	Partial Denitritation - Anammox	

PEG	PolyEthylene Glycol (gel carrier reactor)	
PITSF	Phased Isolation Tank Step Feed (technology)	
PN	Partial Nitritation	
PN-AMX	Partial Nitritation - Anammox	
PN-DN	Partial Nitritation - Denitritation	
PP	Pilot Plant	
PSBR	Pulsed SBR	
RA	Relative Abundance	
RBC	Rotating Biological Contactor	
RT	Room Temperature	
RWW	Reject Wastewater	
SAA	Specific Anammox Activity	
SAA_{OFMSW}	SAA with OFMSW as liquid medium	
SAA_{PBS}	SAA with PBS as liquid medium	
SAA_{slurry}	SAA with pig slurry as liquid medium	
SABF	Submerged Aerated Biological Filter	
SB	Swim-Bed (reactor)	
SBAR	Sequencing Batch Airlift Reactor	
SBR	Sequencing Batch Reactor	
SBBGR	Sequencing Batch Biofilter Granular Reactor	
SBBR	Sequencing Batch Biofilm Reactor	
sCOD	soluble COD	g sCOD/L
sCOD/N	sCOD to Nitrogen ratio	g sCOD/g N
$sCOD_{rem}$	sCOD removed	%
SD	Sulfur Denitrification	
SDA	Specific Denitrifying Activity	
SDA_{OFMSW}	SDA with OFMSW as liquid medium	
$SDA_{PBS\text{-}NO2}$	SDA with PBS as liquid medium and NO_2^- as nitrogen substrate	
$SDA_{PBS\text{-}NO3}$	SDA with PBS as liquid medium and NO_3^- as nitrogen substrate	
SE	Short Experiment	
SE	steam explosion	
SEM	Scanning Electron Microscopy; Scan Electron Microscope	
SFBR	Submerged Fixed-bed Biofilm bioReactor	
SHARON	Single reactor for High activity Ammonia Removal Over Nitrite	
SNA	Specific Nitritation Activity	
SNAD	Simultaneous partial Nitritation, Anammox and Denitrification	
SNAP	Single stage Nitrogen removal using Anammox and Partial nitrification	
SOD®	Sulfur Oxidation Denitrification	
SRT	Solids Retention Time	

STAR	Stirred Tank Anammox Reactor	
STIR1/2	stirring [batch assays including -]	
TAF	Thermophilic Anaerobic Filter	
TC	Total Carbon	g TC/L
tCOD	total COD	g tCOD/L
TN	Total Nitrogen	g TN/L
TN_{rem}	TN removed	%
TOC	Total Organic Carbon	g TOC/L
TOC/N	TOC to Nitrogen ratio	g TOC/g N
TSS	Total Suspended Solids	g TSS/L
UAC	Upflow Anammox Column (reactor)	
UAF	Upflow Anaerobic Filter	
UASB	Upflow Anaerobic Sludge Blanket (reactor)	
UBF	Upflow BioFilm (reactor)	
UC	Upflow Column (reactor)	
UF	UltraFiltration	
UFM	UltraFiltration Membrane	
UFBC	Upflow Fixed Bed Column (reactor)	
UMBR	Upflow Microaerobic Biofilm Reactor	
UMSR	Upflow Microaerobic Sludge Reactor	
USBF	Upflow Sludge Bed-Filter (reactor)	
USBR	Upflow Sludge Bed Reactor	
USI	Underground Soil Infiltration (system)	
VER	Volume Exchange Ratio	%
VIF	Variance Inflation Factor	
VS	Volatile Solids	
VSS	Volatile Suspended Solids	g VSS/L
VSS/TSS	VSS to TSS ratio	g VSS/g TSS
WW	Wastewater	
WWTP	Wastewater Treatment Plant	

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Chapter 1

Introduction

Summary

In Chapter 1, the current context for the treatment of industrial wastewater effluents is briefly described. Global population growth, water scarcity, climate change and more stringent effluent quality requirements (for example: nitrogen) are pushing the boundaries of the current state-of-the-art wastewater treatment for an improved management of emissions before discharge

The present thesis was focused on the application of the partial nitritation-anammox (PN-AMX) processes to the removal of nitrogen from industrial effluents.

The anammox bacteria, the existence of which was unveiled more than four decades ago, had its wealth of knowledge continuously growing for the last twenty years, and emerged (coupled with the partial nitritation process) as one of the most promising alternatives for nitrogen removal from industrial effluents. Still, some characteristics of the wastewater need further research and supervision to ensure a good performance and synergy of the biological processes.

The application of the PN-AMX processes to some hot-topic, challenging wastewater liquors such as fish-canning effluents, pig slurry or the organic fraction of municipal solid waste, as well as the technologies currently implemented, was also reviewed in this Chapter.

OUTLINE

1.1. Biological Nitrogen Removal (BNR) of the wastewater

The wastewater treatment is a relatively low-cost (e.g., 50 - 70 euro/(person·year) in The Netherlands) and low-energy demanding (< 7 W/person) process (Van Loosdrecht and Brdjanovic, 2014), with a potential energy content 5 times higher than its own electricity requirements (Liu et al., 2018). However, global phenomena, such as population growth, water scarcity and climate change, determine more stringent effluent norms for the wastewater treatment, pushing the boundaries of the current state-of-the-art sewage treatment plants for an improved management of the harmful pollution and emissions before its final discharge to the water bodies (Van Loosdrecht and Brdjanovic, 2014). On the other hand, both the increasing energy prices and the environmental sustainability through wastewater resource recovery have focused the attention over the efficient recovery of its energy content, with the final goal of achieving a self-sufficient (the so called autarky) and even energy-producing wastewater treatment (Liu et al., 2018; Schaubroeck et al., 2015).

The nitrogen is naturally abundant in the environment, and it is provided for plants and animals nutrition through the nitrogen cycle. However, the dynamic balance between different nitrogen species and the composition of the biosphere has been significantly altered by the human manipulation of the environment. As an example, the use of nitrogen fertilizers and its incorporation into the soil contribute to environmental problems such as the global acidification (Steffen and Rockström, 2015). Regarding the incorporation of nitrogen to the water, the ammonia is the most abundant inorganic nitrogen present in wastewater streams (Chaali et al., 2018). The discharge of excessive amounts of nitrogenous compounds to the final water bodies provokes the excessive growth of algae, known as eutrophication of algal blooms (Shumway, 1990), which limit the amount of dissolved oxygen required for respiration by other aquatic animal and plant species (Gonzalez-Martinez et al., 2018; Soliman and Eldyasti, 2018).

Chemical, physicochemical and biological processes are broadly used for the treatment of nitrogen-rich wastewater (Ni et al., 2017). Mulder (2003) differentiates three concentration ranges:

- < 100 mg N/L (e.g.: domestic wastewater), in which biological nitrogen removal is the preferred process based on cost-effectiveness;
- 100 - 5000 mg N/L (e.g.: sludge digestion liquor), in which biological treatment is preferred after extensive investigation; ammonia stripping and the production of $MgNH_4PO_4$ were identified as interesting alternatives for resource recovery (although not cost-effective in general);
- > 5000 mg N/L (e.g.: steam stripping of wastewater followed by ammonia recovery), in which physicochemical methods are technically and economically feasible.

The biological nitrogen removal (BNR) has been progressively more adopted compared to the physicochemical processes, since it is more effective and relatively inexpensive (Ahn, 2006), although its cost-effectiveness has increased dramatically in the past years, as more processes have become available (Schmidt et al., 2003). Conventional BNR processes, like the activated sludge technology and the combined autotrophic nitrification and heterotrophic denitrification, has been widely applied at full scale for wastewater treatment (Van Hulle et al., 2010). However, the Directive 91/271/EEC highlights the development of new biotechnological processes for water management solutions (EEC Council, 1991). The detection of the anammox bacteria biological pathway more than four decades ago led to the their identification and actual discovery in the 1990s, promoting the appearance of technologies for the autotrophic removal of nitrogen, which are characterized for being more cost-effective and environment friendly than the conventional BNR systems (Gonzalez-Martinez et al., 2018).

Between 30 - 70% of all molecular nitrogen released into the atmosphere is produced by microorganisms which existence has been not detected for a long-time, the anaerobic ammonium-oxidizing (anammox) bacteria (Kartal and Keltjens, 2016). The anammox biochemical reaction was predicted in the 1970s based on thermodynamic calculations (Broda, 1977), but it was not until Mulder et al. (1995) acknowledged a gap in the nitrogen mass balance of a denitrifying fluidized-bed reactor, that they were actually discovered. The stoichiometry of the process was further identified by Strous et al. (1998) (eq. 1.4), and recently revised by Lotti et al. (2014) (eq. 1.5).

Figure 1.1 summarizes the main biological processes within the nitrogen cycle, which will be described in the following sections, focusing in the different bacterial groups (squares) and substrates/products (circles) involved. For example, the anammox bacteria (red) takes as substrates both the ammonium present in the wastewater and the nitrite produced in the partial nitritation by the ammonium oxidizing bacteria (AOB, blue) to produce dinitrogen gas, while the heterotrophic denitrifying bacteria (HDB) use the nitrate produced by either the anammox bacteria or the nitrite oxidizing bacteria (NOB) to produce nitrogen and carbon dioxide.

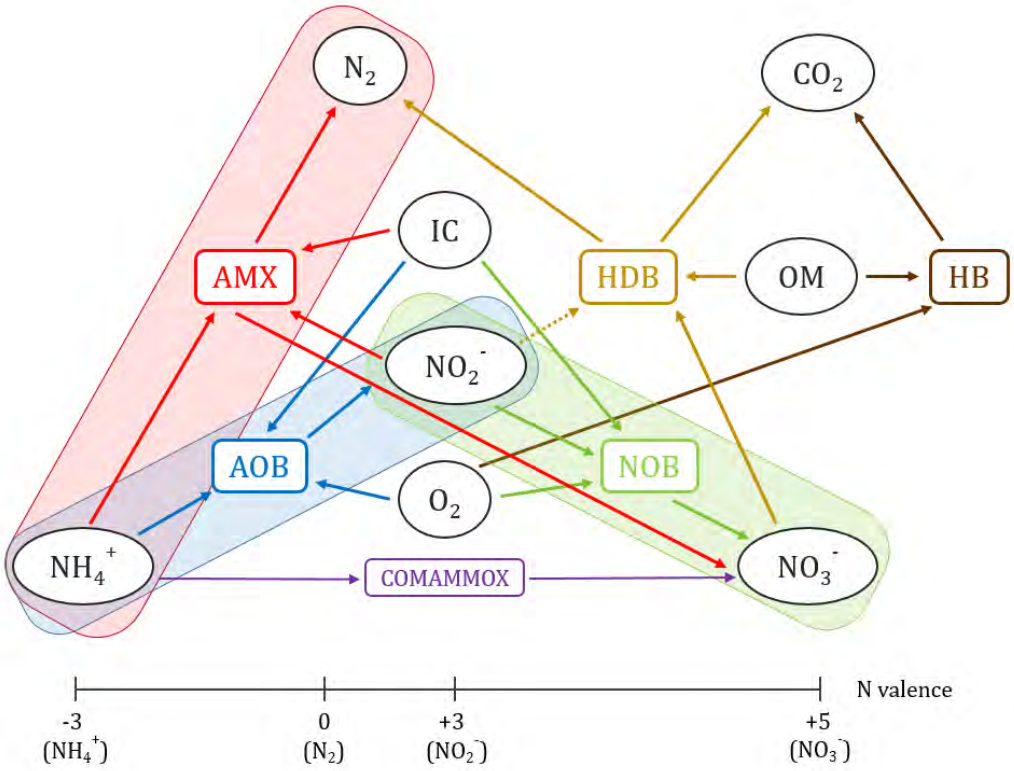

Figure 1.1. Bacterial groups (squares) and substrates/products (circles) for the main biological processes within the nitrogen cycle.

AMX: anammox, AOB: ammonium oxidizing bacteria, COMAMMOX: COMplete AMMonia Oxidiser, HB: (aerobic) heterotrophic bacteria, HDB: heterotrophic denitrifying bacteria, IC: inorganic carbon, NOB: nitrite oxidizing bacteria, OM: organic matter.

1.2. Anaerobic digestion of organic wastes and wastewater

Biomass wastes are regarded to be one of the most important renewable resources of the future. The most common techniques for the direct treatment of biomass and organic wastes are landfilling, farmland application, compost, incineration and Anaerobic Digestion (AD) (Ma et al., 2018). In the last decade, the interest has increased on the AD as a thriving and cost-effective technology for the treatment of biomass waste and wastewater, due to the high energy recovery as methane and its limited environmental impacts, providing a low sludge production and deactivating pathogens (Wang et al., 2018b). In fact, the anaerobic oxidation of organic compounds and its transformation in biogas constitute the link for actual energy recovery (Ma et al., 2018). As an example, Li and Kobayashi (2010) estimated that the annual potential of methane from the Organic Fraction of Municipal Solid Waste (OFMSW), livestock manure and sewage represents about 3950 Ml of crude oil in Japan.

The AD produces large quantities of digestate, of which the separation into two fractions (solid and liquid) allows for the valorization of each fraction by mechanical, physicochemical or biological means. The biological processes constitute an efficient, simple and economical way to recover nutrients, remove undesirable compounds and upgrade the quality of the digestate from both fractions (Monfet et al., 2018). For the solid fraction, the most common biological treatments are composting, enzymatic hydrolysis and biodrying, whereas a second AD step for the liquid fraction of digestate allows for further oxidation of organics in the wastewater.

After solid-liquid separation and AD of the liquid fraction of digestate (LFD), between 70 and 80% of the nitrogen (mainly in the form of ammonium) remains in the LFD (Fuchs and Drosg, 2013), since it is not oxidized or recovered during the AD. Thus, in order to prevent eutrophication problems in the final water bodies, the wastewater requires further post-treatment before discharge. Biological processes such as advanced BNR, algae biomass production and constructed wetlands can remove or recover these nutrients. In this sense, the wastewater begin to be regarded as a substantial supply route for nutrient resources (Ma et al., 2018). On the other hand, digestates (also known as reject waters or centrates) are characterized by low COD/N ratios (1.0 - 2.0 g COD/g N, Lackner et al. (2014)), which makes more economically favorable the advanced BNR.

Regarding the application of conventional AD to the treatment of wastewater, the main disadvantages are the long HRT and the larger reaction volumes required. However, the implementation of membrane technologies, resulting in the anaerobic membrane bioreactors (AnMBR), can be an interesting alternative due to the high organic matter removal efficiencies and complete suspended solids removal, which facilitate the application of nitrogen removal post-treatments (Ma et al., 2018).

1.3. Conventional BNR: nitrification-denitrification (N-DN)

In a conventional wastewater treatment plant (WWTP), the full nitrification-denitrification (N-DN) processes constitute the traditional flagship for the BNR in activated sludge systems. The nitrification comprises two steps for the BNR (see Figure 1.1): in the first one (partial nitritation), the ammonium is oxidized to nitrite by the ammonium oxidizing bacteria (AOB), while in the second step (partial nitratation) the nitrite is oxidized to nitrate by the nitrite oxidizing bacteria (NOB). On the other hand, the heterotrophic denitrification (HD) is the reduction of nitrate to nitrite and further reduction of the produced nitrite to dinitrogen gas in anoxic conditions, by a group of heterotrophic denitrifying bacteria (HDB) (Figure 1.1). The stoichiometry for the combined N-DN process (with acetic acid as organic matter source) follows eq. 1.1:

$$NH_4^+ + 0.625\ CH_3COOH + 2\ O_2 + HCO_3^- \rightarrow 0.5\ N_2 + 3.75\ H_2O + 2.25\ CO_2 \quad \text{eq. 1.1}$$

The HDB require a biodegradable organic carbon source for the successful completion of the HD process. These can be classified into three types (expressed as the chemical oxygen demand, or COD) based on the origin of the carbon source (Ni et al., 2017): COD in the influent wastewater, COD generated by the biomass itself (death and lysis of organisms), and external carbon sources. The preferred carbon sources in the wastewater for HDB are those readily biodegradable, while the HD rates for microorganisms-decay organics are much slower.

For wastewater streams with low content of organic matter, such as municipal solid waste and animal husbandry sewage water, the application of N-DN is not cost-effective (Nozhevnikova et al., 2012), and an external carbon source is necessary, being the methanol the most commonly used external carbon source for denitrification (Ni et al., 2017). This, together with the need for high aeration during the nitrification, as well as the high sludge production of the overall process, make the N-DN chemical- and energy-intensive processes. Thus, other environmentally friendly technologies for BNR are necessary to comply with the long-term sustainability (Van Loosdrecht and Brdjanovic, 2014).

1.4. Innovative BNR: partial nitritation-anammox (PN-AMX)

The innovative implementation of mature technologies such as the high-rate activated sludge, PN-AMX and partial denitritation-anammox (PD-AMX) systems, allows achieving an energy-positive characterization of the water resource recovery facilities, since they are less energy demanding and maximizing energy recovery. Nowadays, the innovation relies more in the way these processes are incorporated into the existing WWTPs (Solon et al., 2019).

The partial denitrification-anammox (PD-AMX) constitute a novel anammox-based process for the treatment of sewage, since the PD is an alternative to the partial nitritation for providing substrate nitrite to the anammox bacteria. This process can reduce by 79% the organic matter required for BNR, handling through denitrification the nitrate released by the anammox reaction, which would not comply with wastewater discharge standards (Li et al., 2016b). However, as it will be later discussed, this can also be achieved through the synergy of PN-AMX and HD processes.

The autotrophic PN-AMX combined process constitute an innovative alternative for the shortcut BNR, which has been intensively studied in the past several years due to the increasingly strict nitrogen discharge restrictions (Li et al., 2018c; Muñoz-Palazon et al., 2018). Up to date, over 2200 papers and 450 patents have been published related with the anammox process (Mao et al., 2017; Zhang and Liu, 2014), strongly suggesting that the anammox biotechnology is growing as a hot, mature research field.

Furthermore, the PN-AMX is often considered essential for the achievement of energy self-autarky, especially when integrated in the main line of WWTPs (Solon et al., 2019).

1.4.1. Characterization of the PN-AMX process

The cooperation among the key functional microbes present in the one-stage PN-AMX processes (AOB, NOB, HB, HDB and anammox bacteria) is critical for its stability as post-treatment of anaerobically pre-treated industrial wastewater (see Figure 1.1). The key functional bacteria (anammox) require a continuous supply of substrates ammonium (from the wastewater) and nitrite, which can be supplied by either the AOB (partial nitritation) or the HDB (partial denitritation). In order to achieve a stable PN-AMX process, the AOB and anammox bacteria must be properly balanced, while simultaneously inhibiting the NOB growth and carefully suppressing or exploiting HDB.

1.4.1.1. Ammonium Oxidizing Bacteria (AOB)

Since the nitrite is not present in the wastewater for the implementation of the anammox process, the previous conversion of ammonium to nitrite by AOB during the PN is required (eq. 1.2). The optimal pH and temperature range for the AOB is 7.5 - 7.8 and 30 - 35 °C (Soliman and Eldyasti, 2018).

$$NH_4^+ + 1.982\ HCO_3^- + 1.382\ O_2 \rightarrow 0.982\ NO_2^- + 1.891\ CO_2 + 2.927\ H_2O + 0.018\ C_5H_7O_2N \quad \text{eq. 1.2}$$

In the PN, which corresponds to the first half of the full nitrification, the AOB oxidize ammonium to nitrite, whereas in the second one the NOB further oxidize the nitrite produced to nitrate during the nitratation process (eq. 1.3) (Figure 1.1). Given the stoichiometry of NOB, they can be potential competitors with the AOB for the substrate DO and with the anammox for the substrate nitrite.

$$NO_2^- + 0.003\ NH_4^+ + 0.488\ O_2 + 0.010\ H_2CO_3 + 0.003\ HCO_3^- \rightarrow 0.003\ C_5H_7O_2N + NO_3^- + 0.008\ H_2O \quad \text{eq. 1.3}$$

Furthermore, the nitrogen cycle has been recently expanded with the discovery of a new *Nitrospira* species, the so-called comammox (COMplete AMMonia OXidiser) bacteria, that can solely perform the complete ammonium oxidation to nitrate in one step (Van Kessel et al., 2015).

Both chemolithotrophic NOB (*Nitrospira* and *Nitrobacter* spp.) and comammox (COMplete AMMonia OXidiser) bacteria (*Nitrospira* spp.) can use nitrite and ammonia, respectively, as sole sources of energy and cellular growth. The AOB comprise chemolithotrophic members of *Betaproteobacteria* class, such as *Nitrosomonas*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus* spp., and *Gammaproteobacteria* class, such as *Nitrosococcus* (Ge et al., 2015). *Nitrosomonas eutropha*, *Nitrosomonas. europaea* and *Nitrosospira* are the dominant AOB genera in the partial nitritation processes (González-Martínez et al., 2014). The temperature is one of the most important factors affecting the AOB abundance and the balance between *Nitrosospira* spp. and *Nitrosomonas* spp. in WWPTs: as an example, a combination of low temperature and high SRT may favour *Nitrosospira* spp. (Cydzik-Kwiatkowska and Zielińska, 2016).

The Ammonium Oxidizing Archaea (AOA), classified in the phylum *Thaumarchaeota*, are also present in wastewater and can take part in the PN. The balance between AOB and AOA depends on the ammonium concentration, favouring the AOB abundance for nitrogen-rich wastewater streams, and the AOA for municipal wastewater streams with lower ammonium concentrations (Bai et al., 2012; Limpiyakorn et al., 2011; Muñoz-Palazon et al., 2018).

1.4.1.2. Anaerobic Ammonium Oxidizing (anammox) bacteria

Between 30 - 70% of all molecular nitrogen released into the atmosphere is produced by microorganisms which existence has been not detected for a long-time, the anaerobic ammonium-oxidizing (anammox) bacteria (Kartal and Keltjens, 2016). The anammox biochemical reaction was predicted in the 1970s based on thermodynamic calculations (Broda, 1977), but it was not until Mulder et al. (1995) acknowledged a gap in the nitrogen mass balance of a denitrifying fluidized-bed reactor, that they were actually discovered. The stoichiometry of the process was further identified by Strous et al. (1998) (eq. 1.4), and recently revised by Lotti et al. (2014) (eq. 1.5).

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O \quad \text{eq. 1.4}$$

$$NH_4^+ + 1.146\ NO_2^- + 0.071\ HCO_3^- + 0.057\ H^+ \rightarrow 0.986\ N_2 + 0.161\ NO_3^- + 0.071\ CH_2O_{0.5}N_{0.15} + 2.002\ H_2O \quad \text{eq. 1.5}$$

The anammox bacteria have been shown to exist in diverse environments, such as oceanic anoxic basins, marine sponges, freshwater ecosystems, terrestrial ecosystems, river sediments, estuaries and

even sea ice (Gao and Tao, 2011). In fact, they play a major role in the global nitrogen cycle, since they are responsible for a 30 - 50% of the nitrogen loss from low oxygen zones and oceans (Devol, 2003; Lam and Kuypers, 2011).

The chemolithotrophic anammox bacteria are able to oxidize ammonium in the absence of oxygen with nitrite as the terminal electron acceptor. For this reason, and besides being in the nature, the anammox bacteria can be used in innovative technologies for wastewater treatment, being ecologically beneficial since they can remove the ammonium in the wastewater without producing nitrous oxide (Peeters and van Niftrik, 2019).

The anaerobic conversion of the substrates ammonium and nitrite to dinitrogen gas, which proceeds via two unusual and toxic intermediates: nitric oxide (NO) and the "rocket fuel" hydrazine (N_2H_4), takes place in a specialized intracellular organelle called the anammoxosome (Peeters and van Niftrik, 2019). Recent research works suggested, on the other hand, the existence of a modified anammox pathway, in which hydroxylamine (NH_2OH) is used instead of NO for the oxidation of ammonium. The reddish colour of the anammox bacteria relates to the high content of cytochrome (Jetten et al., 1998).

Currently, there are six known genera of anammox bacteria, all of which belong to the order Plantomycetales: Candidatus *Brocadia*, *Kuenenia*, *Jettenia*, *Scalindua*, *Anammoxoglobus* and *Anammoximicrobium* (Peeters and van Niftrik, 2019). The majority of the 21 anammox species identified so far can be found in WWTPs or lab-scale reactors, being Candidatus *Brocadia* the most reported genus in wastewater (Muñoz-Palazon et al., 2018), and Candidatus *Scalindua* in marine sediments (Mao et al., 2017). Other genera have special features: *Kuenenia stuttgartiensis* can adapt to salinity conditions up to 3% (w/v) (Kartal et al., 2006), whereas *Brocadia fulgida* and *Anammoxoglobus propinicus* can use acetate or propionate organic acids to grow (Viancelli et al., 2017).

The optimal pH and temperature range for the anammox bacteria is 7 - 8 and 30 - 40 °C (Jin et al., 2012). On the other hand, the growth of anammox bacteria is slow (μ_{max} of 0.065 d^{-1}) and duplication times range between 11 and 22 days (Sobotka et al., 2017), although some genera (*Brocadia sinica*, *Jettenia caeni* and *Scalindua*) showed μ_{max} values between 0.18 - 0.33 d^{-1} (Zhang et al., 2017a). However, Lotti et al. (2015a) demonstrated that the anammox enrichments can become "faster through training", and that the anammox bacteria should not be regarded anymore as an intrinsically slow growing microorganism.

A large part of the current research on anammox focuses on the extraction and characterization of extracellular polymeric substances (EPS), its relation with the performance of the PN-AMX processes, as well as its potential use as bioplastics (Peeters and van Niftrik, 2019). The versatility of anammox bacteria makes them useful for novel applications such as electrode biofilms (Yin et al.,

2015) and wastewater treatment in space, together with other bacteria (Lindeboom et al., 2018; Pichel, 2014).

1.4.1.3. Combination of PN and anammox processes

When combining both processes, the AOB first produce nitrite from the oxidation of the ammonium present in the wastewater (eq. 1.2), and this nitrite (together with the remaining ammonium) is transformed to dinitrogen gas by the anammox bacteria (eq. 1.4); the nitrite can also be supplied by HDB, as will be discussed in Section 1.4.2.2. The global stoichiometry for the PN-AMX processes is represented in eq. 1.6:

$$NH_4^+ + 0.85\ O_2 + 1.11\ HCO_3^- \rightarrow 0.44\ N_2 + 0.11 NO_3^- + 2.56\ H_2O + 1.11\ CO_2 \qquad \text{eq. 1.6}$$

In other words, the ammonium acts as the electron donor for both AOB and anammox bacteria, while the nitrite can act as the electron acceptor for the anammox, the electron donor for NOB, and can also be reduced by HDB. The dissolved oxygen, on the other hand, is an electron acceptor for both AOB and NOB, and can be used by the heterotrophic bacteria (HB) to remove organic matter.

1.4.2. Relationship between PN-AMX and HD processes

1.4.2.1. Heterotrophic Denitrifying Bacteria (HDB)

During the heterotrophic denitrification (HD), the nitrate generated from either the anammox or nitratation (NOB) processes is reduced to dinitrogen gas during four consecutive reductions (with NO_2^-, NO and N_2O as intermediates) by the heterotrophic denitrifying bacteria (HDB) (see eq. 1.7 to eq. 1.10, acetic acid as organic carbon source). The nitrite produced by the AOB could also be used in eq. 1.8. This process requires the presence of a source of organic carbon as electron donor, and the nitrate acts as the last electron acceptor in the respiratory chain, substituting the molecular oxygen. The global stoichiometry for the HD is represented in eq. 1.11, while eq. 1.12 shows the stoichiometry for the conventional N-DN combined process:

$$2\ CH_3COOH + 8\ NO_3^- \rightarrow 4\ CO_2 + 8\ NO_2^- + 4\ H_2O \qquad \text{eq. 1.7} \quad \text{Denitratation}$$

$$CH_3COOH + 8\ NO_2^- + 2\ H_2O \rightarrow 2\ CO_2 + 8\ NO + 8\ OH^- \qquad \text{eq. 1.8} \quad \text{Denitritation}$$

$$CH_3COOH + 8\,NO \rightarrow 2\,CO_2 + 4\,N_2O + 2\,H_2O \quad \text{eq. 1.9}$$

$$CH_3COOH + 4\,N_2O \rightarrow 2\,CO_2 + 4\,N_2 + 2\,H_2O \quad \text{eq. 1.10}$$

$$NO_3^- + 0.625\,CH_3COOH \rightarrow HCO_3^- + 0.25\,CO_2 + 0.5\,N_2 + 0.75\,H_2O \quad \text{eq. 1.11} \quad \text{Denitrification}$$

$$NH_4^+ + 0.625\,CH_3COOH + 2\,O_2 + HCO_3^- \rightarrow 0.5\,N_2 + 3.75\,H_2O + 2.25\,CO_2 \quad \text{eq. 1.12}$$

The nitrate reduction to nitrite is used for respiration (also known as dissimilatory nitrate reduction), and for nitrogen assimilation into biomass. This nitrite may be a complementary source of substrate nitrite for other nitrite-consuming bacteria (NOB and anammox bacteria) (Kuypers et al., 2018). Together with the anammox bacteria, the HDB are the major mechanisms converting the combined nitrogen (NH_4^+, NO_2^-, NO_3^-) to molecular nitrogen (N_2), thereby completing the nitrogen cycle.

The HDB belong to a broad variety of phylogenetic groups (*Pseudomonas*, *Paracoccus*, *Ralstonia* and *Rhodobacter* spp.), being *Thauera* spp. the genera usually detected in WWTPs (Jiang et al., 2008). The optimal pH and temperature range for HD is 7 - 9 and 20 - 35 °C. Similarly to the anammox bacteria, the nitrite can be toxic for the HDB (Ni et al., 2017).

1.4.2.2. Competition for substrate nitrite between anammox and HDB

As previously explained, the different bacterial groups present in the wastewater treatment may compete with the main bacteria involved in the PN-AMX processes (AOB and anammox bacteria) for common substrates. As an example, the NOB may oxidize the nitrite produced in the PN, instead of being reduced by the anammox reaction, and the HB can compete for DO with the AOB or for nitrite with the anammox if sufficient organic matter is present (see Figure 1.1). These undesirable side-pathways within the PN-AMX processes may end up destabilizing the process.

However, the most reviewed disturbance for the anammox bacteria is related with the presence of organic matter promoting the activity of HDB (Chamchoi et al., 2008; Jenni et al., 2014; Kumar and Lin, 2010; Molinuevo et al., 2009). Following the stoichiometric reactions for the anammox (eq. 1.4) and HD (eq. 1.8), both share the nitrite as substrate and electron acceptor, while only the HDB (as opposed to the autotrophic anammox) consume organic matter for their growth (Figure 1.1). Since the HD pathway is thermodynamically more favourable than the anaerobic oxidation of ammonium (Gibbs free energy of -427 kJ/mol and -355 kJ/mol, respectively), the HDB have comparatively higher growth rates and cell yields when organic carbon is available (Kuenen, 2008).

This may end up in the outcompetition of anammox bacteria by HDB, favouring the shift of the PN-AMX process towards the conventional N-DN (Li et al., 2018b). Therefore, the limitation of the HDB activity within a PN-AMX system is a challenging task, especially for those industrial wastewater streams with high concentrations of organic carbon and high COD/N ratios. In these conditions, the potential decrease in the anammox activity can remain unnoticed since the HDB can take over a considerable part of the nitrogen removal (Jenni et al., 2014). The overgrowth of HDB could be regulated by monitoring the amount of COD and the COD/N ratio available in the system. However, the presence of HDB can be used as an advantage to complete the nitrogen removal from the effluent of PN-AMX systems.

1.4.2.3. Improved PN-AMX by synergy with HD

If the characteristics of the wastewater are suitable (sufficiently low COD concentration and COD/N ratio), the synergy and cooperation, rather than competition, between the anammox bacteria and HDB can be achieved (Lawson et al., 2017). In this sense, the implementation of pre-treatment processes to achieve low organic loading rates can help improving this synergy in downstream post-treatments for BNR such as the PN-AMX processes (Tomar and Gupta, 2017).

When organic carbon is available, the nitrate (mainly produced by the anammox bacteria) can be converted to dinitrogen gas by HDB, thus enhancing the removal efficiency of total nitrogen. On the other hand, the HDB can couple with the anammox bacteria, either removing the extra nitrite (potentially toxic for the anammox), or consuming the nitrate produced by anammox (10% of inlet ammonium concentration, eq. 1.4) and concurrently supplying them with substrate nitrite (see eq. 1.7) (Wang et al., 2018b). In this way, higher nitrogen removal efficiencies can be achieved compared to the PN-AMX alone, leading to a better compliance with the discharge limits (Cao et al., 2016; Li et al., 2018b).

The combination of the PN-AMX and HD processes, sometimes named in the literature as the SNAD (Simultaneous partial Nitritation, Anammox and Denitrification) process, have raised the interest in the recent years (Chen et al., 2009; Langone et al., 2014). Most SNAD systems are operated in SBRs, with granular sludge that promotes the co-existence of several bacterial populations and favours the stratification of the substrate and toxic compounds inside the granule; however, the "SNAD" nomenclature is not prevalent in the literature. In the present thesis the "PN-AMX and HD (coupled) processes" terminology was maintained for two reasons: (1) to emphasize the main studied pathways, which are the combined partial nitritation and anammox processes, and (2) because with the SNAD nomenclature the synergy between PN, anammox and HD is taken for granted, which strongly depends on the characteristics of the industrial wastewater.

1.4.3. Advantages of PN-AMX compared to conventional processes

The shortcut BNR takes advantage of the nitrite being directly used as electron acceptor and reduced to dinitrogen gas by the anaerobic ammonium oxidizing (anammox) bacteria, instead of its further oxidation to nitrate in the nitratation (Soliman and Eldyasti, 2018). The combined PN-AMX process, compared with the combined conventional N-DN process, can achieve a total reduction in the DO requirements for the oxidation of the ammonium nitrogen of 60%, and up to 90% of overall operational costs based on aeration (Jetten et al., 2001). Table 1.1 summarizes the O_2 requirements per g of ammonium substrate for the different nitrogen removal processes.

Table 1.1. Reported values for energy expenditures comparing conventional and innovative (PN-AMX) BNR processes.

Process	Equation	g O_2/g NH_4^+-N	% O_2 savings[a]
nitritation	eq. 1.2	3.16	38.5
nitratation	eq. 1.3	1.10	-43.6[b]
nitrification	eq. 1.2 + eq. 1.3	4.25	54.3
nitrification-denitrification	eq. 1.12	4.57	57.5
partial nitritation-anammox	eq. 1.6	1.94	-

[a] Savings in the O_2 and HCO_3^- requirements (%) for the different pathways with respect to the partial nitritation-anammox combined process. [b] The negative sign indicates less O_2 requirements for nitration compared to the PN-AMX processes.

As seen in Table 1.1, the combined PN-AMX process save 57.5% of DO compared to the N-DN processes, close to the extensively reported 60% value. This value is also close to the DO savings with respect to the nitrification process (nitritation + nitratation), which is in accordance with the absence of DO requirements for heterotrophic denitrification (eq. 1.7 to eq. 1.10). On the other hand, disregarding only the second step in the nitrification process (nitratation) to couple the nitritation to the anammox process saves 38.5% of the DO requirements. Furthermore, the CO_2 emissions and sludge production are decreased by 88% by 80%, respectively (Antwi et al., 2019; Flores-Alsina et al., 2019; Soliman and Eldyasti, 2018).

Given the autotrophic nature of the partial nitritation process (PN) and the avoidance of electron donor (organic carbon) supply for HD, the PN-AMX combined process is completely autotrophic, and thus a 100% reduction in the amount of external organic matter can be achieved (Ma et al., 2016; Muñoz-Palazon et al., 2018; Solon et al., 2019). On the other hand, the carbon source for the HD, whose synergy with the PN-AMX might enhance the removal of nitrogen, can be satisfied with the organic matter content in the digestate of the anaerobic digestion (Cui et al., 2016).

Table 1.2. Reported values for energy expenditures comparing conventional and innovative (PN-AMX) BNR processes.

Reference	BNR process	Value
Energy consumption (kWh/kg N)		
Mulder (2003)	PN-AMX	0.86
Wett (2006)	PN-AMX	0.79
Lackner et al. (2014)	PN-AMX	0.8 - 1.92
Van Loosdrecht and Brdjanovic (2014)	N-DN	3.5 - 5.7
Beck and Speers (2015)	N-DN	3.07
Mulder (2003)	N-DN	11.7
Mulder (2003)	Algal ponds	0.1 - 1
Mulder (2003)	Constructed wetlands	< 0.1
Total costs (€/kg N treated)		
Fux and Siegrist (2004)	PN-AMX	2.5
Wett (2006)	PN-AMX	0.79
Fernández (2010)	PN-AMX	0.22
Hauck et al. (2016)	PN-AMX	0.40[a]
Henze et al. (2015)	PN-AMX	1 - 2
Fux and Siegrist (2004)	N-DN	3 - 4
Fernández (2010)	N-DN	3 - 5
Henze et al. (2015)	N-DN	1.41
Fux and Siegrist (2004)	Activated sludge	8

[a] This value correspond to the sum of the electricity burdens of separate and subsequent PN and AMX units

Regarding the energy consumption for the removal of nitrogen, the values for the N-DN processes (Table 1.2) are similar to the energy use for the production of nitrogen fertilizer, 9.6 - 12.4 kWh/kg N (Mulder, 2003), while the energy expenditures for the PN-AMX processes account for only 6.7 - 16.4% compared to N-DN (Table 1.2). Only algal ponds and constructed wetlands have lower energy use than the autotrophic BNR processes (Mulder, 2003). Solon et al. (2019), on the other hand, indicated that the PN-AMX processes reduce the aeration energy consumption to 0.24 kWh/m^3, while Molinos-Senante et al. (2010) considers a market price for treated water of 0.345 €/m^3.

1.5. Inhibition sources for the PN-AMX process

The industrial wastewater streams often contain high concentrations of ammonium and organic matter (despite the previous anaerobic digestion step), which can limit the activity of the anammox bacteria (Xie et al., 2017). Besides, some industrial effluents are also characterized by the instantaneous or sudden changes in the conditions of the wastewater, depending on the seasonality of the associated industry outputs, which can disturb the PN-AMX processes temporarily. Long-term changes in the characterization of the wastewater can even lead to the transformation of key functional groups in the PN-AMX system, including activity, abundance and structure of the microbial community.

In order to apply the PN-AMX process to nitrogen-rich industrial effluents, it is thus important to address the presence and potential effect of a wide range of inhibitory factors (Xie et al., 2017). Several examples of these factors are indexed in the following sections, focusing in the sensitive anammox bacteria. Most of the available studies only addressed a research based on single factors, but a research focused on multiple factors is of great interest due to the characteristics of wastewater streams such as the fish cannery, manure or OFSMW (Gonzalez-Martinez et al., 2018).

1.5.1. pH and temperature

The pH is one of the most important control factors for the PN-AMX processes, especially for nitrogen-rich wastewater streams and high temperature conditions. While the PN depresses the pH, the anammox reaction elevates it, and thus this parameter provides a straightforward glimpse of the current state of the overall process (Mao et al., 2017). The physiological pH range for anammox bacteria is 6.7 - 8.3 (Strous et al., 1999). Too low (below 6.8) or high (above 8.0) pH values entail loss of anammox activity, as reported by Lackner et al. (2014). Puyol et al. (2014a), on the other hand, found an optimal pH value of 6.97, stating that above 7.6 the pH may exert a greater inhibition for anammox than the concentration of free ammonia (< 10%).

The optimal temperature for the anammox bacteria is in the range 30 °C - 40 °C (Lotti et al., 2014; Van Der Star et al., 2007). Above 45 °C, the irreversible loss of activity due to lysis of bacterial cells takes place (Dosta et al., 2008). Some anammox species (Candidatus *Kuenenia* and *Brocadia*) show the highest anammox activity at 37 °C (Narita et al., 2017) and 40 °C (Strous et al., 1999), respectively.

In agreement with the optimal temperature range, most of the autotrophic nitrogen removal processes treating urban or industrial wastewater at full-scale have been operated at sidestream temperature conditions (30 °C - 35 °C), Lackner et al. (2014)). These conditions, which are normally related to the temperature of the wastewater after an anaerobic digestion step, characterize also

certain wastewater streams that are currently focusing the application of the PN-AMX processes, such as the fish cannery, swine slurry and leachate wastewater.

1.5.2. Dissolved Oxygen (DO)

The DO concentration is one of the main control parameters for the full-scale application of the PN-AMX processes (Lackner et al., 2014; Van Dongen et al., 2001), which delimits the interaction between the PN and anammox pathways (Cema et al., 2011; Li et al., 2018b). On one hand, if the DO concentration is too low, the AOB are not able to produce sufficient substrate nitrite for the anammox and the AOB activity may decrease, with a subsequent lower depletion of DO. High DO concentrations may in turn inhibit the anammox bacteria, consequently increasing the nitrite concentrations in the effluent. This inhibition is often reversible except for very high DO levels (Li et al., 2017c), although alive anammox bacteria can be found in environments such as aerobic tanks in WWTPs (> 2 mg O_2/L, Wang et al. (2015)).

As it will be further reviewed in Section 1.7.3, and when not being in suspension, the aerobic and anaerobic ammonium oxidizing bacteria can grow mainly in granular/flocculent aggregates, or attached biofilm. The optimal DO concentration for the granular/biofilm PN-AMX biomass may rely upon several factors, such as the biofilm thickness, density, boundary layer thickness, ammonium and COD surface load and temperature (Hao et al., 2002). Table 1.3 summarizes previous reported values for the optimal DO concentration.

Table 1.3. Reported optimal DO concentrations for the anammox and PN-AMX processes at lab-scale.

Ref.	WW	Process	Reactor	Biomass	DO (mg O_2/L)	Observations
[1]	synthetic	AMX	IASBR	granular	< 0.152[a]	Reversible inhibition below 2% air saturation
[2]	synthetic	AMX	batch	granular	< 0.032	AMX activity temporarily inhibited
[3]	synthetic	AMX	UASB	granular	< 0.2	Required concentration for AMX
[4]	leachate	AMX	RBC	biofilm	0.076 - 1.368	Reversible (<1%) and irreversible (<18%) inhibition
[5]	model	PN-AMX	model	biofilm	1.0	Optimal bulk DO
[6]	AD supern.	PN-AMX	SBR	gran./floc.	< 1.0	Suitable range
[7]	AD black water	OLAND	RBC	biofilm	0.2 - 1.0	Recommended range to facilitate AMX process

Table 1.3 (continued).

[8]	synthetic	CANON	UBF	biofilm	0.2 - 1.0	Recommended range to facilitate AMX process
[9]	synthetic	CANON	SABF	biofilm	0.2 - 0.3	Optimum range

AD: anaerobically digested, AMX: anammox, CANON: Completely Autotrophic Nitrogen removal Over Nitrite, floc.: flocculent, gran.: granular, IASBR: intermittently aerated sequencing batch reactor, OLAND: Oxygen Limited Autotrophic Nitrification Denitrification, PN-AMX: partial nitritation-anammox, RBC: rotating biological contactor, SABF: submerged aerated biological filter, supern.: supernatant, UASB: upflow anaerobic sludge blanket, UBF: upflow biofilm reactor.

References: [1] Strous et al. (1997), [2] Strous et al. (1999), [3] Jung et al. (2007), [4] Egli et al. (2001), [5] Hao et al. (2002), [6] Joss et al. (2009), [7] Vlaeminck et al. (2009), [8] Liu et al. (2013), [9] Yue et al. (2018).

Hao et al. (2002) further determined through modeling that small variations in the range of 0.2 mg O_2/L did not have significant influence on the process performance. Bagchi et al. (2010), on the other hand, focused in the DO loading rate, stating that values lower than 0.06 mg O_2/(mg N·d) were essential to maintain a sufficient nitrite supply.

However, the reported DO optimum for the anammox and PN-AMX processes at lab-scale (Table 1.3) may not agree when scaling up the process. Joss et al. (2011), for instance, reported inhibition of anammox bacteria in PN-AMX flocculent biomass already at 0.2 mg O_2/L in a 400 L pilot-scale reactor. For this reason, they proposed the airflow rate supplied instead of the DO concentration as a better and more reliable control parameter, especially when low DO concentrations are required. In this sense, other authors suggested using both the oxidation-reduction potential (ORP) and the DO concentration for AMX suspended biomass (Lackner et al., 2012; Lackner and Horn, 2012).

1.5.3. Ratio COD/N

The effect of the organic matter over the autotrophic anammox bacteria has been addressed in many research studies (Molinuevo et al., 2009; Ni et al., 2012; Tang et al., 2010b). When high amounts of organic matter are available, the HD has the potential to be the most important metabolic pathway in terms of nitrogen removal efficiency (Sabumon, 2007; Zhang et al., 2015a), competing with the anammox bacteria for the substrate nitrite (Bi et al., 2015; Molinuevo et al., 2009; Takekawa et al., 2014), as seen in Section 1.4.2.2. The lower competition capacity of anammox bacteria for substrate nitrite compared with the HDB may be due to the comparatively higher affinity for nitrite of the latter, leading to its depletion (Gonzalez-Martinez et al., 2018; Tomar and Gupta, 2017).

Besides the concentration of organic matter (as g COD/L) itself, the COD/TN ratio (often abbreviated as C/N ratio in the literature) is extensively reported as one of the key parameters that governs the predominance of bacterial communities in a PN-AMX system and the process selection

between anammox and HD (Chamchoi et al., 2008; Ni et al., 2012; Takekawa et al., 2014; Tomar and Gupta, 2017). Table 1.4 summarizes reported values for both parameters, showing the effect over the anammox and HD processes.

Table 1.4. Reported suitable and inhibiting COD concentrations (g COD/L) and COD/N ratios (g COD/g N) for the anammox-based process.

Ref.	Wastewater	Process	Reactor	Biomass	**mg COD/L**	Observations
[1]	synthetic	AMX	UASB	granular	> 300	Inactivation of AMX bacteria
[2]	synthetic	AMX	UAF	granular	> 300	IC_{50} for 50% AMX activity loss
[3]	synthetic	AMX	UASB	granular	> 400	Suppression of AMX activity
[4]	MRWW	AMX	STAR	granular	50	Inhibition of N removal
[5]	pig slurry	AMX	UASB	granular	292	Complete AMX inhibition
[6]	pig slurry	AMX	UMSR	flocculent	> 200	AMX activity suppressed
					< 150	AMX pathway predominant
[7]	synthetic	PN-AMX	SFBR	carrier biofilm	< 100	Co-existence of AOB-AMX-HDB
[8]	mature LL	PN + AMX	3-stage SBR	granular	< 150	No significant AMX inhibition
[9]	mature LL	PN + AMX	3-stage SBR	granular	800	Good balance of AMX and HDB
Ref.	Wastewater	Process	Reactor	Biomass	**g COD/g N**	Observations
[10]	synthetic	AMX	CSTR	suspended	> 1.0	AMX outcompeted by HDB
[1]	synthetic	AMX	UASB	granular	0.9 - 2.0	Inactivation of AMX bacteria
[3]	synthetic	AMX	UASB	granular	1.0 - 2.0	AMX competing with HDB
					< 3.1	Threshold for AMX inhibition
					> 4.0	Suppression of AMX activity
[11]	synthetic	AMX + HD + AnD	AHR	suspended	0.04 - 0.75	AMX outcompeted HD and AnD
					0.83 - 1.67	AnD outcompeted AMX and HD
[12]	synthetic	PD-AMX	SBR	granular	< 2.0	No anammox inhibition
[13]	synthetic	PN-DN	MBBR	carrier biofilm	< 6.0	Maximum nitrification rate
[14]	model	PN-AMX	MABR	biofilm	> 2.0	AMX process not sustainable
					≥ 2.0	Step increases causes irreversible AMX activity loss
[15]	Industrial-MRWW(review)	PN-AMX	SBR, MBBR	granular	< 2.0	Predominance of AMX and simultaneous HD
[16]	MRWW	PN-AMX	SBR	suspended	1.4	Decrease of AMX activity
					> 1.71	Threshold ratio for HD (NO_2^-)

Table 1.4 (continued).

[17]	MRWW (review)	PN-AMX	SBR, RBC, UASB, ABF	biofilm, granular	2.0 - 3.5	Stable operation of PN-AMX

ABF: anaerobic biological filtrated reactor, AHR: anammox-hybrid reactor, AMX: anammox, AnD: anaerobic degradation, CSTR: continuously stirred tank reactor, HD: heterotrophic denitrification, HDB: heterotrophic denitrifying bacteria, IC_{50}: half maximal inhibitory concentration, LL: landfill leachate, MABR: membrane-aerated biofilm reactor, MBBR: moving bed biofilm reactor, MRWW: municipal reject wastewater, PD-AMX: partial denitritation-anammox, PN + AMX: 2-stage partial nitritation-anammox, PN-DN: partial nitritation-denitritation, RBC: rotating biological contactor, SBR: sequencing batch reactor, SFBR: submerged fixed-bed biofilm bioreactor, STAR: stirred tank anammox reactor, UAF: upflow anaerobic filter, UASB: upflow anaerobic sludge blanket reactor, UMSR: upflow microaerobic sludge reactors.

References: [1] Chamchoi et al. (2008), [2] Wang et al. (2019b), [3] Ni et al. (2012), [4] Li et al. (2011), [5] Molinuevo et al. (2009), [6] Li et al. (2016a), [7] García-Ruiz et al. (2018b), [8] Miao et al. (2014), [9] Miao et al. (2015), [10] Güven et al. (2005), [11] Tomar and Gupta (2017), [12] Du et al. (2014), [13] Zafarzadeh et al. (2011), [14] Lackner et al. (2008), [15] Lackner et al. (2014), [16] Jenni et al. (2014), [17] Nozhevnikova et al. (2012).

As seen in Table 1.4, if the COD/N ratio increases above a threshold range between 1 - 2 g COD/g N, the HDB can grow and compete with AMX for nitrite, inhibiting its activity and decreasing the contribution of the autotrophic nitrogen removal. The reduction of the COD/N ratio in the feeding, as well as increasing the SRT, can mitigate this competition. However, too low COD/N ratios in the long-term may favour an excessive growth of the NOB, which can compete with the anammox for the substrate nitrite, due to the limitation of the aerobic HB and the promotion of the nitrification (Li et al., 2017b).

As previously discussed in Section 1.4.2.3, moderate concentrations of organic matter in the wastewater can also favour the synergy, rather than the competition, between the anammox and HD processes (see Figure 1.1). Still, the organic matter should be properly pre-treated to reach an acceptable concentration level to maintain the long-term stability of the PN-AMX system (Li et al., 2018c).

1.5.4. Free Ammonia (FA) and Free Nitrous Acid (FNA)

The inhibitory effect of ammonium on anammox bacteria was reported only at high concentrations, having little effect below 500 mg NH_4^+/L (Ma et al., 2016) and 1000 mg NH_4^+/L (Ni et al., 2017; Strous et al., 1997). Dapena-Mora et al. (2007) found an IC_{50} value for ammonium of 770 mg/L, stating that the free ammonia (FA) rather than the ammonium itself was the true inhibitor for the anammox bacteria. This is probably because FA can diffuse more easily than ammonium through the cell lipid membrane of the anammox bacteria. Reported FA inhibitory values for AOB and anammox bacteria are summarized in Table 1.5.

Table 1.5. Reported inhibitory values of free ammonia (FA) for AOB and anammox bacteria

Ref.	Wastewater	Reactor	Biomass	pH	T (°C)	FA (mg NH_3-N/L)	Observations
Ammonium Oxidizing Bacteria (AOB)							
[1]	synthetic	batch	NS	8.0	RT	> 10	Start of AOB inhibition
[2]	synthetic	batch	suspended	7 - 8	27	4.93 - 27.2	FA IC_{50} range for AOB
[3]	synthetic	SBR	suspended	6.4	30	> 16	Start of AOB inhibition
[4]	synthetic	SBR	suspended	8.3	30	0.28 - 4.3	FA IC_{50} range for AOB
[5]	synthetic	SBBR	carrier biofilm	8.0	32	30.0 - 32.5	Inhibition in a CANON system
[6]	synthetic, manure	batch	NS	5.5 - 9.0	RT	8.2 - 123.5	Observed inhibitory range
[7]	pig slurry	CSTR	suspended	7.4 - 8.1	25	10	Start of AOB inhibition
[8]	RWW	pilot	NS	7.4 - 8.5	20	> 7	Start of AOB inhibition
[9]	mature LL	HSBR	suspended, carrier	8.2	31	> 10 - 130	Start of AOB inhibition
Anammox bacteria							
[10]	synthetic	UASB	granular	7.8 - 8.5	35	> 2	Minimum threshold
[11]	synthetic	UBF	granular	8.7 - 9.0	35	57 - 187	Observed inhibitory range
[12]	synthetic	batch, SBR	flocculent, biofilm	7.8	30	38[a]	IC_{50} for SAA in batch tests
						> 20 - 25	Long-term AMX instability
						> 35 - 40	Extreme long-term instability
[5]	synthetic	SBBR	carrier biofilm	8.0	32	30.0 - 32.5	Inhibition in a CANON system
[13]	synthetic	MBBR	carrier biofilm			> 150	Start of AMX inhibition
[14]	MRWW	MBBR	carrier biofilm	6.9 - 8.2	35	> 2	Minimum threshold
[15]	pig slurry	batch	granular	7.3 - 8.1	20	13 - 90	Observed inhibitory range
[16]	optoelectronic WW	SBR	carrier biofilm	7.8 - 8.0	27	146	AMX inhibition in the CANON process

[a] Optimal FA concentration: 20 - 25 mg NH3-N/L

CANON: Completely Autotrophic Nitrogen removal Over Nitrite, CSTR: continuously stirred tank reactor, HSBR: hybrid sequencing batch reactor, IC_{50}: half maximal inhibitory concentration, LL: landfill leachate, MBBR: moving bed biofilm reactor, MRWW: municipal reject wastewater, NS: not specified, RT: room temperature, RWW: reject wastewater, SAA: specific anammox activity, SBBR: sequencing batch biofilm reactors, SBR: sequencing batch reactor, UASB: upflow anaerobic sludge blanket reactor, UBF: upflow biofilm reactor, WW: wastewater,

References: [1] Neufeld et al. (1986), [2] Park and Bae (2009), [3] Vadivelu et al. (2006a), [4] Torà et al. (2010), [5] Li et al. (2012a), [6] Anthonisen et al. (1976), [7] Durán et al. (2014), [8] Abeling and Seyfried (1992), [9] Tian et al (2013), [10] Jung et al. (2007), [11] Tang et al. (2010a), [12] Fernández et al. (2012), [13] Aktan et al. (2012), [14] Jaroszynski et al. (2012), [15] Waki et al. (2007), [16] Daverey et al. (2013b).

The differences in the reported inhibitory FA ranges may be due to the different operating conditions, the physical structure of the sludge (flocculent, biofilm, granular) and the microbial populations. On the other hand, and similarly to the ammonium, the anammox bacteria can endure high FA levels (Jin et al., 2012), although excessive concentrations of FA regarding the reported inhibitory thresholds may cause inhibition.

Table 1.6. Reported inhibitory values of free nitrous acid (FNA) for AOB and anammox bacteria

Ref.	Wastewater	Reactor	Biomass	pH	T (°C)	FNA (mg HNO_2-N/L)	Observations
Ammonium Oxidizing Bacteria (AOB)							
[1]	model	SBR	suspended	8.0 - 8.5	30	0.2	FNA IC_{50} for AOB
[2]	review	review	review	review	rev.	0.02 - 0.03	Selective washout of NOB
[3]	synthetic	SBR	suspended	6.4	30	0.40	Inhibition of AOB growth
[4]	synthetic	batch	suspended	7 - 8	27	0.56	Observed inhibitory value
[5]	synthetic	SBR	suspended	8.3	30	0.21 - 1.31	FNA IC_{50} for AOB
[6]	synthetic, manure	batch	NS	5.5 - 9.0	RT	> 0.22 - 2.8	Minimum inhibitory range
[7]	pig slurry	CSTR	suspended	7.4 - 8.1	25	> 0.2	Start of AOB inhibition
[8]	pig slurry	SBR	flocculent	6.2 - 8.5	33	0.08 - 0.37	Start of AOB inhibition
[9]	mature LL	HSBR	carrier	8.2	31	> 0.6 - 1.9	Start of AOB inhibition
Anammox bacteria							
[10]	synthetic	batch	granular	7.0 - 7.8	30	> 0.006	Complete AMX[a] inhibition
[11]	synthetic	batch	suspension	6.5 - 9.0	37	> 0.04	Complete AMX[b] inhibition
[12]	synthetic	batch	carrier	7.8	30	> 0.58 - 3.83	Start of AMX inhibition
[13]	synthetic	batch	granular	6.5 - 7.3	30	0.06 - 0.108	FNA IC_{50} for AMX
						0.2	Complete AMX inhibition
[14]	synthetic	batch	flocculent	7.8	30	0.011	FNA IC_{50} for AMX
						0.5 - 1.5	Safety FNA range for AMX
[15]	synthetic	EGSB	granular	7.7	32	> 0.005	AMX inhibition (CANON)
[16]	optoel. WW	SBR	carrier	7.8 - 8.0	27	0.0067	AMX inhibition (CANON)

[a] Candidatus *Brocadia anammoxidans*, [b] Candidatus *Kuenenia stuttgartiensis*. CANON: completely autotrophic nitrogen removal over nitrite, CSTR: continuously stirred tank reactor, EGSB: expanded granular sludge bed reactor, HSBR: hybrid sequencing batch reactor, IC_{50}: half maximal inhibitory concentration, LL: landfill leachate, NS: not specified, optoel.: optoelectronic, RT: room temperature, SBR: se, WW: wastewater.

References: [1] Hellinga et al. (1999), [2] Zhou et al. (2011), [3] Vadivelu et al. (2006a), [4] Park and Bae (2009), [5] Torà et al. (2010), [6] Anthonisen et al. (1976), [7] Durán et al. (2014), [8] Belmonte et al. (2017), [9] Tian et al (2013), [10] Strous et al. (1999), [11] Egli et al. (2001), [12] Malovanyy et al. (2012), [13] Puyol et al. (2014b), [14] Fernández et al. (2012), [15] Wang and Gao (2016), [16] Daverey et al. (2013b).

Table 1.6 summarizes reported FNA inhibitory values for AOB and anammox bacteria. Regarding the free nitrous acid (FNA), Fernández et al. (2012) stated that this is the true inhibitor for anammox bacteria instead of the nitrite, as opposed to Puyol et al. (2014b), who found that nitrite rather than FNA is the predominant cause of inhibition. Similarly to the case of FA, the NOB are more sensitive than the AOB (Vadivelu et al., 2006a, 2006b).

1.5.5. Ratio N/IC and alkalinity

The ratio between the nitrogen and inorganic carbon (N/IC) conditions the extent of the PN process occurrence, which might be hindered by alkalinity limitations. For a N/IC ratio of 1 g N/g IC, Pedrouso et al. (2017) found that the alkalinity is sufficient to oxidize half the available ammonium in mainstream conditions, if enough DO is provided. If the N/IC is lower than 0.6 g N/g IC, there is an excess of alkalinity for the PN and 100% of the inlet ammonium content can be oxidized to nitrite, whereas between 0.6 and 0.8 g N/g IC the inlet ammonium oxidized vary between 100% and 50%, respectively. For ratios above 0.8 g N/g IC, there is not enough alkalinity for the PN process and only 50% of the ammonium content is oxidized to nitrite.

Durán et al. (2014) assessed several N/IC ratios between 1.19 and 0.82 g/g to achieve a successful partial nitritation when treating pig slurry at 20 °C and 400 mg NH_4^+-N/L. For a nitrogen loading rate of 0.13 g N/(L·d), they found an optimal N/IC ratio of 0.82 g N/g IC to control the pH and inhibitory effects of FA and FNA on the PN, allowing to feed the subsequent anammox reactor with a nitrite to ammonium ratio of 1 g/g and a NLR of 0.1 g N/(L·d). Ganigué et al. (2007), on the other hand, successfully removed high ammonium loads (1 - 1.5 g N/(L·d)) in the start-up of the PN treating landfill leachate by adjusting the molar ammonium to alkalinity ratio to 1:1 with sodium bicarbonate, achieving a 50% nitrite-ammonium effluent for the subsequent anammox process.

1.5.6. Salinity

Besides high concentrations of nitrogenous compounds, many of the wastewater sources produced by the industries are characterized by a high salinity (Malovanyy et al., 2015). Lefebvre and Moletta (2006) indicated that about 5% of industrial effluents are saline or hypersaline. Common saline wastewater streams are the chemical and pharmaceutical, fish canning, seafood processing and aquaculture industry wastewater streams. The fish canning effluents, for example, contain saline levels similar to seawater, up to 19 g Cl^-/L, 12 g Na^+/L and 2.5 g SO_4^{2-}/L (Dapena-Mora et al., 2006). The use of salt for cooking and marine water for toilet flushing also generate effluents with high-salinity concentrations. Comparatively, the salinity concentration for municipal wastewater is in the range between 0.7 and 3.0 g NaCl/L.

Traditionally, as the conventional BNR processes may be inhibited by salt (Lefebvre and Moletta, 2006), most saline wastewater streams were generally treated through physicochemical approaches. However, the BNR anammox-based processes constitute an interesting alternative with good potential to treat saline wastewater streams (Dapena-Mora et al., 2007), which is in agreement with the presence of anammox in marine hypersaline systems (Borin et al., 2013; Kuypers et al., 2003). Table 1.7.a and Table 1.7.b shows information about different salt concentrations affecting the AOB and anammox bacteria, respectively.

Table 1.7.a. Reported inhibitory and non-inhibitory values of salt concentrations (g NaCL/L) for Ammonium Oxidizing Bacteria (AOB).

Ref.	Wastewater	Reactor	Biomass	Dominant species[a]	Salt (g NaCl/L)	Observations
[1]	synthetic	NRBC	biofilm	*Nitrosomonas* spp.	10	Improved performance of the CANON process
[2]	synthetic	batch	flocculent	NS	≤ 13.7	Acclimation of AOB
[3]	synthetic	batch	suspended	*Nitrosom. europaea*	11.7	IC_{50} for AOB (NaCl)
					29.2	96% activity reduction
					22.3	IC_{50} for AOB (KCl)
					37.2	88% activity reduction (KCl)
[4]	synthetic	SBR (PN-AMX)	flocculent	*Nitrosomonas* spp.	0	N_2O emission factor[c]: 0.43%
					5 - 10	N_2O emission factor[c]: 0.75%
					15 - 20	N_2O emission factor[c]: 0.23%
[5]	synthetic	SBR	flocculent	*Nitrosom. Europaea* *Nitrosom. Eutropha*	5 → 10 → 15 → 20	No inhibition of nitrifiers; higher *Nitrosom.* abundance
					10 → 20	No inhibition of nitrifiers; lower *Nitrosom.* abundance
[6]	synthetic	SBR	suspended	NS	10 - 20	Mild inhibition of nitrifiers
[8]	synthetic	RBC	biofilm	NS	20	Start of AOB inhibition
[8]	synthetic	batch	biofilm	NS	30	24% SNA decrease for adapted AOB
					30	43% SNA decrease for non-adapted AOB
[9]	synthetic	SFBR	carrier biofilm	*Nitrosomonas* spp.	25 - 45	Drastically decrease of AOB in the CANON process
[10]	synthetic	SBBGR	biofilm	*Nitrosomonas* spp. *Nitrosovibrio* spp.	30	Adaptation of AMX in the PN-AMX process
					50	Deterioration of AMX in the PN-AMX process
[6]	fish market	SBR	suspended	NS	32	50% decrease N removal
[7]	FGD WW	pilot	suspended	NS	20[b]	Stable nitrification (N-DN)

Table 1.7.b. Reported inhibitory and non-inhibitory values of salt concentrations (g NaCL/L) for anammox bacteria.

Ref.	Wastewater	Reactor	Biomass	Dominant species[a]	Salt (g NaCl/L)	Observations
[11]	synthetic	UASB	suspended	*Brocadia fulgida*	0 - 3	Dominance for low-salinity
				Kuenenia stuttgart.	< 30	Dominance for high-salinity
[9]	synthetic	SFBR	carrier biofilm	*Brocadia* spp.	≤ 3	Adaptation of AMX biomass in the CANON process
[12]	synthetic	SBR	carrier biofilm	NS	≤ 7	Adaptation of AMX bacteria
				NS	↑ 4	AMX endure salt shock loads
[13]	synthetic	batch	suspended	*Brocadia carolini.*	5.44	IC_{50} value for AMX
[14]	synthetic	SBR	granular	*Kuenenia stuttgart.*	3 - 15	SAA increase for adapted / non-adapted AMX biomass
[1]	synthetic	batch	biofilm	*Planctomycet.* spp.	10	Adapted AMX non inhibited
[1]	synthetic	NRBC	biofilm	*Planctomycet.* spp.	10	Improved performance of the CANON process
[17]	synthetic	UASB	granular	NS	13.4	IC_{50} for the AMX (g KCl/L)
[18]	synthetic	batch	granular	*Kuenenia stuttgart.*	13.46	IC_{50} for the AMX (g NaCl/L)
					14.9	IC_{50} for the AMX (g KCl/L)
[19]	synthetic	UAC	granular	*An DHS-2, KU2*	14 → 20	Complete AMX inhibition for this sharp salt increase
					30	Stable AMX performance
					9	Progressive AMX adaptation
[21]	synthetic	UASB	granular	*Kuenenia* spp.	20	Adaptation of AMX bacteria[e]
[22]	synthetic	UASB	suspended	NS	30	AMX adaptation for 5 g NaCl/L increasing salt steps
					20 - 30	Long-term AMX NRR drop
[23]	synthetic	A/O	granular	*Kuenenia* spp., *Brocadia* spp.	20 - 30	Long-term AMX NRR drop
[24]	synthetic	UASB	granular	*Kuenenia* spp., *Anammoxogl.* spp.	29.2	AMX adaptation after long-term stepwise acclimation
[25]	synthetic	UFBC	carrier biofilm	*An DHS-2, KU2*	≤ 30	Stable performance of the AMX process

Table 1.7.b (continued).

Ref.	Wastewater	Reactor	Biomass	Dominant species[a]	Salt (g NaCl/L)	Observations
[26]	synthetic	EGSB	granular	*Kuenenia* spp., *Jettenia* spp.	≤ 30	88% N removal by AMX
[8]	synthetic	batch	biofilm	NS	30	59% SAA decrease for adapted AMX
					30	95% SAA decrease for adapted AMX
[27]	synthetic	EGSB	granular	*Kuenenia stuttgart.*	30	High AMX activities, with EPS composition changes
[28]	synthetic	SBR	granular	*Kuenen. stuttgart.*, *Scalind. wagn.*	30	Threshold for AMX adaptation
					45	Reversible AMX inhibition
[10]	synthetic	SBBGR	biofilm	*Kuenen.*, *Jetten.*, *Brocad.* spp.	30	Adaptation of AMX in the PN-AMX process
					50	Deterioration of AMX in the PN-AMX process
[29]	synthetic	UASB	granular	NS	60 - 75	Low AMX activity observed
[20]	RWW[d]	MBBR	carrier biofilm	*Brocadia fulgida*	15	Inhibition of AMX for 5 g NaCl/L increasing steps
[16]	fish-canning	SBR	granular	NS	10	No long-term AMX inhibition

[a] Some abbreviations are used for AOB and AMX bacterial species: *Nitrosom.*: *Nitrosomonas*, *stuttgart.*: *stuttgartiensis*, *carolini.*: *caroliniensis*, *Planctomycet.*: *Planctomycetes*, *Anammoxogl.*: *Anammoxoglobus*, *Kuenen.*: *Kuenenia*, *Scalind.*: *Scalindua*, *wagn.*: *wagneri*, *Jetten.*: *Jettenia*, *Brocad.*: *Brocadia*. [b] 20 g Cl^-/L [c] The salt concentration in the CANON process is expressed as a function of the N_2O emission factor, directly related to the NO_2^-: higher non-consumed NO_2^- (produced by nitritation) → higher N_2O emission. [d] the reject wastewater was externally supplemented with increasing salt concentrations (0 - 15 g NaCl/L). [e] Co-adaptation to salt and Ni (0.2 mg/L) conditions.

→: steep concentration increase, ↑: sudden concentration load. CANON: completely autotrophic nitrogen removal over nitrite, EGSB: expanded granular sludge bed reactor, EPS: extracellular polymeric substances, FGD: flue gas desulphurization, IC_{50}: half maximal inhibitory concentration, MBBR: moving bed biofilm reactor, N-DN: nitrification-denitrification, NRBC: non-woven rotating biological contactor, NRR: nitrogen removal rate, NS: not specified, RBC: rotating biological contactor, SBBGR: sequencing batch biofilter granular reactor, SBR: sequencing batch reactor, SFBR: submerged fixed-bed biofilm bioreactor, SNA: specific nitritation activity, UAC: upflow anammox column reactor, UASB: upflow anaerobic sludge blanket reactor, UFBC: upflow fixed bed column reactor.

References: [1] Liu et al. (2008), [2] Campos et al. (2002), [3] Hunik et al. (1992), [4] Yan et al. (2016), [5] Bassin et al. (2012), [6] Rene et al. (2008), [7] Dahl et al. (1997), [8] Windey et al. (2005), [9] García-Ruiz et al. (2018a), [10] Ge et al. (2019), [11] Gonzalez-Silva et al. (2017), [12] Yi et al. (2011), [13] Carvajal-Arroyo et al. (2013), [14] Dapena-Mora et al. (2010), [15] Val del Rio et al. (2018), [16] Dapena-Mora et al. (2006), [17] Chen et al. (2014), [18] Dapena-Mora et al. (2007), [19] Yang et al. (2011), [20] Malovanyy et al. (2015), [21] Wu et al. (2019a), [22] Jin et al. (2011), [23] Li et al. (2018e), [24] Lu et al. (2019), [25] Liu et al. (2009), [26] Wang et al. (2019a), [27] Fang et al. (2018), [28] Kartal et al. (2006), [29] Ma et al. (2012).

Despite the increasing research studies on the anammox bacteria and their increasingly widespread features and wastewater applications, Li et al. (2018e) found that only 5.15% of these studies focused on saline wastewater treatment. These authors point out that the results presented in these studies are not consistent, as can also be deduced from Table 1.7, and even contradictory to each other, which may be due to the different operational conditions. On the other hand, only one research study addressed the behavior of the progressive adaptation of the PN-AMX processes to high salinity, as well as against sudden salt shocks, when treating fish canning industrial wastewater (Val del Rio et al., 2018).

Previous reports stated that low salinity levels can benefit the anammox activity up to a certain extent (Dapena-Mora et al., 2010, 2007; Kartal et al., 2006; Malovanyy et al., 2015), as opposed to high salinity levels, which may hinder its application to the treatment of nitrogen-rich wastewater streams (Li et al., 2018e). The high salinity can also provoke the accumulation of nitrite during the PN (Aslan and Simsek, 2012; Ye et al., 2009).

The progressive acclimation of freshwater-derived anammox bacteria (e.g., Candidatus *Kuenenia*) and AOB (e.g., *Nitrosomonas europaea* and *eutropha*) to salt is considered as one of the best ways to overcome inhibition by high salinity (Bassin et al., 2012; Dapena-Mora et al., 2010, 2007; Kartal et al., 2006; Val del Rio et al., 2018). Jin et al. (2011), for example, reported reduction from 67.5% to 43.1% in the salinity inhibition level of anammox through acclimation. The enrichment of halophilic bacteria from marine sediments (e.g., Candidatus *Scalindua*) was also reported (Kawagoshi et al., 2009; Li et al., 2018e).

On the other hand, the study of the sudden salt variations, or salinity shocks is essential since the salinity may vary greatly in most industrial wastewater streams (Vyrides et al., 2010), causing immediate release of cellular constituents and eventually leading to the dramatic decline in the anammox bacteria activity. Li et al. (2018e) defined four stages to help assessing the response in an anammox reactor against salinity shock: sensitive period (unstable anammox against high salinity), interim stable period (neither recovery nor worsening performance), gradual recovery period and final stable period.

Other reported effects of salinity over the anammox bacteria include osmotic stress on bacterial cells and inhibition of the reaction pathway for salinity above 1% (w/v) (Dapena-Mora et al., 2007), as well as changes in the bacterial dynamics and diversity (Gonzalez-Silva et al., 2017; Rodríguez-Sánchez et al., 2017). The salinity may also change the color of granular anammox sludge from red to reddish-brown (Yang et al., 2011), and even promote the granular aggregation of anammox bacteria (Speth et al., 2017). Regarding other ions in the wastewater, both cations K^+ and Ca^{2+} may either benefit the

anammox activity for moderate concentrations (1.75 g K^+/L, Jin et al. (2007), 5.65 mg Ca^{2+}/L, Trigo et al. (2006), 30 g/L NaCl + KCl, Gonzalez-Silva et al. (2017)), or deteriorate it for high concentrations (19.5 g K^+/L, Chen et al. (2014); 226 mg Ca^{2+}/L, Trigo et al. (2006)).

1.5.7. Nitrite

The nitrite, which is the electron acceptor for the oxidation of ammonium, can be toxic for the anammox bacteria (Li et al., 2017a), more vulnerable to nitrite than to ammonium (Carvajal-Arroyo et al., 2014). The reversible inhibition of anammox by nitrite may occur when the capacity of the AOB to oxidize ammonium to nitrite surpasses that of the anammox bacteria to reduce this nitrite, which accumulates in the system. This overcapacity of the PN needs to be watched out during the start-up of the PN-AMX process, since the AOB grow faster. On the other hand, the DO concentration may increase when the AOB activity is low, and this high DO concentration can hinder the activity of anammox bacteria and thus provoke the buildup of nitrite (Mao et al., 2017). Lackner et al. (2014) indicated an average duration of the nitrite accumulation episodes in the order of several days. Table 1.8 summarizes reported values for the nitrite regarding its effect over the anammox process.

Table 1.8. Summary of research studies based on the inhibitory effect of nitrite over the anammox bacteria.

Ref.	Wastewater	Process	Reactor	Biomass	Nitrite (mg NO_2^--N/L)	Observations
Reported non-inhibitory values						
[1]	synthetic	AMX	batch	flocculent	≤ 100	Safe range
[2]	synthetic	AMX	SBR	biofilm	< 240	No inhibition
[3]	synthetic	AMX	FBBR	carrier biofilm	≤ 400	No inhibition
[4]	fertilizer	SNAD	UC	flocculent	20 - 60	Non-inhibitory range
[5]	AD centrate	AMX	MBBR	carrier biofilm	170 - 250	No deactivation (2-day exposure)
[6]	MRWW	PN-AMX	SBR	granular	42	No short-term inhibit.
[7]	fish cannery	AMX	SBR	granular	200	Stable operation

Table 1.8 (continued).

Ref.	Wastewater	Process	Reactor	Biomass	Nitrite (mg NO_2^--N/L)	Observations
Reported inhibitory values						
[8]	synthetic	AMX	MBBR	biofilm	30 - 50	Severe inhibition (6-day exposition)
[9]	synthetic	AMX	batch	suspended	> 60	Short-term inhibition
[1]	synthetic	AMX	batch	flocculent	100 - 280	Complete inhibition
[10]	synthetic	AMX	SBR	flocculent	> 140	Low performance
[11]	synthetic	AMX	batch	suspended	> 185	Inhibition of *Kuenenia stuttgart.* spp.
[12]	synthetic	AMX	ABF	carrier biofilm	280	Inhibition threshold
[10]	synthetic	AMX	SBR	flocculent	> 280	Complete inhibition
[13]	synthetic	AMX	batch	granular	350	50% decrease of AMX activity
[3]	synthetic	AMX	FBBR	carrier biofilm	750	90% (reversible) SAA inhibition
[7]	fish cannery	AMX	SBR	granular	400	Complete inhibition

ABF: anaerobic biological filtrated reactor, AD: anaerobic digestion, FBBR: fixed bed biofilm reactor, MBBR: moving bed biofilm reactor, MRWW: municipal reject wastewater, SBR: sequencing batch reactor, SNAD: simultaneous partial nitritation, anammox and denitrification, UC: upflow column reactor.

References: [1] Strous et al. (1999), [2] Fernández et al. (2012), [3] Kimura et al. (2010), [4] Keluskar et al. (2013), [5] Jaroszynski et al. (2011), [6] Langone et al. (2014), [7] Dapena-Mora et al. (2006), [8] Fux et al. (2004), [9] Bettazzi et al. (2010), [10] Jetten et al. (1998), [11] Egli et al. (2001), [12] Isaka et al. (2007), [13] Dapena-Mora et al. (2007), [14] Kimura et al. (2010).

Regarding the reported values in Table 1.8, Jaroszynski et al. (2011) revealed that nitrite toxicity over the anammox process has been overestimated in some literature studies, and showed no deactivation of the anammox bacteria for a range of 170 - 250 mg NO_2^--N/L during a 2-day exposure time. As suggested by Jin et al. (2012), a safety nitrite concentration would be 100 mg NO_2^--N/L, while 280 mg NO_2^--N/L should generally be considered as a warning value, with compulsory intervention recommended between both values.

1.5.8. Phosphate

Similarly to the nitrite inhibition, the phosphate inhibits the anammox bacteria activity depending on the aggregation degree of the biomass: anammox biofilms are more tolerant to high phosphate concentrations compared to suspended biomass (Carvajal-Arroyo et al., 2013; Van Hulle et al., 2010). Furthermore, the presence of high concentrations of phosphate can also cause its precipitation in the form of struvite (NH_4MgPO_4). Table 1.9 shows some reported values for phosphate concentrations and its effect over the anammox activity.

Table 1.9. Reported phosphate concentrations regarding its effect over the anammox process.

Ref.	Wastewater	Reactor	Biomass	Dominant species	mg PO_4^{3-} /L	Observations
[1]	synthetic	batch	flocculent	NS	> 190	Reversible inhibition
[2]	synthetic	RBC	biofilm	*Kuenenia stuttgartiensis*	168.5	≤ 63% activity decrease
					306.4	≤ 80% activity decrease
					873.4	80% activity decrease
[3]	synthetic	FBR	suspended	*Brocadia anammoxidans*	> 475	Activity loss
[4]	synthetic	UASB	granular	*Kuenenia stuttgartiensis*	≤1532	Long-term (195 days) acclimation
[5]	synthetic	batch	biofilm	*Kuenenia stuttgartiensis*	1900	IC_{50} value
[6]	synthetic	batch	granular	*Brocadia fulgida*	950 - 4750	60% AMX activity stimulation
			suspended	*Brocadia caroliniensis*	2403	IC_{50} value
[7]	AD centrate	SBR	granular	NS	509 – 1462[a]	No inhibition
[8]	LL	RBC	biofilm	*Kuenenia stuttgartiensis, Brocadia anammoxidans*	1900	No inhibition

[a] Orthophosphate concentrations.

AD: anaerobic digestion, FBR: fluidized bed reactor, IC_{50}: half maximal inhibitory concentration, LL: landfill leachate, NS: not specified, RBC: rotating biological contactor, SBR: sequencing batch reactor, UASB: upflow anaerobic sludge blanket reactor.

References: [1] Jetten et al. (1998), [2] Pynaert et al. (2003), [3] Van De Graaf et al. (1996), [4] Zhang et al. (2016a), [5] Dapena-Mora et al. (2007), [6] Carvajal-Arroyo et al. (2013), [7] Galvagno et al. (2016), [8] Egli et al. (2001).

The phosphate can also be a factor of inhibition when precipitated in the form of phosphate salts, causing a decrease in nitrogen removal rate of the system from 100 to only 10 mg N/(L·d) (Trigo et al., 2006).

1.5.9. Metals

The metals can cause toxicity by accumulating in organisms. Nitrogen-rich wastewater streams like the landfill leachate (Table 1.10) usually contain high levels of metal ions. Table 1.11 summarizes reported IC_{50} values for different metals.

Table 1.10. Reported concentration ranges (mg/L) for several metals in leachate-like effluents.

Metal	Range (mg/L)	Ref.	Metal	Range (mg/L)	Ref.	Metal	Range (mg/L)	Ref.
Fe	1.28 - 26	[3]	**Cr**	0.02 - 1.5	[1]	**Co**	0.005 - 1.5	[1]
Al	0.02 - 2	[3]		0.0005 - 1.6	[2]		0.001 - 0.95	[2]
Zn	0.03 - 1000	[1]		0 - 1.98	[4]		0.05 - 0.16	[5]
	0.01 - 155	[2]		0.04 - 0.18	[5]	**Hg**	0.00005 - 0.16	[1]
	0 - 112	[4]	**As**	0.01 - 1	[1]	**Cd**	0.0001 - 0.4	[1]
	0.64 - 10.49	[5]		0.0005 - 1.6	[2]		0.00002 - 0.13	[2]
Cu	0.005 - 10	[1]		0.005 - 0.155	[4]		0 - 0.419	[4]
	0.0005 - 1.4	[2]		0.06 - 0.27	[5]		0 - 0.24	[5]
	0.005 - 0.78	[3]	**Mn**	0.04 - 16.4	[3]	**Ba**	0.006 - 0.164	[3]
	0.003 - 0.49	[4]	**Pb**	0.001 - 5	[1]			
	0.01 - 0.75	[5]		0.0005 - 1.5	[2]			
Ni	0.015 - 1.3	[1]		0 - 0.3	[4]			
	0.001 - 3.2	[2]		0.09 - 3.40	[5]			
	0.30 - 1.15	[5]	**Si**	3.72 - 10.48	[3]			

References: [1] Kjeldsen et al. (2002), [2] Baun and Christensen (2004), [3] Renou et al. (2008), [4] Barlaz et al. (2010), [5] Xie et al. (2015).

Table 1.11. Reported IC_{50} (mg/L) values of several metals for the anammox and nitritation processes

Anammox						**Nitritation**		
Metal	IC_{50} (mg/L)	Ref.	Metal	IC_{50} (mg/L)	Ref.	Metal	IC_{50} (mg/L)	Ref.
Zn	2.7[a] - 6.8[b]	[1]	**Ni**	1.4[a] - 6.0[b]	[1]	**Cu**	26.5	[12]
	3.9 - 4.5	[2]		37.2	[3]		74.3	[13]
	6.4	[3]		48.6	[5]		7.2	[14]

Table 1.11 (continued).

Anammox						Nitritation		
Metal	IC_{50} (mg/L)	Ref.	Metal	IC_{50} (mg/L)	Ref.	Metal	IC_{50} (mg/L)	Ref.
Zn	6.9	[4]	**Ni**	69.2	[7]	**Zn**	5.4	
	2.7[a] - 6.8[b]	[1]		1.4[a] - 6.0[b]	[1]	**Cu**	26.5	[12]
	3.9 - 4.5	[2]		37.2	[3]		74.3	[13]
	6.4	[3]		48.6	[5]		7.2	[14]
	6.9	[4]		69.2	[7]	**Zn**	5.4	
	7.6	[5]	**Ag**	11.52	[11]	**Ni**	5.1	
	20	[6]	**As**	60	[10]	**Co**	12.9	
	25	[6]	**Pb**	NT	[5]	**Cd**	10.4	[15]
	59.1	[7]		6.5	[3]	**Cr(III)**	61.6	
Cu	4.1	[3]		10.4	[10]	**Cr(VI)**	66.2	
	4.2	[5]		40	[11]	**Hg**	9.8	
	4.2	[4]		45.6	[7]	**Ag**	0.6	
	1.9 - 5	[2]	**Hg**	2.33	[10]			
	12.9	[8]		60.35	[11]			
	16.3	[6]	**Cd**	7	[10]			
	19.3	[7]		9.7	[3]			
	32.5	[9]		11.16	[11]			
Cr(II)	9.84	[10]		11.2	[5]			
	26.9	[7]		174.6	[7]			
Mn(II)	175.8	[7]						

[a] Soluble metal concentration. [b] Free-ion metal concentration.

References: [1] Aktan et al. (2018), [2] Lotti et al. (2012), [3] Li et al. (2012b), [4] Daverey et al. (2014), [5] Li et al. (2015), [6] Zhang et al. (2016b), [7] Val del Río et al. (2017), [8] Yang et al. (2013), [9] Zhang et al. (2015c), [10] Yu et al. (2016), [11] Bi et al. (2014), [12] Ochoa-Herrera et al. (2011), [13] Guo et al. (2016), [14] Çeçen et al. (2010a), [15] Çeçen et al. (2010b).

1.5.10. Sulphate and sulphide

The sulphate (SO_4^{2-}) is usually reduced to sulfide (S^{2-}) during the anaerobic digestion process by the sulphate reducing bacteria, and thus the sulfide can lead to the inhibition of anammox in the subsequent nitrogen removal step (Jin et al., 2012). The sulphur compounds can be found especially in wastewater streams such as seafood processing, leather tanning, oil refining and alcohol fermentation wastewater. IC_{50} values were reported for the anammox bacteria: 35.5 g SO_4^{2-}/L (Dapena-Mora et al., 2007) and 11.36 g SO_4^{2-}/L (Chen et al., 2014). Regarding the inhibition by sulfide,

Dapena-Mora et al. (2007) indicated an IC_{50} value for anammox of 9.6 mg S^{2-}/L, while Van De Graaf et al. (1996) showed the resistance of anammox bacteria to concentrations of at least 64 mg S^{2-}/L.

On the other hand, the sulphate anion have a more severe effect on anammox activity than the chloride for the same concentrations (Li et al., 2018b). However, scarce research is available upon long-term effects of the sulphate anion over the anammox activity, and more research is needed in order to understand the possible antagonism or synergism when different cations are brought together in the wastewater.

1.6. Industrial wastewater streams treated by PN-AMX

The first full-scale PN reactor was implemented at the WWTP of Dokhaven, in Rotterdam (Mulder et al., 2001), which was later combined with a subsequent AMX unit in a 2-stage PN-AMX system. On the other hand, the first successful full-scale one-stage PN-AMX reactor with floc-type suspended biomass was the DEMON reactor implemented at the WWTP of Strass, in Austria (Wett, 2006). Currently, there are over 114 full-scale plants worldwide implementing the shortcut BNR, 75% of which are dedicated to the sidestream treatment of municipal wastewater (Ali and Okabe, 2015; Lackner et al., 2014).

The wastewater treated in full-scale anammox-based reactors have ammonium concentrations in the range 500 - 3000 mg N/L, as well as COD/N ratios between 0.2 and 3.8 g COD/g N (Ali and Okabe, 2015). The industrial effluents are also characterized by high nitrogen concentrations (approximately 1000 mg N/L), which are much higher compared to the municipal or domestic wastewater streams (Fernández et al., 2016). Regarding the COD/N ratio range (close to 1 g COD/g N), it strongly depends on the type of industrial wastewater (Tomar and Gupta, 2017), and it may be comparable to the COD/N range for mainstream wastewater in some cases (7 - 12 g COD/g N, Saxena et al. (2019)). With all that, the shortcut BNR is still considered suitable for the treatment of industrial wastewater streams (Jin et al., 2012; Van Hulle et al., 2010).

Since the shortcut BNR involves the use of different microbial communities (see Section 1.4.1), the composition of each community is defined by the complex composition of each wastewater used and its specific characteristics, which may be variable (Kallistova et al., 2016). For this reason, the industrial application of the anammox process requires a case-specific design regarding parameters such as the pH, salinity, temperature, organic matter and nitrogen concentrations, metals and antibiotics (Hu et al., 2013). In this sense, the implementation of efficient pretreatments to lower the concentrations of some of the compounds of the wastewater streams such as the concentration of

organics, is crucial when treating organic carbon-rich effluents (Mao et al., 2017), as well as proper control strategies to optimize the BNR (Saxena et al., 2019).

The PN-AMX processes have been applied for the BNR of a wide range of industrial wastewater. Table 1.12 shows some examples for these wastewater streams.

Table 1.12. Examples of industrial wastewater streams with application of the PN-AMX processes for the removal of nitrogen.

Wastewater	PN-AMX system	WW concentrations (mg/L)		NLR (g N/(L·d))	Maximum % N removal	Ref.
		NH_4^+-N	COD			
AcS supernatant	2-stage	657	NS	0.33	90	[1]
AcS supernatant	IFAS	255 - 705	180 - 550	0.26 - 0.70	80	[2]
Fish-canning	2-stage, CANON	780 - 1670	≤ 1000[a]	0.34 - 0.67	80	[3]
Fish-canning	CANON	206 - 291	45 - 200[a]	0.19 - 0.26	80	[4]
Pig manure	2-stage	3240 - 4960	17340 - 38810	0.93 - 1.42	86	[5]
Pig slurry	2-stage	2000 - 4000	8000 - 17000	1	73	[6]
Pig slurry	CANON	245	420	0.46	75	[7]
Landfill leachate	2-stage	250	600 - 800	0.29 - 0.37	93	[8]
Landfill leachate	2-stage	1330	2250	0.5 - 1.2	94	[9]
Fertilizer WW	SNAD	700 - 800	46.6	0.30 - 0.35	98	[10]
Black water	OLAND	1023 - 1065	581 - 597	1.0 - 2.5	76	[11]
MSG	2-stage	70 - 75	100 - 125	0.024	74	[12]
Potato process.	DEMON	1208	2472	NS	85	[13]
Turtle breeding	AMX	97 - 289	194 - 578	0.19 - 0.58	80	[14]
Tannery	SNAP	250	250	0.024	82	[15]
Optoelectronic	CANON	3712	13.5	0.93	89	[16]
Semiconductor	2-stage	250 - 400	< 5	0.24 - 0.38	88	[17]

[a] expressed as mg TOC/L here.

AcS: activated sludge, CANON: Completely Autotrophic Nitrogen removal Over Nitrite, COD: chemical oxygen demand, DEMON: DEaMmONification process, IFAS: Integrated Fixed-film and Activated Sludge reactor, MSG: monosodium glutamate wastewater, NLR: nitrogen loading rate, OLAND: Oxygen Limited Autotrophic Nitrification Denitrification process, SNAD: Simultaneous partial Nitritation, Anammox and Denitrification process, SNAP: Single stage Nitrogen removal using Anammox and Partial nitrification process, WW: wastewater.

References: [1] Fux et al. (2002), [2] Zhang et al. (2015b), [3] Vázquez-Padín et al. (2009), [4] Val del Rio et al. (2018), [5] Hwang et al. (2006), [6] Yamamoto et al. (2008), [7] Figueroa et al. (2012), [8] Miao et al. (2015), [9] Wang et al. (2016), [10] Keluskar et al. (2013), [11] Vlaeminck et al. (2009), [12] Shen et al. (2012), [13] Gonzalez-Martinez et al. (2015), [14] Chen et al. (2013), [15] Anjali and Sabumon (2017), [16] Daverey et al. (2013b), [17] Tokutomi et al. (2011).

The advantages of the combined PN-AMX process (mainly, reduction of aeration and external carbon source addition) are most pronounced during the treatment of wastewater characterized by high ammonium concentrations and low biodegradable organic matter content. Nowadays, the industrial wastewater streams that withdraw more attention for the application of anammox-based processes are the fish canning industry wastewater, the pig slurry from the livestock manure, and the leachate-like (landfill leachate, OFMSW) effluents (Saxena et al., 2019), as seen from the increase of related research studies over the last years, as it will be reviewed hereafter.

1.6.1. Fish canning industry effluents

Due to the developing world, the growth in the global consumption of fish has doubled since 1973 (7.3 kg/(person·year)) (Chowdhury et al., 2010), and the projected estimations of global fish food consumption for the year 2020 are between 16.2 and 21.5 kg/(person·year)) (Delgado et al., 2003). The fish processing operations, which vary between plants in terms of raw material, source of utility water and unit processes, generate wastewater streams from the different common processes (filleting, freezing, drying, fermenting, smoking and canning) (Chowdhury et al., 2010; Cristóvaõ et al., 2015). The activity of the fish canning industries, which constitute an important economic sector in Galicia (Northwest of Spain), generates large wastewater volumes containing high concentrations of solids, organic matter and nitrogen (Mosquera-Corral et al., 2005). As can be seen in Table 1.13.a, the AD is widely used to treat wastewater fish-canning effluents for the removal of organic matter (Mosquera-Corral et al., 2005), while the PN-AMX processes have gained much interest in the recent years for its application to the removal of nitrogen from these effluents after its treatment in the AD (Li et al., 2018c) (Table 1.13.b).

Table 1.13.a. Summary of different biological processes treating fish-canning effluents for the removal of carbon and nitrogen.

Ref.	Wastewater	Process	Reactor	Biomass	Concentrations (g/L)				Organic matter (COD)	
					TN	COD	VS	Salt (Cl^-)	OLR (g COD/L·d)	% removal[a]
[1]	herring	AD	AFF/AFB	carrier/suspended	3	90	7	65	1.7 - 12.5	85/86
[2]	tuna, mussel	AD	CAD	suspended	4 - 5	10 - 50	1.2 - 3.6	10 - 14	4	75 - 80
[3]	tuna	AD	AFF	carrier biofilm	0.2	3.3	1.9	4[b]	0.3 - 1.8	75
[4]	tuna	AD	AD/DSFF	suspended	0.1 - 0.2	12	0.5	1.6 - 7.6	3 - 13	75/70
[5]	mussel	AD	TAF/MAF	carrier biofilm	0.12 - 0.31	12.4 - 16.9	1.0 - 1.4	10 - 16	9 /24	73/64
[6]	tuna, mussel	AD	UASB	granular	NS	18.5 - 29.5	1.2 - 3.6	NS	1 - 4	80 - 95
[7]	tuna, sardine	AD	UASB	suspended	0.27 - 0.41	1.7 - 2.8	5 - 16	3.2	5 - 8	78 - 92
[8]	tuna	AD + AcS	UASB + AcS	suspended	0.44	5.5	1.57	1.8	1.2	95
[9]	tuna	AD-N-DN	UASB	flocculent	0.15 - 0.23	3.8 - 4.3	NS	NS	15	60
[10]	NS	PN-DN	SBR	flocculent	0.235	2.1	0.5	30	0.7 - 2.1	90
[11]	fish cannery	aerobic	RBC	biofilm	0.28	3 - 5	2	NS	22.13[c]	94
[12]	NS	CNP	SBR	AGS	0.11	0.9 - 1.7	0.16 - 0.22	10.4	5.4	80
[13]	anchovy	CN	GSBAR	AGS	0.05 - 0.38	2.5 - 6.1	4.9 - 5.8	30 - 75	3.2 - 8.0	NS
[14]	anchovy	CN	SBR	flocculent, AGS	0.15	0.8	NS	30 - 50	0.8	98

Table 1.13.b. Summary of different biological processes treating anaerobically digested fish-canning effluents for the removal of carbon and nitrogen.

Ref.	Wastewater	Process	Reactor	Biomass	Concentrations (g/L)				Nitrogen	
					NH_4^+	COD	VS	Salt (NaCl)	NLR (g N/L·d)	% removal[a]
[15]	NS	AcS	batch	flocculent	0.051	9.2	2.8	6.5[d]	NA	NA
[16]	fish cannery	AD-DN	USBF	granul./flocc.	0.38 - 0.41	1.1 - 1.3	NS	NS	1.00 - 1.25	80
[9]	tuna	AD-N-DN	NAS	flocculent	0.07 - 0.59	0.2 - 0.8	NS	NS	0.1 - 0.2	100
[17]	tuna	Aer. + N-DN	CBR + UFM	carrier biofilm, suspended	0.34	0.45	NS	NS	0.8 - 1.7	76
[18]	NS	N-DN	SBR	AGS	0.6 - 2.0	4.0 - 15.8	1.3 - 2.0	7 - 9[d]	0.16 - 0.28	20 - 45
[19]	NS	N-ADN	AcS + SBR	flocculent	0.32	0.31	0.29	3.8[c]	0.02 - 0.08	13 - 33
[20]	anchovy	N-DN	SBAR	AGS	0.288	0.011	4.62	50	0.05	90
[21]	NS	PN	CSTR	flocculent	1.06	0.25[d]	0.15	6.62[d]	1.06	30 - 40
[22]	NS	AMX	SBR	granular	0.7 - 1.0	1.0 - 1.3[e]	NS	8 - 10	0.5	68
[23]	NS	PN-AMX (1/2-stage)	SBR/ SBR	granular	0.78 - 1.67	1	NS	5.9[d]	0.20 - 0.65	40 - 80
[24]	tuna, mussel, sardine	PN-AMX	SBR	granular	0.11 - 0.27	0.30 - 0.52	NS	18.0	0.08 - 0.14	30 - 80
[25]	tuna, mussel, sardine	PN-AMX	SBR	granular	0.21 - 0.29	0.20[d]	NS	7 - 9[f]	0.19 - 0.26	80

[a] The total nitrogen and carbon removal (%) values refer to the maximum values achieved during the operation, or the observed range in stationary state. [b] g/L NaCl. [c] $g/(m^2 \cdot d)$. [d] g Cl^-/L. [e] g TOC/L. [f] Sudden salt peaks of 16 g NaCl/L.

AcS: activated sludge, AD: anaerobic digestion, AFB: anaerobic fluidized bed reactor, AFF: anaerobic fixed filter reactor, AGS: aerobic granular sludge, AMX: anammox, CAD: central activity digester, CANON: Completely Autotrophic Nitrogen removal Over Nitrite, CBR: circulating bed reactor, CN: carbon-nitrogen aerobic removal system, CNP: carbon-nitrogen-phosphorus aerobic removal system, DSFF: downflow stationary fixed film reactor, GSBAR: granular sequencing batch airlift reactor, MAF: mesophilic anaerobic filter, NA: not applicable, NAS: nitrification activated sludge, NS: not specified, PN-DN: partial nitritation-denitritation, RBC: rotating biological contactor, SBAR: sequencing batch airlift reactor, SBR: sequencing batch reactor, SHARON: Single reactor for High activity Ammonia Removal Over Nitrite, TAF: thermophilic anaerobic filter, UASB: upflow anaerobic sludge blanket, UFM: ultrafiltration membrane, USBF: upflow sludge bed-filter reactor.

References: [1] Balslev-Olesen et al. (1990), [2] Mendez et al. (1992), [3] Prasertsan et al. (1994), [4] Veiga et al. (1994), [5] Mendez et al. (1995), [6] Puñal and Lema (1999), [7] Palenzuela-Rollon et al. (2002), [8] Achour et al. (2000), [9] Mosquera-Corral et al. (2003), [10] Capodici et al. (2018), [11] Najafpour et al. (2006), [12] Campo et al. (2017), [13] Corsino et al. (2017), [14] Corsino et al. (2019), [15] Cristóvão et al. (2016), [16] Mosquera-Corral et al. (2001), [17] Oyanedel et al. (2003), [18] Figueroa et al. (2008), [19] Fajardo et al. (2013), [20] Corsino et al. (2016), [21] Mosquera-Corral et al. (2005), [22] Dapena-Mora et al. (2006), [23] Vázquez-Padín et al. (2009), [24] Giustinianovich et al. (2018), [25] Val del Rio et al. (2018).

On the other hand, these effluents are also characterized by a higher salinity compared to other wastewater streams, due to the origin of the raw processing material and the common use of seawater in washing steps during the manufacturing process (Val del Rio et al., 2018). As can be seen in Table 1.13.b, both the conventional N-DN as well as the innovative PN-AMX combined processes have been applied to saline fish cannery wastewaters with salt concentrations in the range of 7 - 18 g NaCl/L, with concentrations up to 50 g NaCl/L (Corsino et al., 2016), and ammonium concentrations in the range 0.05 - 1.67 mg NH_4^+-N/L. Regarding the fish canning effluents before the aerobic/anaerobic digestion (Table 1.13.a), where a broader salinity range was reported (1.6 - 75 g Cl^-/L), the average salinity seems to be reduced before the biological removal of nitrogen. Furthermore, the characterization of the wastewater is highly variable due to the seasonality of the fish canning industries, and the raw materials processed in each moment at the factory (Cristóvão et al., 2016).

1.6.2. Livestock manure and pig slurry

The livestock manure is an inevitable by-product of the farmyard and animal industries, characterized by high concentrations of nitrogen, phosphorus, potassium and solids (Ma et al., 2018). The nitrogen alone from the livestock manure may account for up to 710 thousand ton/y, which means 1.5 times the agricultural demand for nitrogen (Li and Kobayashi, 2010). However, the abusive use of manure as fertilizer can rise the concentration of nutrients in the soil (Riaño and García-González, 2015), and the increasingly restrictive regulations require sustainable solutions for treating the generated pig slurry. The livestock manure has great potential as co-substrate in the AD of carbon-rich waste biomass; it has been reported to constitute the second largest estimated potential for annual methane recovery (1.6 billion m^3) after municipal sewage waste (1.7 billion m^3) (Li and Kobayashi, 2010).

The AD and the BNR are the most attractive alternatives for both biogas production from the organic matter contained in the pig manure, and ammonium removal contained in the pre-digested pig slurry, respectively (Figueroa et al., 2012). The main research works addressing the application of AD and BNR processes are summarized in Table 1.14.a and Table 1.14.b, respectively.

Table 1.14.a. Summary of different biological processes treating the pig manure and pre-digested pig slurry for the removal of carbon.

Reference	Wastewater	Process	Reactor	Biomass	Concentrations (g/L)			COD/N ratio (g/g)	Organic matter (COD)	
					NH_4^+-N	P	K		OLR (g COD/L·d)	% removal[a]
[1]	raw	AD	ICAnR	flocculent	0.71	NS	NS	9.01	6 - 7	80
[2]	raw/AD	aerobic	aeration tank	flocculent	1.1	0.43	NS	14.5	1.37	98
[3]	AD	aerobic	SBR	flocculent	1.53	NS	NS	2.09	0.64	82
[4]	AD	aerobic	CAB	biofilm	0.19	0.15	NS	1.75	0.03 - 0.13	90
[5]	raw	N-DN	2-stage SBR	flocculent	0.21 - 0.25	0.15 - 0.38	NS	15.6	0.75	97
[6]	raw	ANARWIA	UASB	flocculent	0.4 - 1.0	NS	NS	8 - 15	2 - 5	90
[7]	AD	PN	SB	flocculent	0.9 - 1.5	NS	NS	1.7 - 4.3	1.14[b]	84.9[b]

Table 1.14.b. Summary of different biological processes treating the pig manure and pre-digested pig slurry for the removal of nitrogen.

Ref.	WW	Process	Reactor	Biomass	Concentrations (g/L)			COD/N ratio (g/g)	Nitrogen	
					NH_4^+-N	P	K		NLR (g N/L·d)	% removal[a]
[2]	raw/AD	aerobic	aeration tank	flocculent	1.1	0.43	NS	14.5	0.25	97
[8]	raw	aerobic	SBR	flocculent	3.8	20	NS	2.89	0.14 - 0.43	95
[1]	AD	aerobic	SBR	flocculent	0.72	NS	NS	3.90	0.12 - 0.14	93.4
[3]	AD	aerobic	SBR	flocculent	1.53	NS	NS	2.09	0.31	68
[4]	AD	aerobic	CAB	biofilm	0.19	0.15	NS	1.75	0.01 - 0.04	20 - 48
[9]	raw	aerobic	UMSR	flocculent	0.30	NS	NS	0.84	1.1	87
[10]	AD	aerobic	IASBR/SBR	flocculent	0.52	0.033	NS	1.6 - 3.4	0.10 - 0.21	83/75
[5]	raw	N-DN	2-stage SBR	flocculent	0.21 - 0.25	0.15 - 0.38	NS	15.6	0.04 - 0.05	98
[11]	raw	N-DN[c]	A/O	flocculent	2.5 - 4.0	0.17 - 0.28	NS	5.9 - 7.2	0.15 - 0.23	30[c]
[12]	AD	N-DN	A/O SBR	flocculent	3.82 - 4.20	0.55 - 0.69	0.69 - 0.98	11 - 12	0.25 - 0.51	71 - 98
[13]	raw	N-DN	SBR	flocculent	0.71	0.092	NS	9.93	0.21	97.5
[14]	raw/AD	N-DN	SBR	flocculent	0.9	0.09	NS	1.93	0.9	99.8
[15]	AD	N-DN	SBR	flocculent	0.62 - 1.62	NS	NS	0.9 - 6.3	0.31 - 0.52	63 - 80
[16]	raw	N-DN	UMSR	flocculent	0.29 - 0.31	NS	NS	0.3 - 1.3	0.9 - 1.1	80
[17]	raw	N-DN	CF/AcS	carrier biofilm/floc.	0.69	NS	NS	1.52	0.086	62 - 70
[18]	raw	N-DN	UMBR	biofilm	0.23 - 0.31	0.015 - 0.018	NS	0.7 - 1.5	0.68 - 1.12	93.1
[19]	raw	N-DN	USMR	flocculent	0.06 - 0.11	NS	NS	4.5 - 6.2	0.22 - 0.40	40
[20]	raw	N-DN-AMX	UMSR	granular	0.24 - 0.34	0.006 - 0.021	NS	1.0 - 1.3	0.88	86.6
[21]	AD	N-DN-P	SBR	flocculent	1.65	0.144	2.13	2.41	1.65	99.7[d]
[6]	AD	ANARWIA	SBR	flocculent	0.5 - 0.8	NS	NS	1.2 - 2.0	NS	90

Table 1.14.b (continued).

Ref.	Wastewater	Process	Reactor	Biomass	Concentrations (g/L)			COD/N ratio (g/g)	Nitrogen	
					NH_4^+-N	P	K		NLR (g N/L·d)	% removal[a]
[22]	raw	PN-DN	SBR	flocculent	1.5 - 3.4	0.07 - 0.37	NS	2.7 - 6.0	0.3 - 2.0	90
[23]	AD	PN	SB	biofilm	2 - 4	NS	NS	2.5 - 2.8	1.9	50 - 62
[24]	AD	PN	UBF	biofilm	2 - 4	NS	NS	4.0 - 4.2	1.0	58
[25]	AD	PN	batch	flocculent	0.4 - 1.2	NS	NS	3.03	NA	90
[26]	AD	PN	SBR	flocculent	1.65 - 1.92	NS	NS	3.1 - 4.3	0.33 - 0.38	86
[27]	AD	PN	A/O SBR	flocculent	0.35 - 0.88	NS	NS	1.4 - 2.2	0.06 - 0.15	21 - 47
[28]	AD	AMX	batch	granular	0.097	0.032	0.3	2.78[a]	NA	89
[29]	AD	AMX	UASB	granular	0.67 - 3.78	NS	NS	1.2 - 3.6	0.32 - 1.80	92 - 98
[30]	AD	AMX	UASB	granular	0.61	NS	NS	3.8	0.61	72 - 95
[31]	AD	AMX	SBR	granular	1.0 - 2.2	0.36	NS	0.4 - 0.5	1.0 - 7.2	85
[32]	AD	AMX	UC	granular	0.17 - 0.22	NS	NS	0.1 - 0.3	10 - 12	32 - 42
[33]	AD	AMX	airlift	granular	0.28 - 0.66	0.006 - 0.008	NS	1.1 - 1.5	3.9	73
[34]	raw	AMX-DN	UASB	granular	5	0.25 - 0.63	NS	11.2	0.83	71 - 74
[35]	AD	AMX-SD	AMX + SOD®	floc./granular	0.41 - 0.66	0.022	NS	1.52	0.45 - 0.70	80
[36]	AD	2-st. PN-AMX	SBR/USBR	floc./granular	0.89 - 1.07	0.07 - 0.16	NS	1.26	1.36	66.7
[37]	AD	2-st. PN-AMX	SBR/USBR	floc./granular	1.0 - 1.5	0.20	NS	0.82	1.25	94.1
[38]	AD	2-st. PN-AMX	PEG/CSTR	biof./granular	1.58	NS	NS	4.67	3.8/4.1	57 - 75
[39]	AD	2-st. PN-AMX	SB/UASB	biof./granular	1.6 - 2.2	NS	NS	4.5 - 5.5	2.6	95
[40]	AD	2-st. PN-AMX	SBR/SBR	floc./granular	1.02 - 1.43	0.029 - 0.086	1.96	1.0 - 3.3	1.47	57
[41]	AD	2-st. PN-AMX	SBR/SBR	floc./granular	1.22	0.0089	0.985	1.93	0.59 - 0.61	93

Table 1.14.b (continued).

Ref.	Wastewater	Process	Reactor	Biomass	Concentrations (g/L)			COD/N ratio (g/g)	Nitrogen	
					NH_4^+-N	P	K		NLR (g N/L·d)	% removal[a]
[42]	AD	1-st. PN-AMX	SBR	floc./granular	2.08	NS	NS	1.17	0.052	90
[43]	AerD	CANON	SBR	granular	0.24	NS	NS	1.71	0.6	75
[44]	AD	CANON	SBR	granular	0.43 - 0.51	0.015 - 0.052	0.01 - 0.35	0.2 - 1.1	0.25 - 0.35	50 - 70
[45]	AD	CANON-DN	SBBR	carrier biofilm	0.42	0.059	NS	0.6 - 1.2	0.20 - 0.26	95
[46]	AD	SNAD	SBR	flocculent	0.30 - 0.77	0.002 - 0.064	NS	0.6 - 0.9	0.04 - 0.17	96

[a] The total nitrogen and carbon removal (%) values refer to the maximum values achieved during the operation, or the observed range in stationary state. [b] Carbon expressed as TOC (mg/L). [c] The complete treatment scheme include ammonia stripping, A/O biological units, chemical coagulation and activated carbon; the % N removal refers only to the biological step. [d] 97.3% of P removal efficiency.

A/O: anoxic/oxic, AcS: activated sludge, AD: anaerobically digested [influent] (i.e., pre-digested pig slurry feeding), AerD: aerobically digested, ANARWIA: ANaerobic-Adding Raw Wastewater-Intermittent Aeration, CAB: contact aeration basin, CANON: Completely Autotrophic Nitrogen removal Over Nitrite, CF: carbon filter reactor, CSTR: continuously stirred tank reactor, IASBR: intermittently aerated sequencing batch reactor, ICAnR: internal circulation anaerobic reactor, N-DN: nitrification-denitrification, P: phosphorus, PEG: polyethylene glycol gel carrier reactor, PN: partial nitritation, PN-DN: partial nitritation-denitritation, raw: piggery wastewater with no previous aerobic/anaerobic digestion treatment step, usually only solid/liquid separation, SBBR: sequencing biofilm batch reactor, SBR: sequencing batch reactor, SD: sulfur denitrification, SNAD: Simultaneous partial Nitritation, Anammox and Denitrification, SOD®: Sulfur Oxidation Denitrification, UASB: upflow anaerobic sludge blanket reactor, UBF: upflow biofilm reactor, UC: upflow column reactor, UMBR: upflow microaerobic biofilm reactor, UMSR: upflow microaerobic sludge reactor, USBR: upflow sludge bed reactor.

References: [1] Deng et al. (2006), [2] Bicudo and Svoboda (1995), [3] Lo and Liao (2007), [4] Su et al. (1997), [5] Ra et al. (2000), [6] Deng et al. (2007), [7] De Prá et al. (2012), [8] Poo et al. (2005), [9] Meng et al. (2015), [10] Song et al. (2017), [11] Choi and Eum (2002), [12] Kim et al. (2004), [13] Zhang et al. (2006), [14] Obaja et al. (2005), [15] Scaglione et al. (2013), [16] Li et al. (2016a), [17] Yamashita et al. (2016), [18] Meng et al. (2018a), [19] Meng et al. (2018b), [20] Meng et al. (2017), [21] Obaja et al. (2003), [22] Eum and Choi (2002), [23] Yamamoto et al. (2006), [24] Yamamoto et al. (2008), [25] Xu et al. (2014), [26] Im and Gil (2011), [27] Belmonte et al. (2017), [28] Waki et al. (2007), [29] Molinuevo et al. (2009), [30] Ni and Yang (2014), [31] Jin and Zhang (2016), [32] Kindaichi et al. (2016), [33] Wang et al. (2017a), [34] Ahn et al. (2004), [35] Kwon et al. (2019), [36] Hwang et al. (2005), [37] Hwang et al. (2006), [38] Qiao et al. (2009), [39] Yamamoto et al. (2011), [40] Magrí et al. (2012), [41] Scaglione et al. (2015a), [42] Staunton and Aitken (2015), [43] Figueroa et al. (2012), [44] Pichel et al. (2019), [45] Zhang et al. (2012a), [46] Daverey et al. (2013a).

As can be seen in Table 1.14.b, the aerobic and nitrification-denitrification processes were extensively applied for the removal of nitrogen from pig slurry effluents, although in the last decade the autotrophic nitrogen removal with anammox-based process is increasingly withdrawing more attention (Bernet and Béline, 2009). Regarding the application of the coupled partial nitritation to anammox processes (PN-AMX), only a handful of studies have been reported with the two-stage (Hwang et al., 2005, 2006; Magrí et al., 2012; Qiao et al., 2009; Scaglione et al., 2015a; Yamamoto et al., 2011) and one-stage (Daverey et al., 2013a; Figueroa et al., 2012; Pichel et al., 2019; Staunton and Aitken, 2015; Zhang et al., 2012a) configuration. The ammonium concentration of the pig slurry treated in these research works was on average 1.1 ± 0.6 mg NH_4^+-N/L, with a COD/N range of 0.2 - 5.5 g/g, while the observed concentration ranges for phosphorus and potassium were 2 - 250 mg P/L and 10 - 1960 mg K/L, respectively.

1.6.3. Organic Fraction of Municipal Solid Waste (OFMSW)

The OFMSW comprises the largest fraction of the food wastes, which includes the food discarded or lost uneaten at the producing, processing and consuming stages. The potential burden of this waste source is enormous, since it is estimated that one-third of the total global food production is wasted (FAO, 2011). Due to the high content of biodegradable organic matter, the OFMSW is a desirable substrate for the AD (Ma et al., 2018), which can divert a significant part of the organic waste away from sanitary landfills (Renou et al., 2008). The OFMSW can be directly treated in solid-state AD with no dilution, or in liquid AD after being diluted. Similarly to the livestock manure, the food waste can be co-digested along with landfill leachate or waste activated sludge. Still, OFMSW (rather than food waste) is mainly investigated as the unique treatment source for the AD (Ma et al., 2018). After the liquid fraction of the OFMSW is anaerobically digested, BNR processes can be applied for the removal of nitrogen (Pedizzi et al., 2018; Wu et al., 2016), as summarized in Table 1.15.a for landfill leachate and Table 1.15.b for OFMSW digestates.

Table 1.15.a. Summary of pre-digested landfill leachate treated with BNR processes for the removal of nitrogen.

Ref.	LL age	Process	Reactor	Biomass	NH_4^+-N[a] (mg/L)	COD/N ratio (g/g)	k (mS/cm)	Alkalinity (g HCO_3^-/L)	Nitrogen	
									NLR[a] (g N/L·d)	% removal[b]
[1]	mat.	1/2-st. N-DN	SBR	flocculent	0.31 - 0.36	2.1 - 2.2	NS	NS	0.10 - 0.18	92 - 99
[2]	mat.	N-DN + UF + AMX	A/O / GAC	floc./carrier, granul.	0.5 - 1.0	1 - 5	1.1 - 25.2	NS	0.4 - 0.9	94
[3]	NS	PN	FBBR	biofilm	1.6 - 3.1	0.9 - 5.2	NS	4.7 - 8.7	0.2 - 1.0	60 - 74
[4]	NS	PN	SBR	flocculent	1.15 - 3.01	2.0 - 2.8	NS	5.1 - 14.6	1.0 - 1.5	40 - 60
[5]	NS	PN	SBR	flocculent	2.24 - 4.94	1.1 - 1.4	60.6 - 70.5	2.0 - 11.2	0.7 - 1.5	60 - 70
[6]	NS	PN	SBR	flocculent	0.48	1.2 - 3.4	NS	3.1 - 5.2	0.48	98.9
[7]	NS	PN	CSTR	flocculent	3.4 - 5.7	1.8 - 2.4	NS	16.4 - 18.8	1.11	> 50
[8]	mat.	PN	SBR	flocculent	5.97	0.66	74.1	5.49	1.06 - 1.10	50
[9]	mat.	PN	SBR	granular	0.69 - 1.75	2.2 - 3.8	NS	0.41 - 0.90	0.71	63
[10]	mat.	PN	SBR	flocculent	1.89	1.46	NS	NS	0.19	55 - 94
[11]	mat.	PN	SBR	flocculent	1.17	1.86	NS	6.2	0.30 - 0.89	62.5
[12]	old	PN	CSTR	flocculent	2.18	2.58	NS	8.21	0.27 - 0.63	50 - 70
[13]	old	PN	SBR	flocculent	2.83 - 3.51	0.89	NS	12.9	0.74 - 0.91	60
[14]	mat.	PN + IFD	SBR (x2)	flocculent	1.7	1.21	NS	3 - 8	0.09 - 0.38	95
[15]	mat.	PN-DN	A/O	flocculent	1.2 - 1.8	1.7 - 2.5	NS	NS	0.28 - 0.60	90 - 100
[16]	NS	PN-DN	A/O	flocculent	1.1 - 2.0	6.2 - 7.8	NS	4.9 - 6.8	0.20 - 0.46	99.5
[17]	NS	PN-DN	SBR	flocculent	1.5 - 2.5	4.4 - 5.3	NS	NS	NS	97
[18]	NS	PN-DN	SBR	flocculent	0.34	2.15	NS	NS	0.10	98 - 99
[19]	mat.	PN-DN	SBR	flocculent	0.12 - 1.03	5.6	NS	8.2 - 11.3	0.06 - 0.51	99
[20]	immat	PN-DN	PSBR	flocculent	1.05 - 1.30	7 - 8	NS	8.1 - 11.3	0.52 - 0.65	90
[21]	mat.	PN-DN	A/O	flocculent	1.32 - 2.04	1.9	NS	3.8 - 4.3	0.11 - 0.17	66.4

Table 1.15.a (continued).

Ref.	LL age	Process	Reactor	Biomass	NH_4^+-N[a] (mg/L)	COD/N ratio (g/g)	k (mS/cm)	Alkalinity (g HCO_3^-/L)	Nitrogen	
									NLR[a] (g N/L·d)	% removal[b]
[22]	mat.	PN-DN-AMX	ARB	solid	1.68 - 2.60	1.19	NS	4.8 - 6.7	0.74 - 2.03	62 - 90
[23]	NS	PN-DN-AMX	ARB	solid	1.24 - 1.51	1.8 - 1.9	NS	NS	NS	77 - 85
[24]	mat.	PN-DN-AMX	ARB	solid	1.0 - 1.3	1.0 - 1.2	NS	NS	0.04	90
[25]	mat.	DN-PN-AMX	SBAR	floc./granular	1.2 - 1.9	1.1 - 1.5	NS	6 - 8	1.5 - 2.4	99
[26]	mat.	AMX	UASB	granular	0.9 - 1.5	2.2 - 2.7	NS	6 - 10	2.1 - 3.2	87
[27]	NS	AMX-HD	SBR	granular	0.17	2.37	NS	NS	0.23 - 0.53	> 90
[28]	NS	AMX-DN	SBR	granular	0.07 - 0.37	1.6 - 2.1	10.2 - 17.0	0.64 - 0.59	0.24	89
[29]	NS	AMX + N-DN	UBF	carrier biofilm	0.24 - 0.63	2.2 - 2.9	NS	NS	0.12 - 0.27	30 - 90
[30]	NS	2-st. PN-AMX	AUSB/UASB	floc./carrier biofilm	0.63 - 0.80	0.9 - 1.0	NS	6.8 - 8.0	1.47/0.91	80/95
[31]	young	2-st. PN-AMX	AUSB/UAC	floc./granular	1.83 - 2.56	5.8 - 14.7	NS	5.0 - 7.3	1.83 - 2.56	97
[32]	mat.	2-st. PN-AMX	Panammox®	floc./granular	2.31	2.69	NS	2.03	1.78	84
[33]	mat.	2-st. PN-AMX	SBR/UASB	floc./granular	1.04	1.75	NS	3.94	1 - 4	99.7
[34]	mat.	2-st. PN-AMX	SBR (x2)	floc./granular	0.5	3.8	NS	7	0.72 - 0.81	99
[35]	NS	2-st. PN-AMX	SBR/HAR	floc./carrier biofilm	0.5 - 1.0	1.05	NS	1.3 - 2.7	1.02/4.20	99
[36]	mat.	2-st. PN-AMX	A/O / SBR	flocculent	0.40 - 0.41	1.46	NS	NS	0.22	82.6
[37]	mat.	2-st. PN-AMX	SBBR (x2)	floc./granular	3.0	1.0	NS	7.0	0.51 - 1.65	84 - 95
[38]	mat.	2-st. PN-AMX	A/O / CSTR	floc./granular	1.2 - 1.5	1.0 - 1.7	13.5 - 16.3	3.7 - 9.9	0.6	89
[39]	mat.	2-st. PN-AMX	A/O / UASB	floc./granular	1.11 - 1.53	1.6 - 1.9	NS	11.3 - 13.5	0.5 - 1.2	94
[40]	mat.	2-st. PN-AMX	NS/UC	NS/granular	0.57	0.37	NS	1.0	5.95	95
[41]	mat.	2-st. PN-AMX	SBR/SBBR	floc./biofilm	0.01 - 2.50	1.0 - 2.0	NS	NS	NS	90 - 95

Table 1.15.a (continued).

Ref.	LL age	Process	Reactor	Biomass	NH_4^+-N[a] (mg/L)	COD/N ratio (g/g)	k (mS/cm)	Alkalinity (g HCO_3^-/L)	Nitrogen	
									NLR[a] (g N/L·d)	% removal[b]
[42]	old	2-st. PN-AMX	ICAR	granular	2.83 - 3.10	0.5 - 1.9	NS	NS	0.8 - 0.9	76 - 96
[43]	mat.	2-st. PN-AMX + PN-DN	A/O + SBR	floc./granular	1.2 - 1.3	1.8 - 1.9	NS	NS	0.36 - 0.39	70
[44]	old	2-st. PN-AMX + USI	FBBR (x2)	carrier biofilm	1.43 - 2.72	0.8 - 0.9	NS	4.7 - 8.7	0.27 - 1.2	97
[45]	mat.	2-st. PN-AMX + EO	A/O / UAC	floc./granular	0.38 - 0.40	2.9 - 3.7	NS	NS	0.07 - 0.08	90.3
[46]	mat.	1-st. PN-AMX	LFBR	floc./granular	0.25 - 0.65	2.8 - 7.2	NS	0.18 - 0.56	1.2	84
[47]	NS	1-st. PN-AMX + DN	SBR	floc./biofilm	1.45	2.67	NS	9.62	2.9	80 - 90
[48]	NS[c]	1-st. PN-AMX + O_3 + GAC	UBF	carrier biofilm	0.24 - 0.63	2.2 - 2.9	5.59 - 9.36	NS	0.12 - 0.27	75
[49]	old	SNAP	UC	carrier biofilm	0.1 - 0.7	0.80	NS	9.61	0.2 - 1.4	85
[50]	NS	SNAD	aerated tank	granular	0.63	0.87	NS	NS	0.50	80
[51]	NS	SNAD	SBR	flocculent	0.29 - 0.70	0.5 - 0.8	NS	NS	0.12 - 0.28	69 - 88
[52]	mat.	SNAD	SBR	floc./granular	1.95	0.97	NS	9 - 10	0.39	99
[53]	mat.	SNAD	SBBR	biofilm	1.93	0.54	NS	NS	0.72	81 - 97
[54]	mat.	SNAD	SBR	floc./granular	1.2	0.92	NS	3 - 5	0.18	98.7

Table 1.15.b. Summary of pre-digested OFMSW effluents treated with BNR processes for the removal of nitrogen.

Ref.	Process	Reactor	Biomass	NH_4^+-N[a] (mg/L)	COD/N ratio (g/g)	k (mS/cm)	Alkalinity (g HCO_3^-/L)	Nitrogen	
								NLR[a] (g N/L·d)	% removal[b]
[55]	N-DN	A/O	flocculent	2.2 - 3.4	4.6	NS	NS	NS	80
[56]	N-DN	SBR	flocculent	0.69 - 1.33	3.9 - 5.1	1.47 - 28.3	8.8	0.23 - 0.44	> 90
[57]	N-DN	SBR	flocculent	1.12	4.29	NS	NS	0.37	98
[58]	N-DN	SBR	flocculent	2.16 - 2.98	4.2 - 4.8	27 - 35	NS	0.1 - 0.5	95
[59]	PN-DN	SBR	flocculent	0.35 - 0.53	0.1 - 0.2	3 - 6	0.66 - 1.27	1.1	90
[60]	PN-DN	MBR	flocculent	2.16 - 3.89	4.7 - 5.0	27 - 29	13.2 - 18.1	0.18 - 0.32	98
[61]	PN-DN	SBR	-	-	-	-	-	0.2	-
[62]	AMX	batch	granular	0.57 - 2.9	0.8 - 4.1	NS	NS	NA	NA
[63]	AMX	batch	granular	-	-	-	-	NA	NA
[64]	2-st. PN-AMX	MBBR/SBR	carrier biofilm	0.6	NS	NS	NS	1.2	94.7
[65]	2-st. PN-AMX	GSBAR	granular	-	-	-	-	1.0	-

[a] Both the ammonium concentration and the NLR values include the dilution when applicable. [b] The total nitrogen removal (%) values refer to the maximum values achieved during the operation, or the observed range in stationary state. [c] Raw leachate with no previous digestion.

A/O: anoxic/oxic reactor, Alk.: alkalinity, ARB: aged refuse bioreactor, AUSB: aerated upflow sludge bed reactor, CSTR: continuously stirred tank reactor, FBBR: fixed bed biofilm reactor, GAC: granular activated carbon, GSBAR: granular sequencing batch airlift reactor, HAR: hybrid anammox reactor, HD: heterotrophic denitrification, ICAR: internal circulation anammox reactor, immat.: immature, k: conductivity, LFBR: landfill bioreactor, LL: landfill leachate, mat.: mature, MBBR: moving bed biofilm reactor, MBR: membrane bioreactor, NA: not applicable, N-DN: nitrification-denitrification, NS: not specified, PN: partial nitritation, PN-DN: partial nitritation-denitritation, PSBR: pulsed SBR, SBAR: sequencing batch airlift reactor, SBBR: sequencing batch biofilm reactor, SBR: sequencing batch reactor, SNAD: Simultaneous partial Nitritation, Anammox and Denitrification, SNAP: Single stage Nitrogen removal using Anammox and Partial nitrification, st.: stage, UAC: upflow anammox column reactor, UASB: upflow anaerobic sludge blanket reactor, UBF: upflow biofilm reactor, UC: upflow column reactor, USI: underground soil infiltration system.

References: [1] Klimiuk and Kulikowska (2004), [2] Azari et al. (2017), [3] Liang and Liu (2007), [4] Ganigué et al. (2007), [5] Ganigué et al. (2009), [6] Sun et al. (2010), [7] Vilar et al. (2010), [8] Gabarró et al. (2012), [9] Li et al. (2013), [10] Fudala-Ksiazek et al. (2014), [11] Li et al. (2014a), [12] Spagni et al. (2014), [13] Phan et al. (2017a), [14] Zhang et al. (2019), [15] Zhang et al. (2007), [16] Peng et al. (2008), [17] Wu et al. (2009), [18] Kulikowska and Bernat (2013), [19] Wang et al. (2013), [20] Zhu et al. (2013), [21] Chen et al. (2016), [22] Xie et al. (2013), [23] Wang et al. (2014), [24] Wang et al. (2017b), [25] Li et al. (2018d), [26] Yun et al. (2016), [27] Ruscalleda et al. (2010), [28] Ruscalleda et al. (2010), [29] Gao et al. (2015a), [30] Liu et al. (2010), [31] Akgul et al. (2013), [32] Anfruns et al. (2013), [33] Li et al. (2014b), [34] Miao et al. (2014), [35] Phan et al. (2014), [36] Wu et al. (2015), [37] Miao et al. (2016), [38] Sun et al. (2016), [39] Wang et al. (2016), [40] Li et al. (2018a), [41] Miao et al. (2018b), [42] Phan et al. (2017b), [43] Wu et al. (2019b), [44] Liang and Liu (2008), [45] Wu et al. (2016), [46] Shalini and Joseph (2018), [47] Xu et al. (2010), [48] Gao et al. (2015b), [49] Vo and Nguyen (2016), [50] Wang et al. (2010), [51] Wang et al. (2011), [52] Zhang et al. (2017b), [53] Wang et al. (2018a), [54] Zhang et al. (2018), [55] Kübler et al. (2000), [56] Macé et al. (2006), [57] Dosta et al. (2007), [58] Mayer et al. (2009), [59] Fatone et al. (2011), [60] Wäger-Baumann and Fuchs (2011), [61] Frison et al. (2012), [62] Scaglione et al. (2017), [63] Scaglione et al. (2015b), [64] Caffaz et al. (2008), [65] Lotti et al. (2019).

As can be seen in Table 1.15, most of the reported literature on BNR processes addressed the treatment of mature/old landfill leachate, rather than OFMSW effluents. The reported mature landfill leachates treated with BNR processes are characterized by COD/N ratios in the range 0.9 - 7.8 g/g, as opposed to immature leachates where the COD concentration and COD/N ratio could reach above 10 g/L and 10 g/g, respectively (Zhu et al., 2013). The OFMSW, on the other hand, are characterized by lower COD/N ratios (0.8 - 5.1 g/g), as well as conductivity values between 1.5 - 35 mS/cm (Table 1.15.b). Up to date, only two research works (Caffaz et al., 2008; Lotti et al., 2019) addressed the application of the combined PN-AMX process to the nitrogen removal from OFMSW effluents.

1.6.4. Main challenges for the application of PN-AMX

The major current challenges in the application of the PN-AMX processes include the inhibition of the BNR by the compounds present in the wastewater and substrates (see Section 1.5), the suppression of the NOB activity, the microbial retention, slow start-up of the anammox process (Saxena et al., 2019), and the floatation of the biomass (Mao et al., 2017).

1.6.4.1. Start-up

Due to the long doubling time (11 - 20 days) of the anammox bacteria, the period going from the start-up to a stable operation of the PN-AMX processes is much larger than for the conventional N-DN processes. Although some authors reported high nitrogen removal efficiencies (91%) after a start-up of 27 days from activated sludge (Zhang et al., 2014), the most extended seeding practice in the current research studies to overcome the usually long start-up issues consists in the inoculation with anammox bacteria already treating nitrogen-rich wastewater streams (Chi et al., 2018). Still, the current research on fast start-up strategies are mainly focused at lab-scale, and thus the application to full-scale need further verification (Mao et al., 2017).

1.6.4.2. Floatation

The production of dinitrogen gas inside the PN-AMX granules from the anammox reaction forms internal gas tunnels, which may be obstructed due to the growth of the granule and the EPS secreted. The resulting trapped gas pockets can lead to increased buoyant forces in the granule and floatation issues (An et al., 2013). Different diameter values delimiting settling from floating granules were reported: 2.92 mm (Chen et al., 2010), 2.20 mm (Ma et al., 2016) and 1.35 mm (Lu et al., 2012). Chen et al. (2010) proposed the mechanical break-up of the granules as a strategy to re-establish the settling properties of the granular biomass. Dapena-Mora et al. (2004), on the other hand, found that the

overload of the anammox process (i.e., higher applied nitrogen loading rate (NLR) than the anammox specific activity) is responsible for the floatation of the granular biomass; the related lower granule density due to the inner accumulation of dinitrogen gas can thus be avoided by controlling the applied NLR.

1.6.4.3. N_2O emission in PN-AMX processes

As previously said, one of the drawbacks and hot topics of the PN-AMX processes is the emission of nitrous oxide (N_2O) from the microbial reduction of nitric oxide (NO) during the denitrification and nitritation processes (Eskicioglu et al., 2018; Kuypers et al., 2018). This greenhouse gas, with a warming potential 310 times higher than CO_2 on a 100-year horizon (Kartal et al., 2011; Ravishankara et al., 2009), may account for 0.01 - 1.8% of the influent nitrogen load of a WWTP (Ni et al., 2017). The key parameters that determine the emission of N_2O are the aeration cycle, aeration rate, DO concentrations and nitrite concentrations (Kampschreur et al., 2008). The concentration of nitrite, which is a necessary intermediate product for the formation of N_2O, should be controlled below 5 mg N/L for a stable performance of the PN-AMX processes and lower N_2O emissions.

1.6.4.4. Competition of the PN-AMX processes by NOB

The competitive growth of NOB is an important factor that can deteriorate the removal of nitrogen in a PN-AMX system. Li et al. (2018b) indicated that 40% of the one-stage PN-AMX processes treating industrial wastewater reported increasing concentrations of nitrate due to NOB activity. As previously said, in order to stop the total nitrification (nitritation + nitratation) at the PN, the activity of AOB must be favoured rather than the NOB one (see Figure 1.1). When treating high-strength industrial wastewater, the NOB can be selectively inhibited by controlling parameters like the free ammonia (FA) and DO concentration, temperature, pH, alkalinity and hydraulic and solids retention time (HRT - SRT) (Table 1.16).

Inhibition of NOB by FA, pH, temperature and HRT

The FA and FNA, rather than the ammonium and nitrite, diffuse easily across the cell membrane and may be toxic for NOB (Li et al., 2018e). The inhibition by FA is favoured by the high nitrogen concentrations of the industrial wastewater, and may support long-term stable operations (Zhang et al., 2012b). As shown in Table 1.16, the NOB are more sensitive to FA concentrations than the AOB.

Table 1.16. Inhibitory values of free ammonia (FA), and optimal temperature and pH ranges, for NOB.

Ref.	WW	Reactor	Biomass	pH	T (°C)	HRT (d)	Value
FA inhibition values (mg NH_3-N/L)							
[1]	synthetic, manure	batch	NS	5.5 - 9.0	RT	NA	0.082 - 0.82[a]
[2]	synthetic	SBR	flocculent	7.0 - 7.4	30	3.8	10 - 22
[3]	synthetic	batch	flocculent	7.5	26 - 28	NA	0.8 - 22
Optimal temperature range (°C)							
[4]	synthetic	SBR	flocculent	7.2 - 7.5	20	0.5	< 17
[5]	AD supern.	A/O	flocculent	7.52	28.7	1.6	< 24
[6]	synthetic	batch	flocculent	8.1	25	NA	10 - 20
Optimal pH range							
[7]	synthetic	batch	Flocculent	6.8 - 10.2	RT	NA	7.2 - 7.6[b]

[a] Values for an optimal pH of 7.5.
[b] Values for *Nitrobacter* spp.

A/O: anoxic/oxic reactor, AD supern.: anaerobically digested supernatant, HRT: hydraulic retention time, NA: not applicable, NS: not specified, RT: room temperature, SBR: sequencing batch reactor.

References: [1] Anthonisen et al. (1976), [2] Jianlong and Ning (2004), [3] Kim et al. (2010), [4] Munz et al. (2011), [5] Fux et al. (2002), [6] Balmelle et al. (1992), [7] Alleman (1985).

The FA rather inhibits than kills the NOB, and thus the NOB have the potential to adapt to long-term FA exposure and recover its activity up to a certain extent (Soliman and Eldyasti, 2018). Jianlong and Ning (2004) reported the ability of NOB to adapt to 10 mg NH_3-N/L, achieving the inhibition for higher concentrations (22 mg NH_3-N/L). Moreover, Kim et al. (2010) showed that the *Nitrobacter* spp. have higher inhibition threshold range (30 - 50 mg NH_3-N/L) than *Nitrospira* spp. (0.8 - 22 mg NH_3-N/L). However, Soliman and Eldyasti (2018) (Table 1.16) reported that *Nitrobacter* spp. were active between 10 and 20 °C despite inhibiting FA concentrations, showing the prevalence of the temperature factor. As opposed to NOB, the AOB have a comparatively higher FA inhibition range (8 - 123 mg NH_3-N/L, Anthonisen et al. (1976)), as well as higher optimal temperature (> 17 °C, Munz et al. (2011); > 24 °C, Fux et al. (2002)) and pH (7.5 - 8.5, Alleman (1985)) ranges.

Given the doubling times for AOB (7 - 8 h) and NOB (10 - 13 h), a shorter HRT and SRT high enough to retain AOB would favour both the AOB activity and the washout of the NOB, since the latter may acclimate to non-favouring conditions. In fact, the NOB are able to grow faster than AOB under low substrate conditions, and thus a sufficient concentration of ammonium in the effluent (> 2 mg NH_4^+-N/L) should be ensured for the AOB to outcompete the NOB (Chandran and Smets, 2000).

Inhibition of NOB by DO and intermittent aeration

When the inhibition of the NOB by FA is not possible, due to the low pH and ammonium concentrations in the wastewater, the kinetic NOB suppression can be achieved by using DO control and intermittent aeration (Ma et al., 2016; Seuntjens et al., 2016).

Given the lower DO half saturation constant of AOB (0.3 mg O_2/L) compared to NOB (1.1 mg O_2/L) at temperatures over 25 - 30 °C, the AOB can outcompete the NOB for low DO concentrations and favour the nitrite accumulation (Ma et al., 2016; Wiesmann, 1994). Chandran and Smets (2000) suggested DO threshold concentrations below 1.5 mg O_2/L for NOB inhibition. However, under long-term low DO conditions, the k-strategists *Nitrospira*-NOB spp. (with low specific growth rates and high substrate affinity) can outcompete the r-strategists *Nitrobacter*-NOB spp. (with high specific growth rates and low substrate affinity), which are usually the dominant NOB species (Ge et al., 2015; Regmi et al., 2014). The latter may thus end up outcompeting AOB for the DO substrate in the range of 21 - 28 °C (Li et al., 2017b; Seuntjens et al., 2016).

The NOB possess a nitrate-production lag phase for aerobic conditions higher than the ammonium-oxidation lag phase of AOB. This delay time can be exploited through (1) air flow regulation (Gilbert et al., 2014) or (2) transient anoxia conditions, in which the oxygen is turned off after the aeration phase before the NOB activity is restored, and the nitrite produced by the AOB are subsequently reduced by the AMX (Ma et al., 2016; Miao et al., 2018a). As opposed to Gilbert et al. (2014), Kornaros et al. (2010) found that the delay time in nitrate production is a function of the length of the anoxic period.

1.7. Current implementation of PN-AMX

The implementation of an efficient and simple PN-AMX combined processes constitutes a motivation for the treatment of industrial, nitrogen-rich wastewater streams (Antwi et al., 2019). Many full-scale PN-AMX system alternatives are currently available, with special emphasize in the granular and biofilm systems, as well as the one- and two-stage configurations.

1.7.1. Implementation of the PN-AMX at full scale

1.7.1.1. Full-scale PN-AMX processes

There are many different technologies for nitrogen removal at full-scale: SHARON/ANAMMOX, DEMON, CANON, OLAND, SNAP and ANITA Mox® (Gonzalez-Martinez et al., 2018; Mao et al., 2017; Van Der Star et al., 2007). They mainly differ in their operation (continuous or discontinuous), the number of stages (one- or two-stage) and in the presentation of biomass.

Some of these systems work with biomass-accumulated flocculent sludge (DEMON), whereas others work with granular sludge (CANON, ELAN®) or with sludge as a biofilm moving bed (ANITA Mox®). However, one of the main problems associated with these technologies is the stability of biomass (Mao et al., 2017).

The ELAN® process is an anammox-based process carried out with granular biomass in one single reactor, based on the establishment of aerobic and anoxic zones within the granule (Morales et al., 2015). In order to avoid ammonium and nitrite limitations and/or inhibitions, the operational strategy is based in the control of HRT and bulk DO concentration. The oxygen supplied to the reactor has to limit the nitrogen removal rate in order to avoid unstable periods due to oxygen and/or nitrite inhibition effects on anammox biomass.

In the CANON process, the nitrogen is removed in a one-stage PN-AMX reactor with granular biomass and in microaerophilic DO concentrations (Gonzalez-Martinez et al., 2016), saving up to 63% in aeration requirements (Liu et al., 2012). The low DO levels allow for the oxidation of ammonium to stop in nitrite, avoiding its further oxidation to nitrate. The CANON process may save up to 90% in operating costs, based on aeration, compared to conventional nitrogen removal systems (Jetten et al., 2001), and has lower N_2O emissions (Okabe et al., 2011).

In the DEMON process, the nitrogen is removed in a one-stage PN-AMX reactor by adjusting the aeration cycles with the pH: after decreasing the pH during the PN to the set-point value, the aeration is turned off to allow the deammonification, and the contrary when the pH increases during the anammox reaction (Val Del Río et al., 2016). This process provides an efficient and stable nitrite production without complex controlling strategy, enhances the anammox granulation due to the alternating aerobic conditions, and saves 25% of energy costs due to the lower aeration requirements, with an energy consumption of 1.16 kWh/kg N compared to 6.5 kWh/kg N for the conventional BNR systems (Wett, 2006).

In the ANITA Mox® process, the nitrogen is removed in a one-stage PN-AMX reactor provided with carriers (Christensson et al., 2013), either in a MBBR or IFAS configuration. In the carriers, which provide high biomass retention, the anammox bacteria are allocated in the inner layer of the biofilm, below the aerobic layer where the DO and ammonium are consumed by the AOB (Furukawa et al., 2006). This technology is used for the treatment of streams with high nitrogen loads, such as effluents from anaerobic sludge digestion processes and industrial wastewater (Gonzalez-Martinez et al., 2018).

1.7.1.2. Full-scale PN-AMX bioreactors

Different types of bioreactor configurations have been used to develop the one-stage PN-AMX processes: SBR, fluidized-bed and fixed-bed reactors, UASB anammox reactors, MBR, RBC, MBBR, FBBR and IFAS (Li et al., 2018b).

The SBR and biofilm reactors are the most widely applied ones for anammox cultivation (Boltz et al., 2017; Mao et al., 2017). They provide 75% (Strous et al., 1998) and 95% (Van Der Star et al., 2008) enrichment of flocculent/granular biomass and planktonic cells, respectively. More than 50% of all the PN-AMX full-scale systems are SBRs, followed by MBBRs (Lackner et al., 2014), in processes such as the ANAMMOX™ (Van Der Star et al., 2007) and DEMON™ (Wett, 2007).

Recently, much attention has been paid to biofilm reactors like the IFAS and MBBRs (Boltz et al., 2017). The Integrated Fixed-film Activated Sludge (IFAS) reactor combines aerated suspended activated sludge and anammox biofilm in carriers, protected from oxygen by an outside layer of AOB (Veuillet et al., 2014), while the Moving Bed Biofilm Reactor (MMBR) relies on moving carriers to attach the biofilm. The latter has been successfully applied for municipal and industrial wastewater including pulp and paper industry, poultry processing and dairy wastewater streams (Barwal and Chaudhary, 2014; Chaali et al., 2018; Szatkowska et al., 2007).

On the other hand, the sequencing batch biofilm reactor (SBBR) system combines the advantages of biofilm reactors and SBRs, offering higher biomass concentrations in the reactor, nitrogen loading rates and increased process stability towards shock loadings and biomass enrichment of slow-growing organisms like the nitrifiers (Chaali et al., 2018).

The membrane technology applied to the anammox process provide faster start-up compared to other reactor systems (Wang et al., 2009), although the membrane fouling may be even more important for anammox MBRs due to the their higher hydrophobic EPS excretions, that can aggregate onto the membrane (Eskicioglu et al., 2018; Trigo et al., 2006). Since they enable the cultivation of free-living planktonic anammox bacterial cells, the MBR technology was recently used to determine the μ_{max} of the anammox bacteria (Zhang et al., 2017a).

1.7.2. One-stage vs. two-stage configuration

For the PN-AMX processes, the one- and two-stage technologies have been proposed for coupling both processes (Connan et al., 2018). In the one-stage configuration (often named as single-stage), both AOB and anammox bacteria work in one single reactor, whereas in a two-stage system the partial nitritation is performed in the first reactor, and the effluent containing nitrite and the remaining

ammonium is subsequently fed to a second reactor where the anammox bacteria produce dinitrogen gas.

The main challenge for the PN-AMX processes in one-stage is to maintain the co-existence of AOB and anammox bacteria, which can be achieved by alternating the aeration conditions (e.g., DEMON system) or with granular/attached growth systems (e.g., CANON process, ELAN®) (Morales et al., 2015; Soliman and Eldyasti, 2018). The different SRT necessary for AOB and anammox bacteria can be achieved with granulation, carrier materials, sieves, gel entrapment (Isaka et al., 2017), sponges and filters to attach AOB and anammox, respectively (Zulkarnaini et al., 2018), or hydrocyclones to impose a shear-stress to induce the washout of unwanted bacterial groups (Seuntjens et al., 2016). Currently, more and more research studies focus on the enhancement, control and optimization of the single-stage PN-AMX process through modeling (Antwi et al., 2019; Flores-Alsina et al., 2019).

In the two-stage configuration, both bioreactors are controlled at different DO, temperature, pH and SRT conditions. In full-scale, usually the first unit is a chemostat without sludge retention (e.g., the SHARON process), while an upflow reactor or a MBBR is used for the second to decouple the HRT-SRT and maintain high anammox biomass concentrations (Soliman and Eldyasti, 2018).

After the initial application of the PN-AMX processes in a two-stage configuration (Lackner et al., 2014), the single-stage reactor became the current mainstream configuration (Ali and Okabe, 2015), and the majority of full-scale installations based on the PN-AMX processes make use of the one-stage configuration for industrial (88%) and municipal wastewater treatment (75%) (Ali and Okabe, 2015). As previously said, the DEMON, OLAND and SNAP are examples of one-stage anammox-based processes, usually applied in SBR or MBBR configuration (Jaroszynski and Oleszkiewicz, 2011). In general, the PN-AMX combined processes are more competitive in a single stage in terms of simplicity, lower capital investment and lower footprint (Ali and Okabe, 2015), while a two-reactor system has more potential for optimization (Monfet et al., 2018).

The one-stage configuration offers as advantages lower construction costs and economic space occupation (including engineering, construction and operation costs), high nitrogen load capacity and lower HRTs (Ali and Okabe, 2015; Joss et al., 2009; Mao et al., 2017). The space limitations and the economic constraints are important and determine the expansion of an existing treatment facility (Ni et al., 2017). Due to the simultaneous PN and anammox processes, there is no need to control the NO_2^--N/ NH_4^+-N ratio after the PN, and the anammox inhibition by nitrite overload is more easily avoided (Jaroszynski and Oleszkiewicz, 2011; Lackner et al., 2014). However, one-stage reactors need longer start-up periods (Lackner et al., 2014), and the complex microbial population consortia may lead to instability against loading shocks (Hao et al., 2002; Nielsen et al., 2005) and long-recovery periods after disturbances (Jaroszynski and Oleszkiewicz, 2011).

Two-stage systems are more suited to treat wastewater streams containing toxic and organic pollutants (Vázquez-Padín et al., 2009), some of which can be removed already in the PN unit (Mao et al., 2017). The removal of COD in this aerobic step, especially for higher COD/N ratios, lower the risk for HDB overgrowth in the anammox reactor (Vlaeminck et al., 2012). Furthermore, the two-stage configuration is more flexible and stable since both processes (PN and anammox) can be adjusted and controlled independently at different conditions (Veys et al., 2010). It also provides potential lower recovery periods (Jaroszynski and Oleszkiewicz, 2011). Although the two-stage configuration usually involves higher capital costs (Jaroszynski and Oleszkiewicz, 2011), these can be compensated with low operating costs when treating high ammonium-content wastewater (Mao et al., 2017).

However, the PN effluent must provide a stable composition to avoid nitrite intoxication of the anammox bacteria in the second reactor, and the high nitrite fed to the anammox must be continuously removed with thorough mixing to avoid toxic concentrations. On the other hand, two-stage PN-AMX systems generate more N_2O emissions, given the separation between both pathways and the production of N_2O during the PN when nitrite accumulates. Furthermore, anammox bacteria do not produce N_2O, (Kartal et al., 2011). Regarding the influent nitrogen load, the N_2O emissions in a two-stage PN-AMX reactor account for 2.3 - 6.6% of the removed nitrogen (Desloover et al., 2011; Kampschreur et al., 2008), compared to one-stage systems where the percentage is of 0.4 - 1.3% (Joss et al., 2009).

1.7.3. Granular vs. biofilm systems

Since the performance of the anammox reaction relies on the amount of anammox biomass, its retention inside the reactor is critical for the stable operation of the one-stage PN-AMX processes (Strous et al., 1999). For engineering applications, the enrichment of PN-AMX bacterial consortia in full-scale reactors is preferably performed with attached growth systems (biofilm or granular sludge), instead of suspended growth systems (Gustavsson, 2010; Zhang et al., 2015b), since they offer higher settling velocity of solids and biomass retention, and lower area requirements and sludge production. Furthermore, biofilms and granules are more resilient to high nitrite levels because of the protective cell layer, even in the presence of potential inhibitors like high DO concentrations (Eskicioglu et al., 2018). On the other hand, the difference in growth rates between nitrifiers and anammox bacteria (at least ten times higher for the AOB and NOB) can be evened in a stratified PN-AMX bacterial consortia in a biofilm or granular structure, which provides a natural SRT selection for both of them (Gustavsson, 2010).

A biofilm is a complex, coherent cellular structure that grows attached either to a static surface or on suspended carriers. In a heterogeneous biofilm, the organisms are distributed in a layered structure

(Soliman and Eldyasti, 2018). Several examples of carriers for biofilm growth are zeolites, AnoxKaldnes™ carriers, submerged hollow fiber membrane modules, non-woven membrane reactors and polyethelene sponge carriers (Li et al., 2018b).

The development of granular sludge in SBRs is becoming a highly promising environmental biotechnology for wastewater treatment (Boltz et al., 2017), capable of achieving higher NRRs in the case of the anammox granular sludge (Fernández et al., 2008; Tang et al., 2011). As opposed to biofilms, the granular sludge is formed when the bacterial cells self-aggregate in dense, fast-settling conglomerates with a large surface area, as a response to high velocities and shear stress conditions (Beun et al., 2000). In general, three phases are hypothesized for the self-immobilization of anammox bacteria: microbial cell cluster formation, anammox subunit formation, and final anammox granule formation (Lu et al., 2012). The EPS matrix plays a crucial role in biomass granulation, maintaining its structural integrity (Ma et al., 2016).

The PN-AMX granules provide an inner gradient distribution of DO, creating both an outer aerobic layer for AOB and an anoxic core for the anammox bacteria (Chu et al., 2015). In this way, the opposite DO demands for both bacterial groups can be satisfied in the granule itself. Besides, the nitrite gradients that develop inside the granule prevent the anammox bacteria from substrate inhibition (Van Hulle et al., 2010). Larger granules favour the anammox process and the outcompetition of NOB, while smaller granules usually have more presence of NOB (Volcke et al., 2012). Ranges for optimal granule size for maximum nitrogen removal were determined by Ni et al. (2009) (1.0 - 1.3 mm) and An et al. (2013) (1.0 - 1.5 mm).

As summarized from this Introduction chapter, the biological nitrogen removal through the PN-AMX processes is one of the most promising alternatives for its application to the treatment of industrial wastewater streams. In Chapters 3 to 6, several application examples will be analyzed in order to shed some light over the applicability of the PN-AMX processes.

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Chapter 2

Materials and Methods

Summary

In Chapter 2, the materials and methods used in the thesis are described. They comprise those used to measure the parameters to characterize the wastewater (nitrogenous compounds and organic matter, inorganic ions, metals, pH, conductivity and dissolved oxygen), the composition of the gas phase in activity batch tests, and the granular sludge.

The granular sludge was characterized by means of parameters such as the solids concentration, specific activity of both anammox and heterotrophic denitrifying biomass, and confocal microscopy with a Scan Electron Microscope (SEM).

The descriptions of the general calculations such as mass balances and nitrogen removal efficiencies are also provided, as well as calculations related to the specific activity of the biomass, and statistical analysis of the experimental data.

Finally, the Fluorescent *In Situ* Hybridization (FISH) technique, applied to the identification of the microbial populations involved in the biological processes (mainly for the nitrite oxidizing bacteria), as well as the bacterial diversity, phylogenesis and taxonomy by Illumina® analysis, are also described.

OUTLINE

2.1. Analysis of the liquid phase

2.1.1. Nitrogen compounds

All liquid samples were filtrated (0.45 μm, MF-Millipore, Millipore) before the determination of the nitrogen compounds to remove the solids, as indicated in the Standard and Methods (APHA/AWWA/WEF, 2012).

2.1.1.1. Ammonium (NH_4^+)

Ammonium was determined spectrophotometrically by an analytical method based on the production of indophenol blue by the reaction of ammonia ion with salicylate and hypochlorite in the presence of sodium nitroprusside (Bower and Holm-Hansen, 1980). This method used less toxic compounds than the phenol-hypochlorite method described in the Standard Methods (APHA/AWWA/WEF, 2012). The characteristic blue color produced makes the assay useful for the direct, visual estimation of ammonium in culture systems. The liquid samples must be filtered prior to its analysis.

Reagents preparation

- Reagent A: this solution was prepared by mixing 0.28 g/L of sodium nitroprusside ($Na_2[Fe(CN)_5NO]$) and 440 g/L of sodium salicylate ($C_7H_5NaO_3$) in distilled water.
- Reagent B: this solution was prepared by mixing 18.5 g/L of sodium hydroxide (NaOH) and 120 g/L of sodium citrate ($Na_3C_6H_5O_7$) in distilled water.
- Reagent C: this was a commercial solution of sodium hypochlorite (NaClO) with a 5% concentration (w/w).
- Reagent D: this solution was prepared by mixing 7 parts of reagent B and 1 part of reagent C. Reagent D was stable for 1 hour after preparation.

Determination procedure

Firstly, 600 μL of reagent A and 1 mL of reagent D were added to 5 mL of filtered sample. Reaction time is fixed between 2 and 3 hours. The samples must be protected from light. The measurement of the colored sample was done with a spectrophotometer (Shimadzu UV-1800) at a wavelength of 640 nm. The concentration was given by comparison of the obtained absorbance with the values of the calibration curve (Figure 2.1).

Calibration curve

Firstly, a stock solution of 10 mg NH_4^+/L was prepared. Secondly, more diluted samples were prepared so as to generate a reliable calibration curve in the range 0.000 - 0.933 mg NH_4^+-N/L. Then, the absorbance of each sample was measured with the spectrophotometer and related to the NH_4^+ concentration (in terms of NH_4^+-N/L) by means of the calibration curve (Figure 2.1).

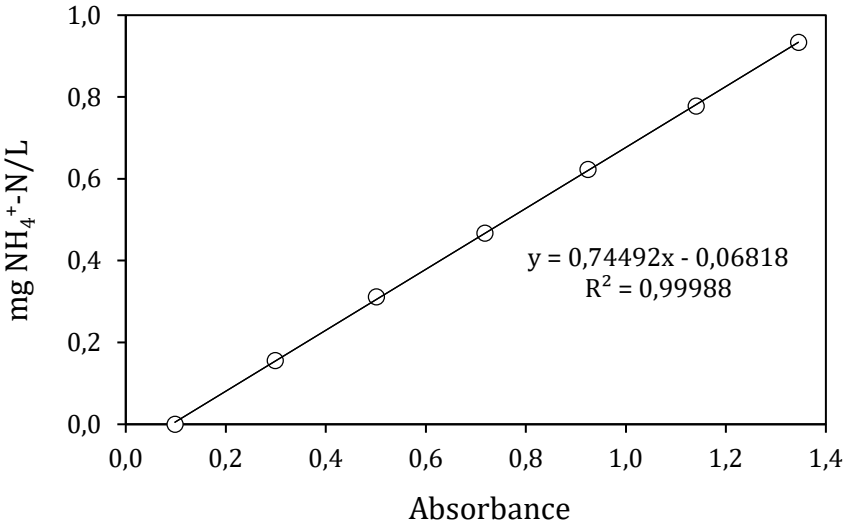

Figure 2.1. Calibration curve for the determination of the ammonium concentration.

where "x" is the absorbance of the measured sample and "y" the ammonium concentration (mg NH_4^+-N/L)

2.1.1.2. Nitrite (NO_2^-)

Nitrite was determined spectrophotometrically by a method based on the production of a reddish purple azo dye at pH 2.0 - 2.5 by coupling diazotized sulphanilamide with N-(1-napthyl)-ethylenediamine dyhydrochloride (NED dihydrochloride). This procedure corresponds to the method 4500-NO_2^--B (Colorimetric Method) described in the Standard Methods (APHA/AWWA/WEF, 2012).

Reagents preparation

- Sulphanilamide: 10 g of sulphanilamide ($C_6H_8N_2O_2S$) were dissolved in 100 mL of concentrated HCl and 600 mL of distilled water. After cooling, the volume was filled up to 1 L with distilled water.

- NED: 0.5 g of NED ($C_{10}H_7NHCH_2CH_2NH_2 \cdot 2HCl$) were dissolved in 500 mL of distilled water.

Determination procedure

A volume of 0.1 mL of each reagent was added to 5 mL of sample. The minimum reaction time for the stabilization of the colour was 20 minutes. The measurement of the colored sample was done in the spectrophotometer (Shimadzu UV-1800) at a wavelength of 543 nm. The concentration was given by comparison of the obtained absorbance with the values of the calibration curve Figure 2.2.

Calibration curve

Firstly, a stock solution of 10 mg NO_2^-/L was prepared. Secondly, more diluted samples were prepared so as to generate a reliable calibration curve, in the range of 0 - 0.25 mg NO_2^--N/L. Then, the absorbance of each sample was measured with the spectrophotometer and related to the NO_2^- concentration (in terms of mg NO_2^--N /L) by means of the calibration curve (Figure 2.2).

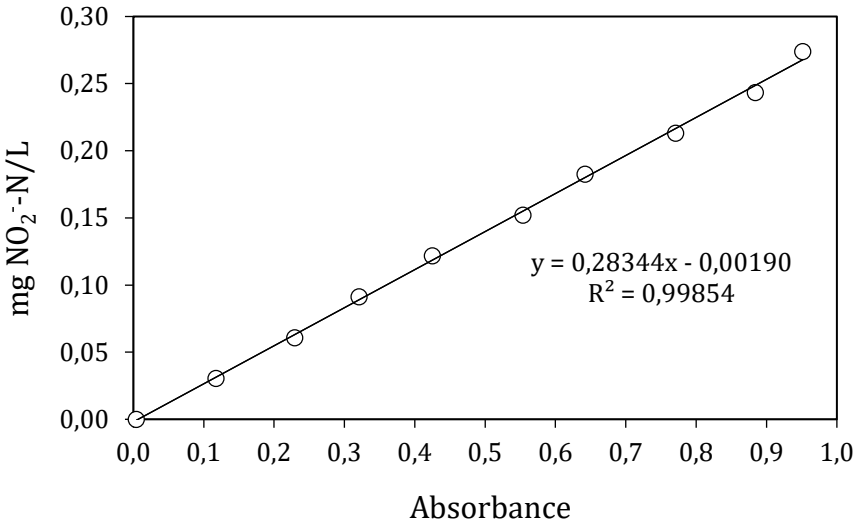

Figure 2.2. Calibration curve for the determination of the nitrite concentration

where "x" is the absorbance of the sample measured and "y" the nitrite concentration (mg NO_2^--N/L).

2.1.1.3. Nitrate (NO_3^-)

Nitrate concentration was determined by the measurement of UV absorption of the liquid sample at a wavelength 220 nm. Because dissolved organic matter may also absorb at 220 nm and NO_3^- does not absorb at 275 nm, a second measurement at 275 nm was used to correct the value of NO_3^-. This procedure corresponds to the methods 4500- NO_3^--B (Ultraviolet Spectrophotometric Screening Method) described in the Standard Methods (APHA/AWWA/WEF, 2012).

Reagents preparation

- HCl 1 N: 8.3 mL of HCl (37%, w/v, 1.19 g/L) are dissolved in 100 mL of distilled water.
- Sulfamic acid: this is a commercial chemical reagent (H_3NSO_3).

Determination procedure

A volume of 100 µL of HCl 1 N was added to 5 mL of sample. Then, the absorbance of each sample was measured at 220 and 275 nm with the spectrophotometer and the NO_3^- concentration (in terms of NO_3^--N /L) was calculated by using the calibration curve (Figure 2.3). The absorbance related to nitrate was obtained by subtracting two times the absorbance reading at 275 nm from the reading at 220 nm, following eq. 2.1:

$$\text{Absorbance } (NO_3^- - N) = Abs_{220} - 2 \cdot Abs_{275} \qquad \text{eq. 2.1}$$

Interferences

The known interferences of this method are the organic matter and the nitrite. The interference due to the presence of the organic matter in the sample is resolved by taking into account the absorbance related to nitrate at the second wavelength (275 nm), while the interference by nitrite is avoided by adding a small amount of sulfamic acid before the addition of the HCl 1 N.

Calibration curve

Firstly, a stock solution of 10 mg NO_3^-/L was prepared. Secondly, more diluted samples were prepared so as to generate a reliable calibration curve, in the range of 0 - 3 mg NO_3^--N/L. Then, the absorbance of each sample was measured with the spectrophotometer and related to the NO_3^- concentration (in terms of NO_3^--N /L) by means of the calibration curve (Figure 2.3).

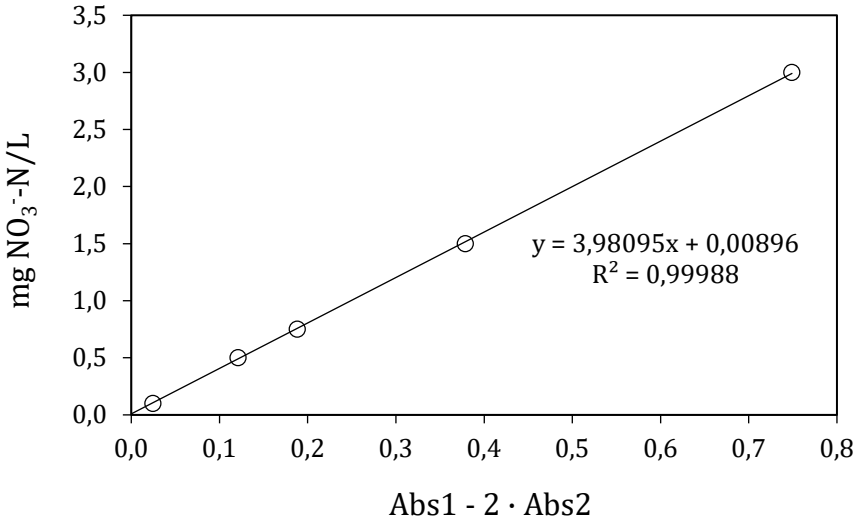

Figure 2.3. Calibration curve for the determination of nitrate

where "x" is the difference between the absorbance at 220 nm and double the absorbance at 275 nm (eq. 2.1) of the sample, and "y" the nitrate concentration (mg NO_3^--N/L).

2.1.1.4. Total nitrogen (TN)

The TN was determined in a total organic nitrogen analyzer (Shimadzu TOC-L_{CSN}) equipped with a quimio-luminiscence detector. All the nitrogen presented in the sample was catalytically oxidized to nitrous oxide (N_2O). The instrument was connected to an automated sampler (Shimadzu, ASI-L). The TN concentration was determined from the amount of N_2O produced during the combustion of the sample at 720 °C, using platinum immobilized over alumina spheres as catalyst. The N_2O produced was optically measured with the quimio-luminiscence detector after being cooled and dried. High purity air was used as carrier gas with a flow of 150 mL/min. A curve comprising at least four calibration points in the range of 0 to 0.7 g NH_4^+/L, using NH_4^+ (1000 mg/L, Merck) as standard, was used for TN quantification.

2.1.2. Carbon compounds

2.1.2.1. Total Organic Carbon (TOC)

The Total Organic Carbon (TOC) is a convenient and direct expression of total organic content. The TOC is independent of the oxidation state of the organic matter and does not measure other organically bound elements, such as nitrogen, hydrogen and inorganics that can contribute to the

oxygen demand measured by COD (APHA-AWWA-WPCF, 2012). To determine the quantity of organically bound carbon, the organic molecules must be broken down and converted to a single carbon molecular form that can be measured quantitatively. The TOC concentration was determined by a Shimadzu analyzer (TOC-L_{CSN}) as the difference between the Total Carbon (TC) and the Inorganic Carbon (IC) concentrations. The instrument was connected to an automated sampler (Shimadzu, ASI-L). The TC concentration was determined from the amount of CO_2 produced during the combustion of the sample at 720 °C, using platinum immobilized over alumina spheres as catalyst. The IC concentration was obtained from the CO_2 produced in the chemical decomposition of the sample with HCl (1 M) at room temperature. The CO_2 produced was optically measured with a non-dispersive infrared analyzer (NDIR) after being cooled and dried. High purity air was used as carrier gas with a flow of 150 mL/min. A curve comprising at least four calibration points in the range of 0 to 1 g C/L, using potassium phthalate ($C_8H_5KO_4$) as standard for TC and a mixture of sodium carbonate and bicarbonate (Na_2CO_3/$NaHCO_3$, 3:4 w/w) for IC, was used for quantification.

2.1.2.2. Chemical Oxygen Demand (COD)

The Chemical Oxygen Demand (COD) is defined as the amount of oxygen required to oxidize the organic matter by the use of a strong chemical oxidant (potassium dichromate) in an acid medium. A catalyst (silver sulphate) was used to improve the oxidation of some organic compounds. After digestion, the remaining amount of unreduced $K_2Cr_2O_7$ was titrated with ferrous ammonium sulphate to determine the amount of $K_2Cr_2O_7$ consumed, being the amount of oxidized organic matter calculated in terms of oxygen equivalents. The main reaction follows eq. 2.2:

$$Cr_2O_7^{2-} + \text{Organic matter} + H^+ \Leftrightarrow Cr_3^+ + CO_2 + H_2O \qquad \text{eq. 2.2}$$

The concentrations of total and soluble Chemical Oxygen Demand (tCOD and sCOD, respectively) were determined following the method 5220C of the Standard Methods (APHA-AWWA-WPCD, 2012). The tCOD was determined using the raw sample and the sCOD using the sample previously filtered through a pore size filter of 0.45 μm (MF-Millipore, Millipore).

Reagents preparation

- Standard potassium dichromate digestion solution: 10.216 g of $K_2Cr_2O_7$ and 33 g of $HgSO_4$ were dissolved in 500 mL of distilled water. Then, 167 mL of concentrated H_2SO_4 were added. Afterwards, the solution was cooled at room temperature and finally diluted to 1000 mL.

- Sulphuric acid reagent: 10.7 g of Ag_2SO_4 were added to 1 L of concentrated H_2SO_4. The solution can be used after 48 hours.

- Ferroin indicator solution: 1.485 g of $C_{18}H_8N_2 \cdot H_2O$ (phenanthroline monohydrate) and 0.695 g of $SO_4Fe \cdot 7\ H_2O$ were dissolved in 100 mL of distilled water.

- Potassium dichromate solution 0.05 N: An amount of 1.226 g of $K_2Cr_2O_7$, previously dried at 105 °C for 2 hours, was dissolved in 500 mL of distilled water.

- Ferrous ammonium sulphate titrant (FAS) 0.035 N: 13.72 g of $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$ were dissolved in distilled water. Then, 20 mL of concentrated H_2SO_4 were added and the solution was finally cooled and diluted to 1000 mL.

Determination procedure

The procedure is applicable to samples with COD concentrations between 90 - 900 mg/L. Volumes of 2.5 mL of each sample were placed in 10-mL Pyrex® glass tubes. After that, 1.5 mL of digestion solution, as well as 3.5 mL of sulphuric acid reagent, were added slowly on the wall of the Pyrex® tube, slightly tilter in order to avoid mixing. A blank sample using distilled water (2.5 mL) was prepared in the same way. This blank sample acted as "reference", providing the COD content of the distilled water, which is supposed to be negligible. After being sealed with Teflon and tightly capped, the Pyrex® tubes were finally mixed completely and placed in the block digester (ECO 16, VELP SCIENTIFICA), preheated to 150 °C. The duration of the digestion period was 2 h. After digestion, the Pyrex® tubes were cooled to room temperature. Then, the content of the tubes was transferred to a beaker and, after addition of 1 - 2 drops of ferroin indicator, the solution was titrated under rapid stirring with the FAS.

The FAS solution was standardized daily as follow: 5 mL of distilled water and 3.5 mL of sulphuric acid were added together in a small beaker. The mixture was cooled to room temperature and 5 mL of potassium dichromate solution (0.05 N) were added. Finally, 1 - 2 drops of ferroin indicator were added and this mixture was titrated with FAS titrant. The end-point corresponded to a colour change

from blue-green to reddish brown. The molarity of the FAS solution and the COD concentration of the samples were calculated with eq. 2.3 and eq. 2.4:

$$M_{FAS} = \frac{5 \times 0.05}{V_{FAS}} \qquad \text{eq. 2.3}$$

$$COD = \frac{(A - B) \times M_{FAS} \times 8000}{V} \qquad \text{eq. 2.4}$$

Where M_{FAS} is the molarity of the FAS solution (mol/L), V_{FAS} is the volume of FAS solution consumed in the titration (mL), COD is the Chemical Oxygen Demand concentration (mg/L), A is the volume of FAS solution consumed by the blank (mL), B is the volume of FAS solution consumed by the sample (mL), V is the volume of sample used before dilution, and 8000 (mg COD/mol FAS) is the conversion factor for the FAS.

Interferences

The most common interference of this method is the chloride ion at concentrations higher than 2 g/L. Chloride reacts with silver ion to precipitate silver chloride, and thus inhibits the catalytic activity of silver. Bromide and Iodide can interfere similarly.

2.1.3. Inorganic ions

The anions nitrite (NO_2^-), nitrate (NO_3^-), chloride (Cl^-), bromide (Br^-), phosphate (PO_4^{3-}), sulphate (SO_4^{2-}), and the cations lithium (Li^+), sodium (Na^+), ammonium (NH_4^+), potassium (K^+), magnesium (Mg^{2+}) and calcium (Ca^{2+}) were determined by ion chromatography (IC) with an Advanced Compact IC system (861, Metrohm), equipped with a CO_2 suppressor (MCS 853, Metrohm) and a sample processor/injector (838, Metrohm). Anions were determined with a Metrosep A column Supp 5 (250 × 4.0 mm) and a mobile phase (buffer) with 3.2 mM Na_2CO_3 and 1.0 mM $NaHCO_3$ at a flow rate of 0.7 mL/min. Cations were determined with a column (250 × 4.0 mm) (Metrosep C3, Metrohm) and nitric acid 3.5 mM as mobile phase at a flow rate of 1 mL/min. The injection volume of the sample was 20 µL and data collection was done by using the Processor software IC Net 2.3.

Reagents

- Mobile phase for anions: Na_2CO_3 3.2 mM (339.2 mg Na_2CO_3 in 100 mL of deionized water) and $NaHCO_3$ 1.0 mM (84 mg $NaHCO_3$ in 1000 mL of deionized water).

- Mobile phase for cations: Nitric acid 3.5 mM (0.243 mL of nitric acid 65% in 1000 mL of deionized water).

- Standard commercial solutions for anions and cations, with commercial stock concentrations of 1 g/L for all the corresponding solutions.

Determination Procedure

Table 2.1 shows the calibration ranges for the different inorganic ion concentrations, which determined the proper dilutions with distilled water of the samples in order to fit to these ranges.

Table 2.1. Calibration ranges for the different inorganic ions (mg/L)

Anions	Lower value	Higher value	Cation	Lower value	Higher value
Cl^-	1.0	100	Li^+	0.05	5
NO_2^-	0.05	5	Na^+	1.5	150
NO_3^-	0.5	50	NH_4^+	0.1	10
Br^-	0.2	20	K^+	0.5	50
PO_4^{3-}	0.5	50	Mg^{2+}	0.5	50
SO_4^{2-}	1.5	150	Ca^{2+}	0.5	50

2.1.4. Metals

The concentration of metals was determined by Inductively-Coupled Plasma - Mass Spectrometry (ICP-MS). The concentration of total metals in the liquid phase was determined from 10 mL of non-filtered sample, whereas the concentration of dissolved metals was determined from 10 mL of filtered sample (0.45 μm). The difference between both measurements provided the concentration of metals in suspension. Before the analysis, the non-filtered samples were digested 20 min at 165 °C in a microwave with high-pressure closed vessel (Millestone, Ethos-1) with 5 mL of HNO_3 and 2 mL of H_2O_2, rinsing with MilliQ water up to 50 mL. Regarding the measurement of metals adsorbed on the biomass, the granular sludge samples were digested 20 min at 190 °C in a microwave with high-pressure closed vessel (Millestone, Ethos-1) with 8 mL of HNO_3 and 2 mL of H_2O_2, rinsing with MilliQ water up to 50 mL. The liofilisation of the sample provided the dry residue content.

In the ICP-MS equipment (Agilent 7700x), the liquid sample is first injected in a Peltier-type refrigerated spray chamber, where it is converted to spray gas by means of a microconcentric nebulizer. The ions from the plasma further generated from the spray gas are detected in a quadrupole mass analyzer and an ORS (octopole reaction system), where the different ions from each isotope are separated according to their mass. The results constitute the average of three consecutive measurements, with a sensitivity in the order of ng/L for most of the elements. The working conditions for the ICP-MS are summarized in Table 2.2.

Table 2.2. Working conditions for the ICP-AMS Agilent 7700x

Parameter	Units	Value	Parameter	Units	Value
Plasma parameters			Ion lenses		
RF Power	W	1550	Extract 1	v	0
Sample depth	mm	8	Extract 2	v	-140
Carrier gas	L/min	1.1	Omega bias	v	-100
Nebulizer pump	rps	0.1	Omega lens	v	11.6
S/C Temperature	°C	2	Cell entrance	v	-40
Reaction Cell			Cell exit	v	-60
He Gas	mL/min	3.6	Deflect	v	4.8
Detector parameters			Plate bias	v	-60
Discriminator	mv	4.5	QP bias	v	-15
Analog HV	v	1746	*Octopole parameters*		
Pulse HV	v	1035	OctP RF	v	160
			OctP bias	v	-18

HV: high voltage, OctP RF: octopole radiofrequency, QP: quadrupole, RF: radiofrequency, rps: revolutions per second, S/C Temp.: temperature of spray chamber, v: volts.

A curve comprising four calibration points for each metal, using different concentration ranges of a reference metal for each metal (Table 2.3), was used for quantification.

Table 2.3. Calibration ranges for the different reference metals determined (μg/L)

Ref. metal	Conc. range	R calibr. curve	Ref. metal	Conc. range	R calibr. curve	Ref. metal	Conc. range	R calibr. curve
^{9}Be	0 - 10	0.9999	^{56}Fe	0 - 10000	0.9999	^{78}Se	0 - 100	0.9999
^{27}Al	0 - 10000	0.9999	^{59}Co	0 - 100	0.9992	^{95}Mo	0 - 100	0.9994
^{47}Ti	0 - 100	0.9992	^{60}Ni	0 - 1000	0.9991	^{107}Ag	0 - 100	0.9983
^{51}V	0 - 100	0.9999	^{63}Cu	0 - 1000	0.9997	^{111}Cd	0 - 100	0.9989
^{52}Cr	0 - 500	0.9998	^{66}Zn	0 - 10000	0.9998	^{202}Hg	0 - 100	1.0000
^{55}Mn	0 - 10000	0.9999	^{75}As	0 - 100	0.9989	^{208}Pb	0 - 100	0.9994

2.1.5. COD fractionation

The total COD of the wastewater (tCOD) was fractionated in the two major divisions: total biodegradable COD (bCOD) and total inert COD (iCOD), and the tbCOD was further classified into the soluble readily biodegradable COD (S_b) and the particulated slowly biodegradable COD (X_b) fractions, whereas the iCOD was subdivided into soluble inert COD (S_i) and particulated inert COD (X_i). The tbCOD and S_b fractions were experimentally determined through independent respirometric bath assays from the profiles of consumed (dissolved) oxygen (CO), in a respirometer (Surcis BM-T Plus 151204). Table 2.4 summarizes the calculations for the estimation of S_b, which was the main fraction considered for the discussion of the experimental results in Chapter 6 with respect to the concentration of soluble COD (sCOD) in the wastewater.

Table 2.4. Calculations for the different COD fractions of the wastewater

Total COD calculations (raw WW)	Soluble COD calculations (filtered WW)
$bCOD = \frac{CO}{1-Y}$	$S_b = \frac{CO}{1-Y}$
$X_b = bCOD - S_b$	$S_i = sCOD - S_b$
$iCOD = tCOD - bCOD$	$X_i = iCOD - S_i$
$tCOD = bCOD + iCOD$	$sCOD = S_b + S_i$
$Y = 1 - \frac{CO}{COD_{ac}}$	

bCOD: (total) biodegradable COD, CO: consumed (dissolved) oxygen, iCOD: inert COD, S_b: soluble readily biodegradable COD, S_i: soluble inert COD, sCOD: soluble COD, tCOD: total COD, X_b: particulated slowly biodegradable COD, X_i: particulated inert COD, Y: heterotrophic growth yield.

The respirometric batch assays were performed in triplicate in a completely mixed system with aeration and recirculation of the liquid medium. The temperature was controlled at 25 °C with a thermostatic bath. The batch assays were carried out with 1 L of activated sludge for the endogenous respiration phase, with a concentration of volatile suspended solids (VSS) between 1.5 and 5.0 g VSS/L for the estimation of bCOD, and between 2.0 and 5.0 g VSS/L for the estimation of S_b. In order to inhibit the nitrification and the related consumption of oxygen, a concentration of allylthiourea of 2 - 3 mg ATU/L was added 30 min before the start-up of the assays.

For bCOD estimation, the raw wastewater was added as substrate, while for S_b the wastewater samples were previously centrifuged at 7500 rpm (10 min, Eppendorf 5430) and filtered at 45 µm. A volume of wastewater of 20 mL was added, according to the manufacturer specifications for substrate addition as a function of the COD concentrations. The batch assays were finished when the oxygen consumption rate decreased and was maintained at zero.

The heterotrophic growth yield (Y), expressed as g COD/g COD, was estimated in the same operational conditions as in the batch assays with sodium acetate (0.4 g/L) as substrate and a 50-mL sample volume. The Y values were calculated as showed in Table 2.4, considering the consumption of oxygen (CO) during the assay and the COD of the acetate solution (COD_{ac}) added as source of organic matter.

2.1.6. Other control parameters

2.1.6.1. pH

The pH is one of the key parameters measured in wastewater biological treatment systems, since its control is important to maintain the activity of the microorganisms involved in the different treatment processes. The pH measurements were performed with an electrode (Hamilton Flushtrode) connected to a pH-meter (GLP-22, Crison Instruments). The sensitivity of the instrument is ± 1 mV, corresponding to 0.01 pH units. The electrode was calibrated at room temperature with two standard buffer solutions of pH 7.02 and 4.00, respectively.

2.1.6.2. Conductivity

The conductivity measurements, expressed in mS/cm, were performed with an electrode (Hach Lange 50 60, Platinum Cell) connected to a portable conductivity meter (Hach Lange sensION™ + EC 5). The electrode can be calibrated at room temperature with one of these three standard buffer solutions: 147 µS/cm, 1413 µS/cm and 12.88 mS/cm. Due to the high conductivities of the tested

industrial wastewater streams, the latter (12.88 mS/cm) was selected as buffer solution for the calibration of the electrode.

2.1.6.3. Dissolved Oxygen (DO)

A dissolved oxygen portable meter (Hach Lange, model HQ40d) with a membrane covered galvanic dissolved oxygen sensor (LDO101 probe) was used to measure the DO concentration in the liquid media inside the operated laboratory scale reactors.

2.2. Analysis of the gas phase

The composition of the gas phase was analyzed at the end of the SAA (specific anammox activity) and SDA (specific denitrifying activity) batch tests (see Section 2.3.2). The knowledge about the composition of the gas phase, specially the proportions of N_2 and CO_2, allows correcting the experimental SAA and SDA values, considering the main gaseous products of the anammox process (N_2) and heterotrophic denitrifying process (N_2 and CO_2). The calculations based on the gas composition are further discussed in Section 2.5.4.

The gas composition (N_2, CH_4, CO_2, N_2O and H_2S) was determined by gas chromatography (HP, 5890 Series II) equipped with a Thermal Conductivity Detector (TCD). The stainless steel column was 2 m long with an external diameter of 1/8" and it was filled with Porapack Q (mesh 80/100). The temperatures of the injector, column and detector were 110, 35 and 110 °C, respectively. Helium was used as carrier gas with a flow of 15 mL/min. The sample volume (1 mL) was injected through a septum into the entrance of the instrument. The calibration was performed with a standard mixture of gases (CH_4: 66%, CO_2: 30%; N_2: 2% and H_2S: 2%) by a response factor method, using the CO_2 as reference gas. A typical gas chromatogram is depicted in Figure 2.4.

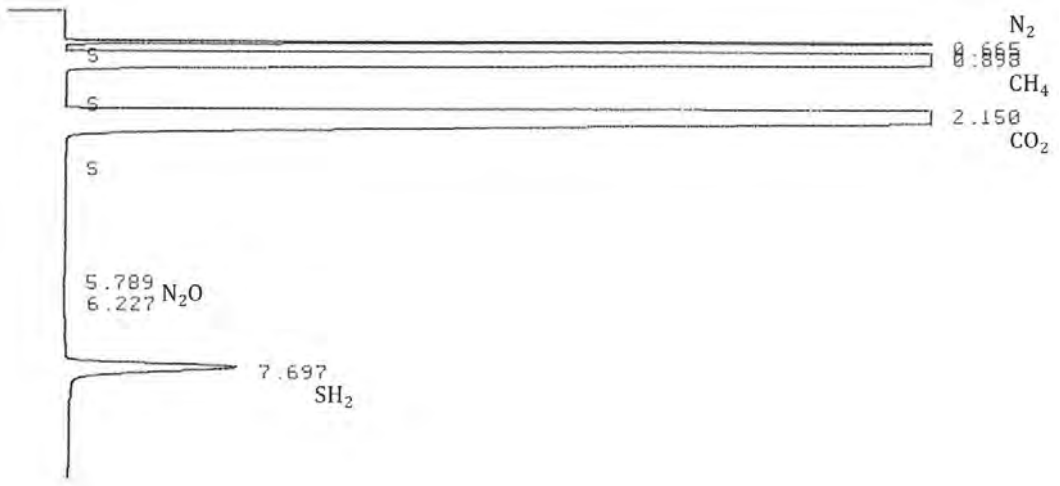

Figure 2.4: Typical gas chromatogram showing the complete composition of the gas phase. The numbers indicate the elution time for each compound.

From the chromatograms like the one in Figure 2.4, the molar fractions for nitrogen (N_2) and carbon dioxide (CO_2) can be calculated to estimate the specific anammox and denitrifying activity (Section 2.5.4.1).

2.3. Biomass Characterization

2.3.1. Solids

Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) were determined following the methods 2540D and 2540E, respectively, described in the Standard Methods (APHA/AWWA/WEF, 2012).

Determination procedure

For the determination of TSS, a selected well-mixed sample volume (in order to yield a residue between 2.5 and 200 mg) was filtered through a weighed glass fiber filter (Whatman, GF/C, 4.7 cm of diameter, 1.2 µm of pore size) and the residue retained on the filter was dried to a constant weight (approximately during 2 h) at 103 - 105 °C until achieving a constant weight. The increase in weight of the filter represents the TSS content of the sample.

To determine the VSS, the residues from method 2540D (APHA/AWWA/WEF, 2012) were burnt to achieve constant weight at 550 °C during half an hour. The weight lost during ignition corresponds to the volatile fraction, since only a small amount of inorganic salts is decomposed and volatilized at

that temperature. This determination offers a rough approximation of the amount of organic matter present in the solid fraction of the wastewater and the sludge.

2.3.2. Specific activity of the biomass

2.3.2.1. Specific Anammox Activity (SAA)

The batch assays used to estimate the specific anammox activity (SAA) of the biomass were performed according to the methodology described by Dapena-Mora et al. (2007). This methodology is based on the measurement throughout time of the overpressure generated by the nitrogen gas produced in completely closed vials with a total volume of 38 mL and a liquid volume of 25 mL. The overpressure in the headspace was measured using a differential pressure transducer 0 - 5 psi, linearity 0.5% of full-scale manufactured by Centerpoint Electronics. The biomass concentration inside the vials was fixed at the beginning of the experiment at approximately 2 - 3 g VSS/L, although higher values were fixed when the SAA was expected to be lower.

Before the beginning of the batch test, the biomass was washed between three and six times with different liquid media depending on the desired activity to be measured:

- Maximum SAA (SAA_{PBS}): phosphate buffer (0.143 g KH_2PO_4/L and 0.747 g K_2HPO_4).
- SAA with industrial wastewater (SAA_{WW}) (Chapters 4 and 5): undiluted pre-digested pig manure and OFMSW liquor, respectively.

The washing step was normally performed by adding the phosphate buffer to the granular biomass, shaking the mixture and allowing the biomass to settle, then withdrawing the supernatant and adding again phosphate buffer. When the granular biomass settle slowly or presented a high flocculent fraction, mild centrifugation steps (5 min, 3000 rpm) were performed instead of settling by gravity to ease the washing steps.

After filling the vials up to 25 mL with the washed biomass, the gas and liquid phases were purged with inert gas (helium) to remove the oxygen from the bulk liquid and headspace, with a minimum purging time of 5 min per vial. The vials were subsequently placed in a thermostatic shaker (HWY-200D, Labolan Technics), fixing the agitation speed at 250 rpm and the temperature to 30 °C. The biomass in the vials was acclimated for at least 2 hours before the batch test began.

Similarly to the washing of the biomass, the initial concentrations of substrates at the beginning of the batch test were fixed depending on the desired activity to be measured:

- Maximum SAA (SAA_{PBS}): 70 mg NH_4^+-N/L and 70 mg NO_2^--N/L (Dapena-Mora et al., 2007).
- SAA with industrial wastewater (SAA_{WW}) (Chapters 4 and 5): 70 mg NO_2^--N/L; no ammonium was added since it was already in excess in the industrial wastewater.

The production of dinitrogen gas (N_2) was determined in the gas phase as the increment of pressure in the headspace of the vials, measured by means of the pressure transducer device. The calculations for the estimation of the SAA values are further detailed in Section 2.5.4.2.

2.3.2.2. Specific Denitrifying Activity (SDA)

The batch assays used to estimate the specific denitrifying activity (SDA) of the biomass were performed according to the methodology described by Buys et al. (2000). This methodology is based on the measurement throughout time of the overpressure generated by the nitrogen gas produced in completely closed vials, although the total and liquid volumes were the same as those used in the SAA bath tests (38 and 25 mL, respectively). The overpressure in the headspace was measured using a differential pressure transducer 0 - 5 psi, linearity 0.5% of full-scale manufactured by Centerpoint Electronics. The biomass concentration inside the vials was fixed at the beginning of the experiment at approximately 3 - 4 g VSS/L, although this value was higher when the SDA was expected to be lower, and usually higher compared to the concentrations in the SAA batch tests due to the comparatively lower SDA activity of the biomass samples.

Before the beginning of the batch test, the biomass was washed between three and six times with different liquid media depending on the desired activity to be measured:

- Maximum SDA (SDA_{PBS}): phosphate buffer (0.143 g KH_2PO_4/L and 0.747 g K_2HPO_4).
- SDA with industrial wastewater (SDA_{WW}) (Chapter 5): undiluted pre-digested OFMSW liquor.

The washing and purging steps were the same as in the case of the SAA batch tests. Regarding the initial concentrations of substrates at the beginning of the batch test, these were also fixed depending on the desired activity to be measured. Both nitrite and nitrate were used in different batch tests to assess the denitritation and denitratation capacity (SDA_{NO2} and SDA_{NO3}, respectively). A minimum substrate ratio of 3.5 g COD/g N was ensured in the liquid medium, according to Buys et al. (2000):

- $SDA_{NO2\text{-}PBS}$: 100 mg NO_2^--N/L and 350 mg COD/L (as $NaHCO_3$)
- $SDA_{NO3\text{-}PBS}$: 100 mg NO_3^--N/L and 350 mg COD/L (as $NaHCO_3$)
- $SDA_{NO2\text{-}WW}$ (Chapter 5): 100 mg NO_2^--N/L; no organic matter was added since it was already in excess in the industrial wastewater
- $SDA_{NO3\text{-}WW}$ (Chapter 5): 100 mg NO_3^--N/L; no organic matter was added since it was already in excess in the industrial wastewater

The production of biogas (N_2 + CO_2) was determined in the gas phase as the increment of pressure in the headspace of the vials, measured by means of the pressure transducer device. The calculations for the estimation of the SDA values are further detailed in Section 2.5.4.2.

2.3.3. Scanning Electron Microscopy - Energy Dispersive X-ray spectroscopy (SEM - EDX)

Morphological studies of the biomass were performed with a scan electron microscope controlled with a computer system and with a magnification capacity ranging from 15 to 290,000 folds. The sludge sample was washed three times for 10 minutes with phosphate buffer 0.05 N at pH 7.4 and subsequently fixed with a solution of glutaraldehyde 3% in phosphate buffer overnight. After fixation, the sample was dehydrated using ethanol solutions with increasing ethanol concentrations (30, 50, 70 and 100%). On the other hand, sludge samples were also prepared for SEM - EDX analysis by simple overnight dehydration at room temperature, with no washing steps or further reagents addition.

To investigate the elemental composition of the granular biomass samples, a micro-analysis was carried out. The instrument used to analyze the samples was the SEM EVO LS15 (Zeiss) with a system of micro-analysis (EDX) at a voltage of 20 kV. Images of the analyzed surface were taken with a BSD (backscatter electron detector) / SE (secondary electrons) detector, in conditions of variable pressure. The samples were shaded with iridium (10 nm thickness) prior to the observation under the SEM for a better quality signal. On the other hand, the elemental composition of the granular surface was determined with the software-controlled EDX detector (INCA-X act, OXFORD). A working distance of 8.5 mm at 20 kV were selected as optimal conditions for a more precise analysis. A typical count of the different atoms detected in a sample is represented in Figure 2.5:

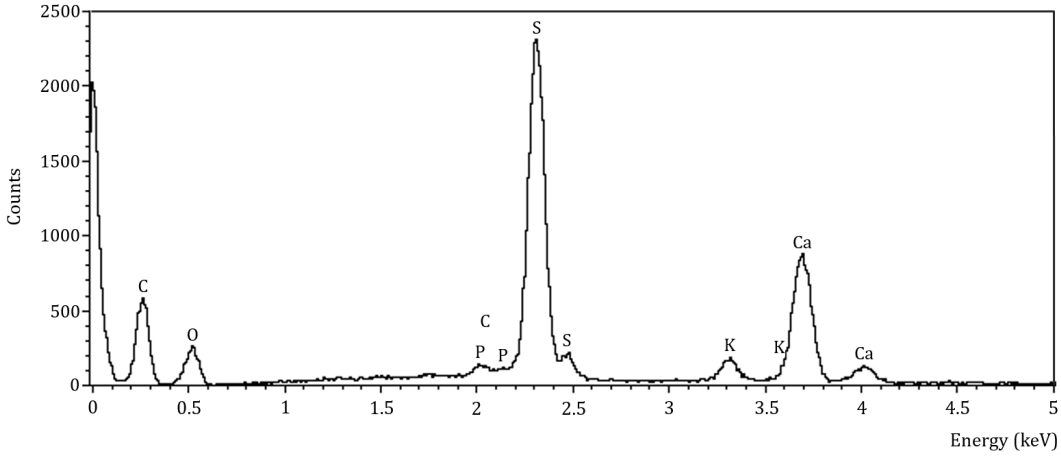

Figure 2.5: Representation of the different atoms present in a sample of granular biomass detected in a micro-analysis

2.4. Microbiological analyses

2.4.1. Identification of bacterial populations by Fluorescence *In Situ* Hybridization (FISH)

The FISH technique allows the identification of microorganisms at any desired taxonomical level, just depending on the specificity of the used probe. Nowadays, it is a semi-quantitative molecular technique commonly used to follow microbial populations in biomass samples. In Chapter 4, only the identification (not the quantification) of the bacterial populations was performed.

The FISH technique uses fluorescent labelled probes to detect specific region 16S rRNA. The probes hybridize with the targeted sequence of any desired taxonomical level (domain, phylum, genus or specie) to be later detected microscopically. The protocol includes four steps (Amann et al., 1995): (1) fixation and permeabilization of the sample; (2) hybridization of the targeted sequence to the probe; (3) washing steps to remove the unbound probe; and (4) detection of labelled cells by microscopy.

Reagents preparation

- PBS (3x): 0.49 g of KH_2PO_4 were dissolved in 80 mL of distilled water. Then, 2.3 g of NaCl were added and the pH value was adjusted to 4.2. Finally, the volume was filled up to 100 mL. PBS (1x) is prepared by 1:3 diluting the PBS (3x) with distilled water.

- Fixative solution: 6.5 mL of distilled water were heated to 60 °C and 0.4 g of paraformaldehyde are added. Then, one drop of NaOH 1 M was added while the solution is vigorously shaken for 1 - 2 minutes until its complete solubilisation. 3.3 mL of PBS (3x) are added and the pH value is adjusted to 7.2 with HCl (about one drop of HCl 1 M). Finally, the solution was filtered through a 0.2 µm membrane filter.
- Hybridization buffer: 360 µL of 5 M NaCl solution and 40 µL of 1 M Tris/HCl (pH 8.0) were mixed in a 2 mL Eppendorf tube. The amount of formamide (vol%) for the hybridization buffer was selected depending on the probe used (Table 2.5). Finally, 4 µL of sodiumdodecylsulphate 10% (w/v) were added to the mixture.

Table 2.5: Formamide and water addition to the hybridization buffer prepared at different concentrations.

Formamide (vol%)	Formamide (µL)	MilliQ (µL)
0	0	1600
5	100	1500
10	200	1400
15	300	1300
20	400	1200
25	500	1100
30	600	1000
35	700	900
40	800	800
45	900	700
50	1000	600
55	1100	500
60	1200	400

- Washing buffer: 1 mL of Tris/HCl (pH 8.0), and the required volume of 5 M NaCl and 0.5 M EDTA (pH 8.0), depending on the percentage of formamide used with the applied probe (Table 2.6), were mixed in a 50 mL Falcon tube. The tube was filled up to 50 mL with distilled water and preheated at 48 °C prior to its use.

Table 2.6: NaCl and EDTA addition to the washing buffer.

Formamide (vol%)	5 M NaCl (µL)	0.5 M EDTA (µL)
0	9000	-
5	6300	-
10	4500	-
15	3180	-
20	2250	500
25	1590	500
30	1120	500
35	800	500
40	560	500
45	400	500
50	280	500
55	200	500
60	80	500

Procedure

1. Fixation of cells. Biomass was washed in PBS (1x), then three volumes of fixative solution were added to one volume of suspension. The solution was kept in ice for 2 h. After this time, it was washed again with PBS (1x) and the cells were resuspended in PBS (1x). Iced 98% ethanol was added to the biomass suspension in a ratio 1.25:1 in volume. Samples were stored at -20 °C.

2. Immobilization of cells on microscope slides. After fixation, the suspension in ethanol of the fixed biomass was spread in each well of a coated Teflon/glass microscope slide (5 - 10 µL). The slide was dried at 46 °C for 10 min. Then, the cells were dehydrated by successively rinsing the slides 3 times for 3 minutes with 50%, 80% and 98% ethanol and dried with air.

3. Hybridization. The hybridization buffer was prepared and kept at room temperature. The hybridization tube was prepared by placing a folded tissue inside a 50 mL Falcon tube. Part of the hybridization buffer (10 µL) was pipetted into the wells of the slides with the biomass and the rest was poured onto the tissue inside the Falcon tube. The FISH probe was added to the wells of the slides (1 µL of stock solution with a final concentration of 30 ng/µL for Cy3 and Cy5-labelled probes and 50 ng/µL for FITC labelled probes). Then, each slide was placed inside the hybridization tube and incubated for 1.5 h at 46 °C. In the meantime, the washing buffer was prepared and preheated in a water bath at 48 °C.

4. Washing. This step should be performed rapidly. The slide was transferred into the Falcon tube containing the washing buffer and incubated for 15 minutes at 48 °C. Then, the slide was transferred from the washing buffer to cold distilled water for few seconds and dried with air.

5. Microscopy and image acquisition. The targeted organisms can be detected by the characteristic fluorescence of the dye (fluorochrome) attached to the probe. The fluorochromes used to detect the hybridized rRNA were FLUOS (5(6)-carboxyfluorescein- N-hydroxysuccinimide ester) and Cy3 (indo-carbocyanine). The stain DAPI (4,6-diamino-2-phenylindole) was used for the visualization of all the cells present in the sample. Slide wells were embedded with Vectashield H-1200, which amplifies the fluorescence, avoids fading and contains DAPI dye, and the cover slip is placed on the slide. For the analysis of the slides, an epifluorescence microscope (Axioskop 2 plus, Carl-Zeiss, USA) in combination with a digital camera (Coolsnap, Roper Scientific Photometrics, USA) was used. An acquisition software RSI image v 1.7.3 (Roper Scientific Photometrics, USA) was used to collect and process the images taken from the analyzed samples.

The FISH technique was applied to characterize the microbial populations in Chapter 4, where the probes specified in Table 2.7 were used:

Table 2.7: Probes used for FISH and the formamide (FA) concentration used during hybridization.

Probe	Target site 16S	Probe sequence (5' → 3')	% FA	Target organisms	Ref. [a]
EUB 338	338-355	GCT GCC TCC CGT AGG AGT	0 - 50	Bacteria domain	[1]
Ntspa712 Competitor	712-732	CGC CTT CGC CAC CGG CCT TCC CGC CTT CGC CAC CGG GTT CC	35	Most members of phylum *Nitrospira*	[2]

[1] Amann et al. (1990); [2] Daims et al. (2001)

2.4.2. Bacterial diversity, phylogenesis and taxonomy

The different procedures followed in Chapter 3 and 5 to determine the bacterial diversity, phylogenetic variations, as well as the bacterial taxonomy and evolution, are described below.

Chapter 3 (fish canning industry wastewater)

Total genomic DNA was extracted using the phenol-chloroform protocol (Alonso-Gutiérrez et al., 2009), quantified in a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and checked for size and integrity by standard electrophoresis. To prepare the 16S rRNA gene amplicon libraries, the V4 hypervariable region of the 16S rRNA bacterial gene was amplified using the 515F-806R primer pair (Gilbert et al., 2010), as described previously by Regueiro et al. (2014). DNA libraries were checked for size quality and integrity using a Bioanalyzer (Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA).

After determining DNA concentrations by quantitative PCR, libraries were pooled in equimolar amounts and sequenced at the genomics unit of the Parque Científico de Madrid (Spain) on an Illumina MiSeq System (Illumina) using MiSeq Reagent Kit v3 (Illumina). Initial sequence quality-filtering and analysis was performed as previously described Regueiro et al. (2014). To determine the alpha diversity of the bacterial community, observed species, Simpson, Chao1, Shannon and Simpson evenness indices were calculated using 100 rarefactions of 116,728 sequences in QIIME v1.9.1 (quantitative insight into microbial ecology) (Caporaso et al., 2010).

Chapter 5 (Organic Fraction of Municipal Solid Waste (OFMSW)

Total genomic DNA was extracted using the Stool DNA Isolation KIT (Norgen. Thorold, Canada). Total DNA concentrations were quantified in a Qubit fluorometer (Thermo Fisher Scientific Waltham, MA, USA) and checked for size integrity by standard electrophoresis. The V3V4 hypervariable region of the 16S rRNA gene was amplified for *Bacteria* with the primer pair S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A (Klindworth et al., 2013). Then, samples were analyzed by Illumina following the procedure described in Braz et al. (2018).

Raw sequences were filtered to remove low-quality reads and then clustered into Operational Taxonomic Units (OTUs) at 97% cutoff for sequence similarity using the QIIME v.1.9.1 pipeline (Caporaso et al., 2011). Bioinformatic assays were performed to the most abundant bacterial OTUs (above 0.5% of the total observed OTUs) with the software R (version 3.5.1). Community structure variations were visualized using the principal component analysis (PCoA). Distance-based constrained redundancy analysis (db-RDA) was applied to find the operational parameters that better explained the diversity observed in the unconstrained PCoA, based on the pairwise weighted UniFrac distances matrix. Both were accomplished using the software R (version 2.13.1), via the capscale function in vegan package (Oksanen et al., 2007). Conductivity, sCOD/N ratio, and NaCl, sCOD and PO_4^{3-} concentrations in the influent were the analyzed parameters.

Analysis of Variance (ANOVA) was used to determine if each constraining parameter added a significant amount of information to the constrained model ($p < 0.05$) (Agler et al., 2012). The Variance Inflation Factors (VIF) were studied to evaluate if the constraints were redundant. Non-significant or redundant parameters were discarded until all the constraining variables in the db-RDA were significant ($p > 0.05$) and non-redundant (VIF score < 10) (Regueiro et al., 2015). The dendrograms for the samples of each reactor were built with the cluster.datasets package in R.

2.5. Calculations

The calculations regarding the nitrogen and carbon mass balances for the reactor with the PN-AMX combined process are presented in this section, together with the calculations related to the batch assays for the determination of the SAA and SDA, and the statistical calculations.

2.5.1. Nitrogen

2.5.1.1. Nitrogen Loading and Removal rates

Nitrogen Loading Rate (NLR)

The nitrogen loading rate (NLR), which indicates the nitrogen load applied to the PN-AMX system, was calculated from the influent concentrations for the ammonium and the HRT (eq. 2.5):

$$\mathrm{NLR}\left(\frac{\mathrm{g\ N}}{\mathrm{L\cdot d}}\right)=\frac{\left(\mathrm{NH_4^+-N}\right)_{\mathrm{inf}}+\left(\mathrm{NO_2^--N}\right)_{\mathrm{inf}}+\left(\mathrm{NO_3^--N}\right)_{\mathrm{inf}}}{\mathrm{HRT}}\cdot\frac{1}{1000} \qquad \text{eq. 2.5}$$

where $(NH_4^+\text{-}N)_{inf}$, $(NO_2^-\text{-}N)_{inf}$ and $(NO_3^-\text{-}N)_{inf}$ are the concentrations of ammonium, nitrite and nitrate in the influent (mg N/L), respectively, and HRT the hydraulic retention time (d).

Nitrogen Effluent Rate (NER)

The nitrogen effluent rate (NER), which indicates the outcome nitrogen load of the PN-AMX system, was calculated from the effluent concentrations for the ammonium and the HRT (eq. 2.6):

$$\mathrm{NER}\left(\frac{\mathrm{g\ N}}{\mathrm{L\cdot d}}\right)=\frac{\left(\mathrm{NH_4^+-N}\right)_{\mathrm{eff}}+\left(\mathrm{NO_2^--N}\right)_{\mathrm{eff}}+\left(\mathrm{NO_3^--N}\right)_{\mathrm{eff}}}{\mathrm{HRT}}\cdot\frac{1}{1000} \qquad \text{eq. 2.6}$$

where $(NH_4^+\text{-}N)_{eff}$, $(NO_2^-\text{-}N)_{eff}$ and $(NO_3^-\text{-}N)_{eff}$ are the concentrations of ammonium, nitrite and nitrate in the effluent (mg N/L), respectively, and HRT the hydraulic retention time (d).

Nitrogen Removal Rate (NRR)

The nitrogen removal rate (NRR), which indicates the nitrogen removal capacity of the PN-AMX system, was calculated as the difference between the NLR and the NER (eq. 2.7):

$$\mathrm{NRR}\left(\frac{\mathrm{g\ N}}{\mathrm{L\cdot d}}\right)=\mathrm{NLR}-\mathrm{NER} \qquad \text{eq. 2.7}$$

Ammonium Oxidizing Rate (AOR)

The ammonium oxidation rate (AOR), which indicates the removal of ammonium by the ammonium oxidizing bacteria (AOB), was calculated from the total nitrogen removed in the system (eq. 2.8) and the influent-effluent mass balances for the ammonium (eq. 2.9):

$$\begin{aligned} TN_{removed}\left(\frac{mg\ N}{L}\right) &= \Delta\left(NH_4^+ - N\right)_{inf-eff} + \Delta(NO_2^- - N)_{inf-eff} + \Delta(NO_3^- - N)_{inf-eff} \\ &= \left(\left(NH_4^+ - N\right)_{inf} - \left(NH_4^+ - N\right)_{eff}\right) \\ &+ ((NO_2^- - N)_{inf} - (NO_2^- - N)_{eff}) + ((NO_3^- - N)_{inf} - (NO_3^- - N)_{eff}) \end{aligned} \qquad \text{eq. 2.8}$$

where $TN_{removed}$ is the concentration of total nitrogen removed in the system, $\Delta(NH_4^+\text{-}N)_{inf\text{-}eff}$ represents the total concentration of ammonium nitrogen oxidized in the system (mg NH_4^+-N/L), $\Delta(NO_2^-\text{-}N)_{inf\text{-}eff}$ represents the total concentration of nitrite nitrogen produced in the system (mg NO_2^--N /L), and $\Delta(NO_3^-\text{-}N)_{inf\text{-}eff}$ represents the total concentration of nitrite nitrogen produced in the system (mg NO_3^--N /L).

$$\begin{aligned} AOR\left(\frac{g\ NH_4^+ - N}{L \cdot d}\right) &= \left(\Delta\left(NH_4^+ - N\right)_{inf-eff} - \Delta\left(NH_4^+ - N\right)_{anammox}\right) \cdot \frac{1}{HRT} \cdot \frac{1}{1000} \\ &= \left(\Delta\left(NH_4^+ - N\right)_{inf-eff} - \frac{TN_{removed}}{2.0499}\right) \cdot \frac{1}{HRT} \cdot \frac{1}{1000} \end{aligned} \qquad \text{eq. 2.9}$$

where $\Delta(NH_4^+\text{-}N)_{anammox}$, or $(TN_{removed}/2.0499)$, represents the concentration of ammonium nitrogen oxidized by anammox bacteria (mg NH_4^+-N/L). According to the stoichiometry of anammox bacteria (eq. 1.4, Chapter 1), 1 mol NH_4^+-N is oxidized to produce 1.02 mol N_2 (i.e., 2.0499 mol N_2-N).

Nitrite Oxidizing Rate (NOR)

The nitrite oxidation rate (NOR), which indicates the total removal of nitrite by nitrite oxidizing bacteria (NOB), was calculated from the total nitrogen removed in the system (eq. 2.8) and the influent-effluent mass balances for the nitrite (eq. 2.10):

$$\mathrm{NOR}\left(\frac{\mathrm{g\ NO_3^- - N}}{\mathrm{L \cdot d}}\right) = (\Delta(\mathrm{NO_3^- - N})_{\mathrm{eff-inf}} - \Delta(\mathrm{NO_3^- - N})_{\mathrm{anammox}}) \cdot \frac{1}{\mathrm{HRT}} \cdot \frac{1}{1000} \qquad \text{eq. 2.10}$$

$$= \left(\Delta(\mathrm{NO_3^- - N})_{\mathrm{inf-eff}} - \frac{\mathrm{TN_{removed}}}{2.0499} \cdot 0.26\right) \cdot \frac{1}{\mathrm{HRT}} \cdot \frac{1}{1000}$$

where $\Delta(NO_3^-N)_{anammox}$, or $(TN_{removed}/2.0499 \cdot 0.26)$, represents the concentration of nitrate nitrogen produced by anammox bacteria (mg NO_3^-N/L), considering the ammonium nitrogen oxidized by anammox ($TN_{removed}/2.0499$) as in eq. 2.9. According to the stoichiometry of anammox bacteria (eq. 1.4, Chapter 1), 0.26 mol NO_3^-N are produced per mol of NH_4^+N oxidized.

2.5.1.2. Nitrogen removal percentage

Ammonium removal (% NH_4^+N)

The ammonium removal percentage, which indicates the total oxidation of ammonium in the system due to both the ammonium oxidizing bacteria (AOB) and the anammox bacteria, was calculated from the influent-effluent mass balances for the ammonium and for the total nitrogen removed in the system (eq. 2.11):

$$\%\ \mathrm{NH_4^+ - N} = \frac{\Delta\left(\mathrm{NH_4^+ - N}\right)_{\mathrm{inf-eff}}}{\mathrm{TN_{inf}}} \cdot 100 \qquad \text{eq. 2.11}$$

where $\Delta\left(\mathrm{NH_4^+ - N}\right)_{\mathrm{inf-eff}}$ represents the total concentration of ammonium nitrogen oxidized in the system (mg NH_4^+N) and TN_{inf} is the concentration of total nitrogen in the influent (mg N).

Total Nitrogen removal (% TN)

The total nitrogen removal percentage, which indicates the global elimination of nitrogen due to the PN-AMX combined process, was calculated from the total nitrogen removed in the system with respect to the total nitrogen in the influent (eq. 2.12):

$$\%\ \mathrm{TN} = \frac{\mathrm{TN_{removed}}}{\mathrm{TN_{inf}}} \cdot 100 \qquad \text{eq. 2.12}$$

where $TN_{removed}$ (mg N) is the concentration of total nitrogen removed in the system, and TN_{inf} is the concentration of total nitrogen in the influent.

Nitrogen removal by Heterotrophic Denitrification (% HD)

The mass-balance estimation of the contribution of heterotrophic denitrification (HD) to the removal of nitrogen is crucial to evaluate the competition between the anammox and HD processes (Schielke-Jenni et al., 2015). In order to do so, assumptions were made considering that the observed removal of organic matter in the reactor was due only to the activity of the heterotrophic denitrifying bacteria (HDB). Although the aerobic heterotrophic bacteria (HB) could also oxidize the organic matter up to a certain point, the proposed assumption provides instead an insight on the maximum capacity and potential removal of nitrogen of the HDB.

The calculation of the percentage removal of nitrogen by HD differs whether the organic matter is expressed as the concentration of total organic carbon (TOC, Chapter 3) in eq. 2.13, or as the concentration of the soluble chemical oxygen demand (sCOD, Chapters 4 - 6) in eq. 2.14.

$$\%\,HD_{TOC} = \frac{(NO_3^- - N)_{HD-TOC}}{TN_{inf}} \cdot 100 = \frac{\Delta TOC \cdot 0.933}{TN_{inf}} \cdot 100 = \frac{(TOC_{inf} - TOC_{eff}) \cdot 0.933}{TN_{inf}} \cdot 100 \qquad \text{eq. 2.13}$$

where TOC_{inf} and TOC_{eff} are the concentrations of TOC in the influent and effluent, respectively (mg TOC/L), TN_{inf} is the concentration of total nitrogen in the influent (mg N/L), $(NO_3^-\text{-N})_{HD\text{ - }TOC}$ represents the proportional removal of nitrate attributed to the HD regarding the observed removal of organic matter (expressed as TOC); and 0.933 g NO_3^--N/g TOC relates the consumption of nitrate and organic matter in the HD.

$$\%\,HD_{sCOD} = \frac{(NO_3^- - N)_{HD-sCOD}}{TN_{inf}} \cdot 100 = \frac{\Delta sCOD/7.24}{TN_{inf}} \cdot 100 = \frac{(sCOD_{inf} - sCOD_{eff})/7.24}{TN_{inf}} \cdot 100 \qquad \text{eq. 2.14}$$

where $sCOD_{inf}$ and $sCOD_{eff}$ are the concentrations of sCOD in the influent and effluent, respectively (mg sCOD/L), TN_{inf} is the concentration of total nitrogen in the influent (mg N/L), $(NO_3^-\text{-N})_{HD\text{ - }sCOD}$

represents the proportional removal of nitrate attributed to the HD regarding the observed removal of organic matter (expressed as sCOD); and 7.24 g NO_3^--N/g sCOD relates the consumption of nitrate and organic matter in the HD.

2.5.1.3. Free Ammonia (FA) and Free Nitrous Acid (FNA) concentrations

The concentrations of free ammonia (NH_3) and free nitrous acid (HNO_2) were calculated in eq. 2.15 and eq. 2.16, according to Anthonisen et al. (1976):

$$\mathrm{FA}\left(\frac{\mathrm{mg\ NH_3-N}}{\mathrm{L}}\right)=\frac{\left(\mathrm{NH_4^+-N}\right)\cdot 10^{\mathrm{pH}}}{\left(\mathrm{e}^{6344/(273+\mathrm{T})}+10^{\mathrm{pH}}\right)} \qquad \text{eq. 2.15}$$

$$\mathrm{FNA}\left(\frac{\mathrm{mg\ HNO_2-N}}{\mathrm{L}}\right)=\frac{\left(\mathrm{NO_2^-}-\mathrm{N}\right)}{\left(\mathrm{e}^{-2300/(273+\mathrm{T})}+10^{\mathrm{pH}}+1\right)} \qquad \text{eq. 2.16}$$

where T is the temperature (°C).

2.5.2. Carbon

Total Organic Carbon (TOC) removal (% TOC)

The total organic carbon removal percentage, which indicates the global total removal of organic matter (expressed as TOC) in the system due to heterotrophic denitrifying bacteria (HDB) and aerobic heterotrophic bacteria (HB), was calculated from the influent-effluent mass balances for the TOC, following eq. 2.17:

$$\%\ \mathrm{TOC}=\frac{\mathrm{TOC_{inf}}-\mathrm{TOC_{eff}}}{\mathrm{TOC_{inf}}}\cdot 100 \qquad \text{eq. 2.17}$$

where TOC_{inf} and TOC_{eff} are the TOC concentrations in the influent and effluent, respectively (mg TOC/L).

Soluble Chemical Oxygen Demand (sCOD) removal (% sCOD)

The total soluble COD (sCOD) removal percentage, which indicates the global total removal of organic matter (expressed as sCOD) in the system due to heterotrophic denitrifying bacteria (HDB) and aerobic heterotrophic bacteria (HB), was calculated from the influent-effluent mass balances for the sCOD, following eq. 2.18:

$$\%\ \mathrm{sCOD} = \frac{\mathrm{sCOD_{inf}} - \mathrm{sCOD_{eff}}}{\mathrm{sCOD_{inf}}} \cdot 100 \qquad \text{eq. 2.18}$$

where $\mathrm{sCOD_{inf}}$ and $\mathrm{sCOD_{eff}}$ are the sCOD concentrations in the influent and effluent, respectively (mg sCOD/L).

2.5.3. Carbon and Nitrogen ratios

TOC/N and sCOD/N ratios

The TOC/N and sCOD/N ratios, which provide an insight into the possible extent of biological processes, such as the heterotrophic denitrification (HD), along with the PN-AMX processes (with a threshold ratio of 1.71 g sCOD/g N for complete HD via nitrite, Jenni et al. (2014), were calculated from the influent concentrations of TOC and sCOD (respectively), as well as the influent concentration for total nitrogen, following eq. 2.19 and eq. 2.20:

$$\mathrm{TOC/N\ ratio}\left(\frac{\mathrm{g\ TOC}}{\mathrm{g\ N}}\right) = \frac{\mathrm{TOC_{inf}}}{\mathrm{TN_{inf}}} \qquad \text{eq. 2.19}$$

$$\mathrm{sCOD/N\ ratio}\left(\frac{\mathrm{g\ sCOD}}{\mathrm{g\ N}}\right) = \frac{\mathrm{sCOD_{inf}}}{\mathrm{TN_{inf}}} \qquad \text{eq. 2.20}$$

where $\mathrm{TOC_{inf}}$, $\mathrm{sCOD_{inf}}$ and $\mathrm{TN_{inf}}$ are the influent concentrations of TOC, sCOD and TN, respectively.

Nitrogen to inorganic carbon ratio (N/IC)

The N/IC ratio, which provides an insight into the possible limitation of the partial nitritation process by alkalinity (with a threshold ratio of 1 g N/g IC, above which the oxidation of ammonium is

limited to the 50% or less, Pedrouso et al. (2017)), were calculated from the influent concentrations total nitrogen and inorganic carbon, following eq. 2.21:

$$\text{N/IC ratio}\left(\frac{\text{g N}}{\text{g IC}}\right) = \frac{\text{TN}_{\text{inf}}}{\text{IC}_{\text{inf}}} \qquad \text{eq. 2.21}$$

where TN_{inf} and IC_{inf} are the influent concentrations of TN and IC, respectively.

Nitrate produced to ammonium oxidized ratio (ΔNO_3^--N/ΔNH_4^+-N)

The nitrate produced to ammonium oxidized ratio, which provides an insight into the possible extent in the activity of either nitrate producing bacteria (NOB) or nitrate consuming bacteria (HDB), and which takes a stoichiometric value of 0.11 g NO_3^--N/g NH_4^+-N) for the PN-AMX combined process, was calculated from the mass balances for nitrate and ammonium, following eq. 2.22:

$$\text{Nitrate: ammonium ratio}\left(\frac{\text{g NO}_3^- - \text{N}}{\text{g NH}_4^+ - \text{N}}\right) = \frac{\Delta(\text{NO}_3^- - \text{N})_{\text{eff-initial}}}{\Delta\left(\text{NH}_4^+ - \text{N}\right)_{\text{inf-eff}}} \qquad \text{eq. 2.22}$$

$$= \frac{(\text{NO}_3^- - \text{N})_{\text{eff}} - (\text{NO}_3^- - \text{N})_{\text{initial}}}{\left(\text{NH}_4^+ - \text{N}\right)_{\text{inf}} - \left(\text{NH}_4^+ - \text{N}\right)_{\text{eff}}}$$

where $(NO_3^-\text{-N})_{eff}$ is the mass of nitrate nitrogen in the effluent (mg NO_3^--N), and $(NO_3^-\text{-N})_{initial}$ is the mass of nitrate nitrogen at the beginning of each reactor cycle (mg NO_3^--N). Thus, the numerator in eq. 2.22 represents the actual nitrate nitrogen produced in the considered cycle, without considering the remaining nitrate nitrogen from the previous cycle.

2.5.4. Specific Anammox and Denitrifying activities (SAA and SDA)

2.5.4.1. Calculation of the molar fraction of N_2 and CO_2 in the biogas

In order to calculate the nitrogen and carbon dioxide production rates from the estimation of the SAA and SDA, the nitrogen and carbon dioxide molar fraction from the biogas (mainly N_2 and CO_2) were calculated following eq. 2.23 and eq. 2.24:

$$X_{N_2}(\%) = \frac{\frac{A_{N_2}}{0.782}}{\frac{A_{N_2}}{0.782} + \frac{A_{CH_4}}{0.644} + \frac{A_{CO_2}}{1} + \frac{A_{N_2O}}{1.061}} \quad \text{eq. 2.23}$$

$$X_{CO_2}(\%) = \frac{\frac{A_{CO_2}}{1}}{\frac{A_{N_2}}{0.782} + \frac{A_{CH_4}}{0.644} + \frac{A_{CO_2}}{1} + \frac{A_{N_2O}}{1.061}} \quad \text{eq. 2.24}$$

where X_{N_2} and X_{CO_2} are the molar fractions of nitrogen and carbon dioxide, respectively (%), A_{N_2}, A_{CH_4}, A_{CO_2} and A_{N_2O} are the corresponding areas to each component of the biogas (obtained from a chromatogram like the one in Figure 2.4), and 0.782, 0.644, 1 and 1.061 are the corresponding conversion factors for the N_2, CH_4, CO_2 and N_2 in the biogas.

2.5.4.2. SAA and SDA with PBS and industrial wastewater as liquid media

The maximum SAA and SDA with phosphate buffer (SAA_{PBS} and SDA_{PBS}) were estimated from the maximum slope of the curve described by the cumulative biogas production throughout time, and related to the concentration of biomass in the vials. The biogas production rate was calculated from the maximum slope of the curve describing the pressure increase in the vial throughout time (α, atm/min), following eq. 2.25:

$$\frac{d(\text{biogas})}{dt}\left(\frac{\text{mol biogas}}{\text{min}}\right) = \alpha\frac{V_G}{R \cdot T} \quad \text{eq. 2.25}$$

where V_G is the volume of the gaseous phase (L), R the ideal gas coefficient (atm·L/mol·K), and T the temperature (K). Since the SAA and SDA are referred to the production of nitrogen gas, its production rate was calculated from the biogas production rate taking into account the molar fraction for the N_2 (eq. 2.23), following eq. 2.26:

$$\frac{d(N_2)}{dt}\left(\frac{\text{mol } (N_2)}{\text{min}}\right) = \frac{d(\text{biogas})}{dt} \cdot X_{N_2} \quad \text{eq. 2.26}$$

The SAA and SDA were calculated from the biogas production rate and the biomass concentration in the vial (X, g VSS/L) using eq. 2.27:

$$SAA\left(\frac{g\ N_2 - N}{g\ VSS \cdot d}\right) = \frac{dN_2/dt}{X \cdot V_L}\frac{28\ g\ N}{1\ mol\ N_2}\frac{1440\ min}{d} \qquad \text{eq. 2.27}$$

where V_L is the volume of the liquid phase (L).

Since the values of the affinity constant of the anammox bacteria for ammonium and nitrite are lower than 10 μM and 5 μM, respectively (Strous et al., 1999), it can be considered that the activity measured is the maximum activity for the range of nitrite and ammonium concentrations used.

Considering the four main biological processes that can produce dinitrogen gas from substrates (anammox, denitritation, denitratation and complete denitrification), and their respective stoichiometries (eqs. 1.4, 1.8 - 1.10, 1.7 and 1.11), the possible contribution of each process as a function of the added substrates for the batch tests was summarized in Table 2.8:

Table 2.8: Summary of those processes which contributed to the production of dinitrogen gas in each batch test for the determination of the SAA and the SDA, as a function of the substrates added or present in the industrial wastewater (main) and the produced substrate (secondary).

		Main substrate			**Secondary substrate**		
Batch Test		AMX	Denitri.	Denitra.	AMX	Denitri.	Denitra.
SAA_{PBS}	$NH_4^+ + NO_2^-$	✓					
SAA_{WW}	$NO_2^- + WW$	✓	✓				NO_3^-
$SDA_{NO2\text{-}PBS}$	$NO_2^- + sCOD$		✓				
$SDA_{NO2\text{-}WW}$	$NO_2^- + WW$	✓	✓				NO_3^-
$SDA_{NO3\text{-}PBS}$	$NO_3^- + sCOD$			✓			
$SDA_{NO3\text{-}WW}$	$NO_3^- + WW$			✓	NO_2^-		

"Main substrate" represents the substrates added to the liquid medium (or already present in the wastewater) necessary for each corresponding biological process. "Secondary substrate" represents the intermediate N-species produced during the biological processes, which may serve as substrate for other bacterial groups (the N-species is indicated in each case). AMX: anammox. Denitri.: denitritation. Denitra.: denitratation. PBS: phosphate buffer solution. SAA: specific anammox activity. SDA: specific denitrifying activity. sCOD: soluble chemical oxygen demand. WW: industrial wastewater.

The industrial wastewater contains both ammonium nitrogen and organic matter (expressed here as sCOD) in excess, compared to the concentrations of added substrates when using PBS as liquid medium. For this reason, the removal of nitrogen by heterotrophic denitrification (HD) is always going to take place up to a certain point when using industrial wastewater as liquid medium to determine de

SAA, and nitrite or nitrate as additional substrates. For example, the expected contribution of the denitrifying activity observed for the batch tests SAA_{WW} and $SDA_{NO2,\,WW}$ (Table 2.8) would be similar.

From Table 2.8, both the maximum SAA, the maximum SDA from nitrite (denitritation) and the maximum SDA from nitrate (denitratation) can be estimated from the batch tests SAA_{PBS}, $SDA_{NO2\text{-}PBS}$ and $SDA_{NO3\text{-}PBS}$, respectively, while the SDA from denitratation with industrial wastewater can be estimated from batch test $SDA_{NO3\text{-}WW}$. Regarding the SDA for denitratation, and although the nitrite produced in this process could act as substrate for the anammox bacteria, the anammox activity was assumed to be negligible since the intermediate NO_2^- from the denitratation ($NO_3^- \rightarrow NO_2^-$) would be directly reduced in the denitritation ($NO_2^- \rightarrow N_2$).

On the other hand, the contribution of the anammox and denitritation processes to the removal of nitrogen when using wastewater as liquid medium for the batch tests must be elucidated in order to estimate both the SAA and the SDA from nitrite. In this case, the nitrite is a primary substrate for the batch test, and its possible uptake by both pathways should be considered. Since the N_2 in the gas phase is produced from the anammox and denitritation stoichiometries (eq. 1.4 and eqs. 1.8 - 1.10 in Chapter 1, respectively), while the CO_2 is only produced in the denitritation (assuming the HD as the only source of CO_2 gas), the production of nitrogen from the latter can be calculated considering the respective molar fractions (eq. 2.28):

$$\left(\frac{dN_2}{dt}\right)_{SDA_{NO2-WW}\text{ by HD}} = \left(\frac{d(biogas)}{dt}\right)_{SDA_{NO2-WW}} X_{CO_2} \cdot \frac{0.50\text{ mol } N_2}{0.25\text{ mol } CO_2} \qquad \text{eq. 2.28}$$

where X_{CO_2} is the molar fraction of carbon dioxide in the gas phase, and the coefficient (0.50/0.25) is the molar relation between the N_2 and CO_2 produced in the denitrification (eq. 1.11, Chapter 1). By subtracting the N_2 production rate by denitritation to the total N_2 production rate, the N_2 production rate by anammox can be deducted (eq. 2.29):

$$\left(\frac{dN_2}{dt}\right)_{SAA\,WW\text{ by anammox}} = \left(\frac{dN_2}{dt}\right)_{SAA\,WW} - \left(\frac{dN_2}{dt}\right)_{SDA_{NO2-WW}\text{ by HD}} \qquad \text{eq. 2.29}$$

Applying the N_2 production rates from eq. 2.28 and eq. 2.29 to eq. 2.27, both the activity of anammox and denitritation bacteria with industrial wastewater can be finally estimated.

2.5.5. Statistical calculations

In order to evaluate the statistically significant differences between experimental data, the IBM® SPSS® Statistics software (v25) was applied to the results from the batch tests in Chapter 5 (see Section 5.4.2). The analysis was applied for the data results between different batch tests and between different feedings for the same batch test. Each batch test was performed in triplicate, and each resulting triplicate data was considered as independent samples. Table 2.9 summarizes the different tests performed for the statistical analysis of the experimental data.

Table 2.9. Statistical tests performed with the IBM® SPSS® Statistics software

Test		Objective of the test	p-value[a]	Description
Shapiro-Wilk normality test		Check whether the data have a normal distribution or not	> 0.05	The data show a normal distribution
One-way ANOVA test	Levene test	Check the homogeneity of variances	< 0.05	There is homogeneity of variances
	Significance level test	Check if the differences between average data are significant	< 0.05	If there is homogeneity of variances (Levene test), the differences between the average data are significant. Otherwise: Brown-Forsythe test
Brown-Forsythe test		Check the equality of variances	< 0.05	The differences between the average data are significant

[a] p-value is the common nomenclature for a statistical significance (or significance level, 'sig.')

2.5.6. Other parameter calculations

Hydraulic Retention Time (HRT)

The Hydraulic Retention Time (HRT), which measures the average length of time that a soluble compound remains in the reactor, was calculated following eq. 2.30:

$$\text{HRT (d)} = \frac{V_{useful}}{F_{cycle} \cdot N} = \frac{V_{min} + V_{eff}}{F_{cycle} \cdot N} \qquad \text{eq. 2.30}$$

where V_{useful} is the useful volume (L) for the biological processes inside the reactor (i.e., the total liquid volume), V_{min} is the minimum liquid volume (L) provided inside the reactor (which depends on the height of the discharge connection), V_{eff} is the exchange volume of the effluent for each cycle, F_{cycle} is the volumetric flow of the feeding for one cycle (L/cycle) and N is the number of cycles in one operational day (cycles/d).

Volatile Suspended (VSS) to Total Suspended Solids (TSS) ratio (VSS/TSS)

$$\text{VSS/TSS ratio}\left(\frac{\text{g VSS/L}}{\text{g TSS/L}}\right) = \frac{\text{VSS}_{\text{inf}}}{\text{TSS}_{\text{inf}}} \qquad \text{eq. 2.31}$$

NaCl and KCl concentrations

The salt concentration, when expressed as NaCl (g/L) or KCl (g/L), were calculated as the average between the NaCl and KCl concentrations from the corresponding anion (Cl^-) and cations (Na^+ or K^+) concentrations, following eq. 2.32 and eq. 2.33, respectively:

$$\text{NaCl}\left(\frac{\text{g NaCl}}{\text{L}}\right) = \frac{\text{NaCl}_{\text{anion}} + \text{NaCl}_{\text{cation}}}{2} = \frac{\left(\frac{\text{Cl}^-_{\text{inf}}}{2} \cdot \frac{58.5}{35.5} \cdot \frac{1}{1000}\right) + \left(\frac{\text{Na}^+_{\text{inf}}}{2} \cdot \frac{58.5}{23} \cdot \frac{1}{1000}\right)}{2} \qquad \text{eq. 2.32}$$

where Cl^-_{inf} is the chloride concentration of the influent (g Cl^-/L), and Na^+_{inf} is the sodium concentration of the influent (g Na^+/L). The molecular masses of Na (23 g/mol), Cl (35.5 g/mol) and NaCl (58.5 g/mol) were used in eq. 2.32.

$$\text{KCl}\left(\frac{\text{g KCl}}{\text{L}}\right) = \frac{\text{KCl}_{\text{anion}} + \text{KCl}_{\text{cation}}}{2} = \frac{\left(\frac{\text{Cl}^-_{\text{inf}}}{2} \cdot \frac{74.5}{35.5} \cdot \frac{1}{1000}\right) + \left(\frac{\text{K}^+_{\text{inf}}}{2} \cdot \frac{74.5}{39} \cdot \frac{1}{1000}\right)}{2} \qquad \text{eq. 2.33}$$

where Cl^-_{inf} is the chloride concentration of the influent (g Cl^-/L), and K^+_{inf} is the sodium concentration of the influent (g K^+/L). The molecular masses of K (39 g/mol), Cl (35.5 g/mol) and KCl (74.5 g/mol) were used in eq. 2.33.

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Chapter 3

Treatment of fish canning industry wastewater with the PN-AMX processes

Summary

In Chapter 3, the application of the partial nitritation-anammox (PN-AMX) processes to fish canning wastewater with high salt (as NaCl) concentrations was evaluated, considering the effect of the organic matter concentration and the shift in the microbial populations. The research work presented hereafter constitutes a direct continuation of the research work found in Val del Rio et al. (2018). The results from the laboratory scale experiments here indicated that the PN-AMX processes may suffer from a concatenation of synergic factors affecting the long-term removal of nitrogen, namely the conductivity (12 - 15 mS/cm), the organic matter concentrations (40 - 120 mg TOC/L) and the nitrogen to inorganic carbon (IC) ratio (0.6 - 1.9 g/g). In these conditions, the PN-AMX processes were able to remove between 0.02 and 0.10 g N/(L·d) treating the fish canning wastewater. Furthermore, the presence of high concentrations of phosphate and sulphate may favour the appearance of whitish precipitates over the granular PN-AMX biomass, composed mainly by sulphur and phosphorus, which may diminish the efficiency of substrate transfer from the liquid medium. A tight control of these factors is strongly advised in order to define more favourable boundaries for the removal of nitrogen with the PN-AMX processes.

OUTLINE

3.1. Introduction

The fish canning industry represents a very important economic sector in the Northwest of Spain (Galicia), with more than 65 factories, located mainly in coastal areas. As a consequence of this industrial activity, large wastewater volumes are produced, which contain high solids, organic matter and nitrogen concentrations (Mosquera-Corral et al., 2005). Therefore, these effluents need to be efficiently handled previous discharge to avoid pressures over the marine environment. As previously seen in Table 1.13.a (Chapter 1), the anaerobic digestion is the most extended technology used for treating fish canning effluents, with the main purpose of reducing the organic matter concentration of the wastewater and produce energy as biogas. The effluents coming from the anaerobic digester (AD) contain high concentrations of nitrogen, mainly from proteins, which have to be removed in a subsequent step to adjust to disposal limits. Conventionally, the combined nitrification-denitrification processes was applied to the removal of nitrogen from the supernatant of the AD (Corsino et al., 2016; Figueroa et al., 2008; Mosquera-Corral et al., 2003). However, these processes require much energy to create aerobic conditions for bacterial nitrification, as well as the availability of organic carbon to remove nitrite and nitrate by heterotrophic denitrifying organisms (Kartal et al., 2010). If a complete autotrophic process is applied, the organic matter can be saved to produce more energy in the AD.

Some works have explored an alternative based on the coupling of nitrification and autotrophic denitrification (Fajardo et al., 2013). However, most of recent research efforts have been focused on other alternatives for nitrogen removal, such as the combination of partial nitritation and anammox (PN-AMX) processes (Li et al., 2018b) (see Table 1.13.b). In the PN-AMX processes, half of the ammonium present is oxidized following the nitritation pathway, combined with the subsequent biological reaction of the produced nitrite and the remaining ammonium to produce nitrogen gas according to the anammox reaction.

The presence of certain inhibitory compounds in the wastewater may hinder the application of the anammox based processes at industrial scale (Jin et al., 2012). For example, high saline concentrations may induce salt stress over the microorganisms involved in the biological treatment (Scaglione et al., 2017), with the subsequent inhibition of many enzymes, decrease in the cell activity and eventually cell death by plasmolysis (Jin et al., 2012). Furthermore, the characterization of these effluents is strongly related to the seasonality in the fish canning industry, which may generate unexpected variations in the wastewater depending on the changes in the raw material processed in the factory (Cristóvão et al., 2016).

Regarding the effect of the salinity over the anammox bacteria, Val del Rio et al. (2018) found that they can withstand and quickly recover after sudden peaks of salt up to 16 g NaCl/L. However, the adaptation of the anammox bacteria to relatively high salinity (7 - 9 g NaCl/L) is feasible but can shape

the PN-AMX bacterial community in the long-term, inhibiting up to a certain point the anammox population (Val del Rio et al., 2018). This may in turn provoke the buildup of the nitrite, substrate for the anammox, process which is produced in the previous nitritation process, leading to the worsening of the overall nitrogen removal.

Besides the high salinity, sufficiently high organic matter concentrations may destabilize the PN-AMX processes (Val del Rio et al., 2018), as anammox is in fact recommended for the treatment of wastewater streams with low carbon to nitrogen ratios, like the supernatant from the AD (Lackner et al., 2014). Nevertheless, a steady blending of the supernatant with wastewater from before the AD treatment would provide a moderate supplement of organic matter, which could stimulate the consumption of accumulated nitrite by heterotrophic denitrification and promote up to a certain extent a supplementary pathway for nitrogen removal. In this sense, scarce research is available addressing the combined effect of salts and organic matter over the partial nitritation (Mosquera-Corral et al., 2005) or the PN-AMX processes (Giustinianovich et al., 2018; Val del Rio et al., 2018).

An important aspect to consider is the possible shift in the microbial populations due to the salt and organic matter concentrations. Despite the advancements of next generation sequencing platforms in microbial ecology, only few studies addressed the influence of salinity in the PN-AMX processes (Val del Rio et al., 2018) and thus the PN-AMX reactor communities using this cutting-edge technology (Agrawal et al., 2017; Wang et al., 2017b). For example, Wang et al. (2017b) studied the microbiological shift in the PN-AMX processes with the progressive increase in the salt concentration from 0 to 20 mg NaCl/L. However, their research work was performed with a synthetic medium ignoring the microorganisms that can be present in industrial effluents and the fluctuations in the composition of the wastewater, such as changes in the concentration of organic matter.

3.2. Objectives

The application of the combined PN-AMX processes in a single unit to treat the supernatant from an anaerobic digester (AD) treating fish canning effluents, blended with non-digested fish canning effluents (prior to the AD), is evaluated in the present Chapter. The objective of this feeding strategy is to stimulate moderately the heterotrophic denitrification as an aid for the performance of the PN-AMX processes in saline conditions. The PN-AMX biomass was previously exposed in the long-term to relatively high concentrations of salt (7 - 9 g NaCl/L) and organic matter (200 mg TOC/L) in a previous research work. The evolution of the main microbial populations in the biomass from the reactor was determined to prove the effects of salinity and organic matter concentration on the performance of the PN-AMX processes and assess the potential destabilization of the process.

3.3. Materials and Methods

3.3.1. Experimental set-up

A laboratory scale sequencing batch reactor (SBR) with a working volume of 1.5 L was used for the lab-scale experiment, already in operation in Val del Rio et al. (2018). The aeration system consisted in a diaphragm pump (Laboport N86, KNF), which supplied air through an air diffuser located at the bottom of the reactor, promoting the aeration with the formation of small bubbles. The aeration inside the reactor provided both a good mixture and the dissolved oxygen (DO) necessary to carry out the biological aerobic processes. The DO concentration was measured periodically with a DO probe (Hach Lange LD01) and was manually regulated by changing the opening degree of an air valve located in the gas inlet conduction. Nitrogen gas was sporadically added to the air flow in order to adjust the DO concentration into the reactor.

The reactor was operated at a temperature range of 31 - 33 °C by means of a thermostatic bath, which is close to the expected range for the reject water of a mesophilic AD. The pH was not controlled inside the reactor, with an average value of 7.9 ± 0.5. Operational cycles of 180 min were distributed as follows: 5 min of feeding, 160 min of aeration, 10 min of settling and 5 min of effluent withdrawal. The hydraulic retention time (HRT) varied between 1.3 and 3.8 d for all the operational period (Table 3.1). The aeration regime was continuous and later changed to pulsed aeration, following the distribution in Table 3.1.

3.3.2. Feeding characterization and operational strategy

The operation of the laboratory scale reactor lasted for 206 days and was divided in three different stages depending on the influent concentration (Table 3.1). In Stage I, the reactor was fed with the supernatant from the anaerobic digester (AD) in operation in the fish canning industry, blended with high strength wastewater collected before its treatment in the AD, with a proportion of 50% and 50%, respectively. During Stage II, the proportion changed to 86% (AD supernatant) and 14% (wastewater prior to AD), respectively, in order to diminish the concentration of organic matter in the feeding. In addition, the HRT was increased from 1.8 days to 3.8 days. Finally, no further wastewater prior to the AD was supplemented in the feeding, and a 1:3 dilution with tap water was applied to AD supernatant fed during Stage III. The HRT was lowered to values similar to those from Stage I, with the objective of decreasing the concentration of ammonium but maintaining the same nitrogen loading rage (NLR).

As the SBR operation presented in this chapter represents a straight continuation of the SBR operation in Val del Rio et al. (2018), no actual inoculation was carried out during Stage I. Thus, the granular PN-AMX biomass here is the same that the one at the end of Stage VI in Val del Rio et al.

(2018). The specific anammox activity (SAA) of the biomass at 30 °C was 0.0623 ± 0.0067 g N/(g VSS·d) at the beginning of Stage I (solids concentration in the reactor of 7.3 g VSS/L), and the salt concentration of the wastewater previously treated was 7.9 ± 0.9 g NaCl/L.

Table 3.1. Operational conditions and characteristics of the wastewater fed to the SBR reactor (AD supernatant + non-digested effluent prior to AD) in the different operational stages.

	Stage I	Stage II	Stage III
Days	0 - 76	77 - 178	179 - 206
Operational conditions			
Temperature (°C)	31.5 ± 2.1	33.8 ± 2.0	31.6 ± 1.6
HRT (d)	1.26 ± 0.19	1.3 - 3.8	1.55 ± 0.11
DO (mg O_2/L)	2.1 ± 1.0	2.7 ± 1.3	2.1 ± 0.6
pH	7.5 ± 0.3	7.7 ± 0.3	7.6 ± 0.2
Aeration pulse (ON:OFF, sec.)	continuous	6:4 [a] / 4:6 [b]	2:8
Dilution ratio	1:1	1:1	1:3
% non-digested effluent	50%	14%	0%
NH_4^+ (mg N/L)	251 ± 20	264 ± 29	156 ± 7
IC (mg IC/L)	389 ± 30	280 ± 19	99 ± 7
TOC (mg TOC/L)	65 ± 20	54 - 21 [c]	27 ± 4
Ratio TOC/N (g/g)	0.16 - 0.53	0.12 - 0.59	0.17 ± 0.03
Ratio N/IC (g/g)	0.65 ± 0.04	0.98 ± 0.14	1.55 ± 0.11
Salt (g NaCl/L)	7.4 ± 0.2	7.8 ± 0.2	0.9 ± 0.1
Conductivity (mS/cm)	14.1 ± 0.5	13.6 ± 0.4	3.3 ± 0.3

[a] Discontinuous aeration from day 118 on: 6:4 sec. (ON:OFF)

[b] Discontinuous aeration from day 133 on: 4:6 sec. (ON:OFF).

[c] Sporadic peaks up to 120 mg TOC/L were observed (day 96) due to fluctuations in the industrial wastewater.

3.3.3. Analytical methods

Analytical determination of ammonium (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N), total suspended solids (TSS) and volatile suspended solids (VSS) concentrations, conductivity and pH, was carried out according to the standard methods (APHA/AWWA/WEF, 2012). Total Organic Carbon (TOC) concentration was determined by a Shimadzu analyser (TOC-L_{CSN}, automatic sample injector Shimazdu ASI-L), as the difference between the Total Carbon (TC) and the Inorganic Carbon (IC) concentrations. Cation and anion concentrations were determined by ion chromatography with an

Advanced Compact IC system (861, Metrohm), CO_2 suppressor (MCS 853, Metrohm) and a sample processor/injector (838, Metrohm). For all these previous measurements the samples were filtered by 0.45 μm, except for VSS. Full description of the analytical methods is provided in Chapter 2 (Section 2.1).

In order to identify the composition both of the outer layers (observed precipitates) and the inner layers (active biomass) of the granules, morphological studies were assessed with the SEM-EDX (Scanning Electron Microscopy - Energy Dispersive X-ray spectroscopy) technique. For SEM-EDX analysis, the granules from the biomass sample were dehydrated in two different ways: (1) using ethanol solutions with increasing concentrations (30, 50, 70 and 100%) after consecutive washing (phosphate buffer) and overnight fixing (3% glutaraldehyde in phosphate buffer) steps (following the procedure in Figueroa et al. (2008)), and (2) overnight dehydration at room temperature with no reagents addition. Full description of the preparation method for SEM - EDX is provided in Chapter 2 (Section 2.3.3).

Biomass samples from the reactor from days 20 and 76 (Stage I) were analyzed with molecular techniques to evaluate the microbial distribution and its evolution inside the reactor. Total genomic DNA was extracted using the phenol-chloroform protocol and the obtained DNA libraries were later sequenced with Illumina® following the methodology in Val del Rio et al. (2018). Full description of the molecular techniques applied to samples from this Chapter is provided in Chapter 2 (Section 2.4.2).

3.3.4. Calculations

The nitrogen loading rate (NLR), the nitrogen removal rate (NRR) and the total nitrogen (TN) removal efficiency were estimated based on nitrogen balances and the anammox process stoichiometry and expressed as g N/(L·d), following eq. 2.5, eq. 2.7 and eq. 2.12, respectively (Chapter 2, Section 2.5.1.1 and 2.5.1.2). The maximum total nitrogen (TN) removal percentage by a possible heterotrophic denitrification (HD) was determined based on the mass balance of the total organic content (TOC), following eq. 2.13 (Section 2.5.1.2). Since part of the organic matter can be consumed for growth and/or aerobic oxidation due to the presence of oxygen, this maximum TN removal percentage considers only the maximum "potential" or contribution for the HD, not the actual.

The concentration of free ammonia (FA, NH_3) was calculated at the operational temperature based on both the NH_4^+ and NO_2^- concentrations and the pH in the bulk liquid (i.e., in the effluent of the reactor) according to Anthonisen et al. (1976) (eq. 2.15, Section 2.5.1.3, Chapter 2).

3.4. Results and discussion

3.4.1. Nitrogen removal

The concentration of ammonium fed to the reactor remained in a similar range for Stages I and II (251 ± 20 and 264 ± 29 mg NH_4^+-N/L, respectively), while it decreased to about half (156 ± 7 mg NH_4^+-N/L) in Stage III (Figure 3.1.a). Despite the efforts to achieve a tight and stable aeration control to limit the accumulation of nitrite, the DO concentration range remained between 1.0 and 4.5 mg O_2/L during Stages I and II (Figure 3.1.a, grey bars), which produced an effluent with variable concentrations of ammonium and nitrite (between 45 - 235 mg NH_4^+-N/L). During Stage III, the DO concentration was maintained at a slightly lower range (1.0 - 2.6 mg O_2/L), obtaining average ammonium concentrations in the effluent of 98 ± 7 mg NH_4^+-N/L, and for nitrite below 50 mg NO_2^--N/L (Figure 3.1.a).

In this sense, both the ammonium and the nitrite concentrations in the effluent showed opposite trends. When the oxidation of ammonium by AOB improved in the system, the nitrite built up in the reactor up to 120 - 170 mg NO_2^--N/L, as observed for example for days 46 - 62 and 67 - 76 (Stage I), and days 84 - 98 and 144 - 152 (Stage II), for DO concentrations between 3 and 4.5 mg O_2/L (Figure 3.1.a). The accumulation of nitrite is reported as one of the main problems regarding the deterioration of anammox systems (Li et al., 2018a), and indicates a concurrent lower capacity of nitrite consumers (namely, anammox and nitrite oxidizing bacteria). Except for some days during Stage II, the potential activity of NOB remained low during the operation of the reactor. Thus, the anammox bacteria were the bottleneck in the PN-AMX processes for the removal of nitrogen, since they were not able to reduce the nitrite produced by AOB when the oxidation of ammonium was high.

These nitrite concentrations values are between the reported inhibitory and non-inhibitory values in the literature. On one hand, Jetten et al. (1998) and Isaka et al. (2007) observed a threshold for complete inhibition of flocculent anammox biomass of 280 mg NO_2^--N/L, while the former indicated a low performance already from 140 mg NO_2^--N/L. In the short-term exposure, Jaroszynski et al. (2011) found no deactivation of anammox biomass in biofilm in the presence of 170 - 250 mg NO_2^--N/L, while Fux et al. (2004) and Bettazzi et al. (2010) observed moderate to severe inhibition for lower concentrations (30 - 60 mg NO_2^--N/L). On the other hand, Fernández et al. (2012) found no inhibition of an anammox biofilm operated with nitrite concentrations below 240 mg NO_2^--N/L, in agreement with the stable operation achieved by Dapena-Mora et al. (2006) treating fish canning wastewater with granular anammox biomass at 200 mg NO_2^--N/L. As a conclusion, the nitrite built-up range (120 - 170 mg NO_2^--N/L) probably had an inhibitory effect over the anammox bacteria.

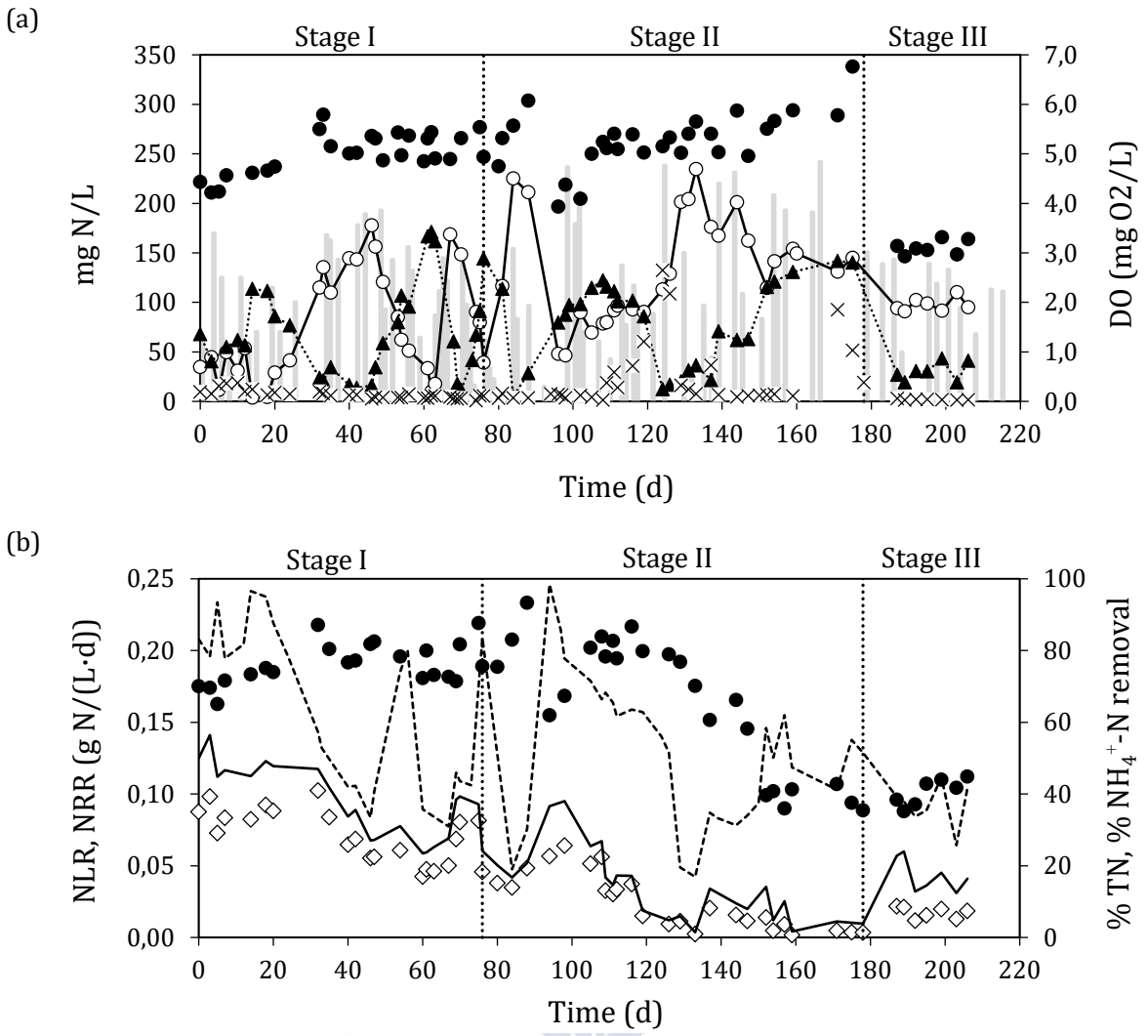


Figure 3.1. Data from the operation of the SBR reactor: (a) concentration of dissolved oxygen, DO (mg O_2/L, ▮), NH_4^+-N in the influent (●), and NH_4^+-N (○), NO_2^--N (▲) and NO_3^--N (×) in the effluent (mg N/L); (b) Nitrogen Loading Rate, NLR (g N/(L·d), ●), Nitrogen Removal Rate, NRR (g N/(L·d), ◇), % of total nitrogen (TN) removal (—) and % of NH_4^+-N removal (– –).

Similarly to the ammonium concentration, the nitrogen loading rate (NLR) was in a similar range during Stage I (0.191 ± 0.014 g N/L·d) and Stage II until day 150 (0.189 ± 0.024 g N/L·d, Figure 3.1.b), corresponding to a hydraulic retention time between 1.3 and 1.7 d. Later, the exchange volume between successive cycles in the reactor progressively decreased until the end of Stage II (with a HRT increase from 1.7 to 3.8 d). Despite trying to achieve a better coupling between the NLR and the actual capacity of the anammox bacteria, the removal of total nitrogen, which was already of 50% at the beginning of Stage I, progressively decreased to 30% and further below 10% during Stage II.

Despite the change in the aeration regime from continuous to discontinuous (6:4 sec. ON:OFF) from day 118 on, the DO range was still broad and the concentration of ammonium in the effluent increased from 100 mg NH_4^+-N/L to 200 mg NH_4^+-N/L (Figure 3.1.a). Maximum values were reached by day 133 (234 mg NH_4^+-N/L) for a 4:6 sec. ON:OFF aeration regime, lowering the ammonium removal from 63% to 20% (Figure 3.1.b). With the decrease in the aeration ratio from day 180 on (2:8 sec. ON:OFF), a more stable DO range was achieved (2.1 ± 0.6 mg O_2/L), although the concentration of ammonium in the effluent did not increase (98 ± 7 mg NH_4^+-N/L), probably because of the lower concentration in the diluted feeding.

The decreasing NLR during Stage II (from 0.189 ± 0.024 g N/L·d to 0.098 ± 0.007 g N/L·d at the end of Stage II) was maintained in the same range during Stage III (0.102 ± 0.009 g N/L·d). The removal of total nitrogen was able to recover up to 20%. This was probably owed to a slight improvement in the anammox activity since the oxidation of ammonium remained between 30 - 50% during Stage III (slightly lower comparing with the end of Stage II), and the maximum contribution of denitrification to the removal of nitrogen was similar to Stage II. The presence of high concentrations of organic matter on the long-term, although its initial use as an aid for the PN-AMX processes, may end up deteriorating the anammox activity.

Scarce research is available regarding the treatment of anaerobically digested fish-canning effluents with the one or two-stage PN-AMX processes (Giustinianovich et al., 2018; Val del Rio et al., 2018; Vázquez-Padín et al., 2009). The NLR applied in the present research work (0.10 - 0.20 g N/(L·d)) is in the same range as in the operation precedent to this Chapter (Val del Rio et al., 2018) (0.19 - 0.26 g N/(L·d)) and in Giustinianovich et al. (2018) (0.08 - 0.14 g N/(L·d)). Vázquez-Padín et al. (2009) applied a NLR of 0.20 - 0.65 g N/(L·d) (780 - 1670 mg NH_4^+-N/L), achieving maximum nitrogen removal efficiencies of 80% for the CANON (Completely Autotrophic Nitrogen removal Over Nitrite) process, similarly to Val del Rio et al. (2018) (80% of nitrogen removal) for fed ammonium concentrations below 300 mg NH_4^+-N/L. However, the nitrogen removal at the end of the operation in Val del Rio et al. (2018) decreased to 50% (which is the starting point in Stage I), probably due to the long-term exposure to the organic matter concentrations in the feeding (30 - 120 mg TOC/L) and the saline concentrations (7.5 - 7.9 g NaCl/L) after more than 200 days of operation.

3.4.2. Influence of salinity and conductivity

During Stages I and II, the conductivity (k, mS/cm) and the salt content (expressed as g NaCl/L) of the fish canning wastewater remained in the same respective ranges (Figure 3.2.a). The salt concentration (7.4 - 7.8 g NaCl/L) was in the same range as in Val del Rio et al. (2018). Regarding the influence of salinity over the PN-AMX processes, Liu et al. (2008) and Campos et al. (2002) observed

an improved performance of the AOB (within the CANON process) and its acclimation to synthetic wastewater with salt concentrations of 10 and 13.7 g NaCl/L, respectively, while Hunik et al. (1992) determined a IC_{50} value of 11.7 g NaCl/L for the AOB. Bassin et al. (2012) found no inhibition of AOB against increasing concentrations of salt from 5 to 20 g NaCl/L. On the other hand, the anammox can progressively adapt to salt concentrations below 10 g NaCl/L (García-Ruiz et al., 2018a; Gonzalez-Silva et al., 2017; Liu et al., 2008; Yang et al., 2011; Yi et al., 2011), with a IC_{50} value of 13.46 g NaCl/L for the anammox (Dapena-Mora et al., 2007). Regarding these reported values, no significant effect of the salinity over the nitrogen removal and the anammox activity should be expected from Stage I to Stage III; however, the long-term exposition (> 200 days) and stability of the PN-AMX processes to salt concentrations in the range of 7.4 - 7.9 g NaCl/L was limited at the end of the previous operation (Val del Rio et al., 2018).

Although the conductivity is normally reported for leachate-like effluents, this parameter, rather than the single salts, may contribute as well to the inhibition of the anammox bacteria (Scaglione et al., 2017). Considering the IC_{50} value (6.1 mS/cm) obtained by these authors, the moderately high conductivity values during Stages I and II (13.9 ± 0.5 mS/cm, Figure 3.2.a) may have contributed to the progressive decrease in the nitrogen removal by anammox.

Besides Na^+ and Cl^-, which were the ions with the highest concentrations during the entire operation (Figure 3.2.a), other ions were present in the fish canning wastewater. Calcium and phosphate were in relatively low average concentrations during the operation (99 ± 39 mg Ca^{2+}/L and 76 ± 40 mg PO_4^{3-}/L, Figure 3.2.b and Figure 3.2.c, respectively), except for some sporadic concentration peaks, and with similar concentrations between influent and effluent. The phosphate is one of the most common inorganic inhibitors of the anammox process, with IC_{50} values for the anammox bacteria of 1900 mg PO_4^{3-}/L (Dapena-Mora et al., 2007) and 2403 mg PO_4^{3-}/L (Carvajal-Arroyo et al., 2013). Jetten et al. (1998) observed reversible inhibition of the anammox bacteria for up to 190 mg PO_4^{3-}/L, while Pynaert et al. (2003) showed a 63% anammox activity decrease for 171 mg PO_4^{3-}/L. Thus, the low phosphate concentrations in the fish-canning effluent treated should not influence negatively the anammox and PN-AMX processes.

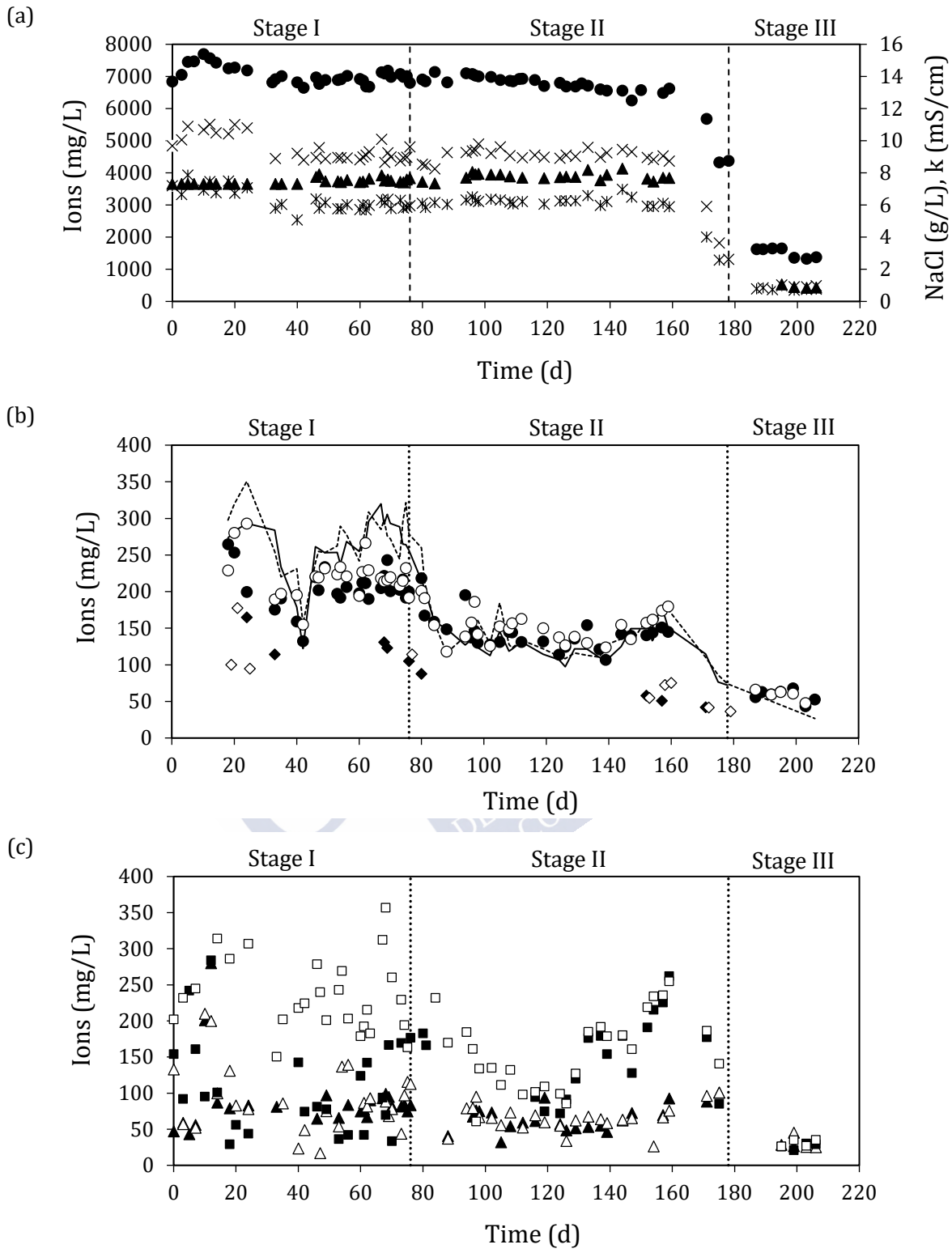


Figure 3.2. Concentration in the SBR feeding of: (a) sodium (Na^+, ✳) and chloride (Cl^-, ×) ions (mg/L), salt (g NaCl/L, ▲), and conductivity, k (mS/cm, ●); (b) influent (●) and effluent (○) potassium (K^+), influent (—) and effluent (– –) magnesium (Mg^{2+}), and influent (◆) and effluent (◇) calcium (Ca^{2+}) cations (mg/L); (c) influent (▲) and effluent (△) phosphate (PO_4^{3-}), and influent (■) and effluent (□) sulphate (SO_4^{2-}) anions (mg/L).

On the other hand, the concentrations of magnesium and potassium were very similar between the influent and effluent for the entire operation. Furthermore, and except for sporadic peaks, the concentrations for K^+ and Mg^{2+} agreed with the decreased organic matter content from Stage I (269 ± 28 mg Mg^{2+}/L and 205 ± 27 mg K^+/L, Figure 3.2.b) to Stage II (147 ± 27 mg Mg^{2+}/L and 126 ± 24 mg K^+/L). The sulphate was in a broader concentration range during Stages I and II (42 - 278 mg SO_4^{2-}/L, Figure 3.2.c). While the concentration in the influent and effluent were similar for the other ions, the influent concentration during Stage I was above 150 mg SO_4^{2-}/L (with sporadic peaks over 350 mg SO_4^{2-}/L). The high concentrations of sulphate may lead to the formation of sulphur, which can in turn contribute to the appearance of precipitates and the inhibition of anammox bacteria.

The 1:3 dilution in the feeding during Stage III favoured lower, non-inhibitory salt concentrations in the feeding (0.9 ± 0.1 g NaCl/L, Figure 3.2.a), as well as an average conductivity below the reported inhibitory threshold (3.3 ± 0.3 mS/cm). These lower values in the feeding, together with the lower concentrations of potassium (57 ± 8 g K^+/L), sulphate (26 ± 4 g SO_4^{2-}/L) and phosphate (28 ± 1 g PO_4^{3-}/L), probably allowed the removal of total nitrogen to slightly increase up to 20% at the end of Stage III (Figure 3.1.b).

3.4.3. Limitation of the nitrite oxidation by NOB

The presence of significant NOB activity is one of the most common problems that reduce the nitrogen removal efficiency in a PN-AMX system (Lackner et al., 2014; Li et al., 2018a, Li et al., 2019). The NOB activity can be inhibited by controlling the concentration of free ammonia (FA), DO, and the pH (Lackner et al., 2014; Li et al., 2019), or through salt stress conditions since NOB are more sensitive than AOB to saline conditions (Ge et al., 2019; She et al., 2016). Because of this, the treatment of saline effluents by the application of the PN-AMX processes is considered as a suitable option (Liu et al., 2008). In the precedent operation of the reactor, the NOB activity remained low for salt concentrations up to 8 g NaCl/L, but a complete wash out of the NOB was not achieved (Val del Rio et al., 2018). Thus, the NOB may have been lethargic at the beginning of the operation (7.4 - 7.8 g NaCl/L during Stage I-II, Figure 3.2.a), but still maintaining its potential activity that could be further developed in only few days if the conditions become favourable (Val del Rio et al., 2018). Thus, an explanation for NOB inhibition should be found elsewhere.

Regarding the inhibition of NOB by FA, no agreement has been reached about the inhibitory threshold, with previously reported values of 0.082 - 0.82 mg NH_3-N/L at room temperature (Anthonisen et al., 1976), 10 - 22 mg NH_3-N/L at 30 °C (Jianlong and Ning, 2004) and 0.8 - 22 mg NH_3-N/L at 28 °C (Kim et al., 2010). During Stage I, the concentration of nitrate in the effluent remained below 20 mg NO_3^--N/L (Figure 3.1.a) despite the varying concentrations of nitrite in the effluent

(between 0 and 140 mg NO_2^--N/L). This suggests a low potential activity of the NOB, since they did not oxidize the nitrite when available in higher concentrations. The intermittent high concentrations of ammonium in the effluent during Stage I favoured FA concentrations in the reactor up to 40 mg NH_3-N (Figure 3.3), which were inhibitory based on the reported values. Furthermore, the ratio between nitrate produced and ammonium oxidized (with a stoichiometric value of 0.11 g NO_3^--N/g NH_4^+-N for the PN-AMX processes) was below this threshold value during Stage I (Figure 3.3), thus confirming the negligible nitrite oxidation by NOB.

During Stage II, even a more straight relation between NOB inhibition and FA concentration can be observed. Between days 77 - 96, the high concentrations of ammonium in the effluent (up to 225 mg NH_4^+-N/L, Figure 3.1.a) provoked an increase in the FA concentration above 90 mg NH_3-N/L (Figure 3.3), and both the concentration of nitrate in the effluent and the ratio between nitrate produced and ammonium oxidized remained low. From day 96 on, the improvement of the partial nitritation favoured lower concentrations of ammonium (below 120 mg NH_4^+-N/L, Figure 3.1.a), and thus lower, non-inhibitory FA concentrations for NOB (below 6 mg NH_3-N/L, Figure 3.3). The concentration of nitrate and the ratio between nitrate produced and ammonium oxidized increased from 5 to 130 mg NO_3^--N/L (Figure 3.1.a) and from 0.04 to 0.91 g/g (Figure 3.3) by day 124, respectively, confirming a peak of NOB activity at this point.

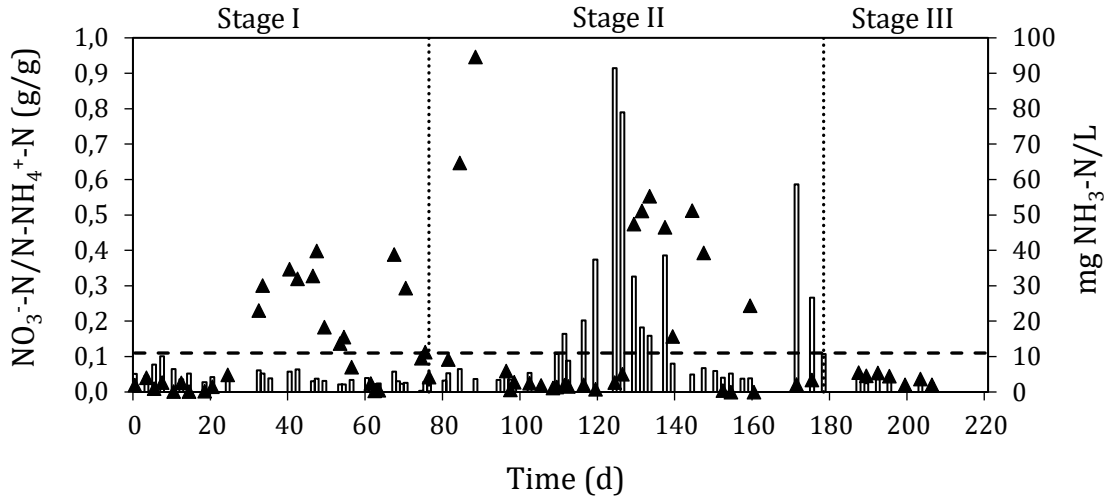


Figure 3.3. Nitrate produced to ammonium oxidized ratio (g NO_3^--N/g NH_4^+-N, □), concentration of free ammonia (mg NH_3-N, ▲) and stoichiometric nitrate produced to ammonium consumed ratio for the PN-AMX processes (0.11 g N/g N) (- - -).

Nevertheless, the increase of the ammonium concentration in the effluent between days 124 - 137 showed the inhibition of NOB already for FA concentrations of 47 - 55 mg NH_3-N/L (Figure 3.3) and the concentration of nitrate depleted again below 5 mg NO_3^--N/L. The same event observed for days 96 - 124 (improved partial nitritation → lower NH_4^+-N effluent concentration + higher NO_2^--N effluent concentration → higher NO_3^--N effluent concentration, i.e. higher potential NOB activity) took place at the end of Stage II (days 147 - 175). On the other hand, the ratio between nitrate produced and ammonium oxidized remained low also for lower concentrations of FA, as seen at the beginning of Stage I (days 1-24, < 4.8 mg NH_3-N/L) and during Stage III (days 187 - 206, < 5.5 mg NH_3-N/L). In the former case, the NOB remained hindered probably due to the previous salt concentrations (7.3 g NaCl/L, Figure 3.2.a), similarly to the NOB limitation found in Val del Rio et al. (2018) for 8 g NaCl/L.

The inhibition of NOB by FA is crucial when low anammox activity is available, due to the absence of direct competitors for the nitrite substrate. However, higher FA concentrations may become a problem since it can also negatively affect the activity of the anammox bacteria and AOB.

The inhibition by FA for the AOB can start from 10 mg NH_3-N/L (Durán et al., 2014; Neufeld et al., 1986), with reported inhibition range of 30.0 - 32.5 mg NH_3-N/L within the CANON process (Li et al., 2012) and IC_{50} range of 4.93 - 27.2 mg NH_3-N/L (Park and Bae, 2009). During Stages I-III, the highest FA concentrations (above 90 mg NH_3-N) were achieved only sporadically at the beginning of Stage II (day 88, Figure 3.3), with values below 40 mg NH_3-N/L between days 23 - 54 (Stage I) and below 55 mg NH_3-N/L between days 129 - 159 (Stage II). However, as previously seen, the occurrence of high FA concentrations (Figure 3.3) is closely related to the high ammonium concentrations in the effluent (Figure 3.1.a), i.e. the former is a consequence of the latter, rather than the cause due to a lower AOB activity. Furthermore, the FA was not determining for the inhibition of AOB, since the oxidation of ammonium was related to the varying DO concentrations supplied, and was able to improve after high FA episodes.

In the case of anammox bacteria, long-term instability of the process was reported between 20 - 40 mg NH_3-N/ L (Fernández et al., 2012) for flocculent biomass and biofilm, with IC_{50} values of 38 mg NH_3-N/ L, while Daverey et al. (2013) reported anammox inhibition within the CANON process at 146 mg NH_3-N/ L. Higher FA inhibitory ranges were reported for granular anammox bacteria: 13 - 90 mg NH_3-N/ L (Waki et al., 2007) and 57 - 187 mg NH_3-N/ L (Tang et al., 2010). Therefore, the FA may have contributed synergistically to the hindering of the anammox activity up to a certain point together with other factors.

Furthermore, it should be noted that the "pace" of nitrate production by NOB was similar to the consumption of that nitrate once the NOB were inhibited by high FA (for example, see the symmetric nitrate peaks around days 124 and 171, Figure 3.1.a). Besides the inhibition nitrite oxidation by NOB,

this highlight the nitrate consumption by third-party bacteria, probably heterotrophic denitrifying bacteria. The rapid consumption of nitrate after its buildup contrasts with the reported duration of the nitrate accumulation episodes (up to several weeks, Lackner et al. (2014)).

3.4.4. Influence of organic matter and heterotrophic denitrification

The process of heterotrophic denitrification (HD) is known to occur simultaneously with the PN-AMX processes when biodegradable organic matter is available. This heterotrophic denitrification facilitates the removal of the nitrate produced by the anammox bacteria (Giustinianovich et al., 2016). However, the development of HD might bring a shift in the microbial populations behavior and compete with anammox bacteria for the nitrite substrate (Jenni et al., 2014; Li et al., 2018a).

During Stage I (and except for the first days), the concentration of organic matter (expressed here as mg TOC/L) in the feeding was in the range 45 - 70 mg TOC/L (Figure 3.4.a), and the TOC removal varied between 20 - 80% during Stage I. The occurrence of slightly higher TOC concentrations in the effluent (between days 42 - 61 and 69 - 76) coincide with the periods of higher ammonium concentrations in the effluent (days 24 - 56 and 70 - 76, Figure 3.1.a). This may suggest that the highly variable DO concentration had a similar effect over the oxidation of both ammonium by AOB and the oxidation of organic matter by aerobic heterotrophs. Furthermore, the ratio between nitrate produced and ammonium oxidized was below the stoichiometric value during Stage I (Figure 3.3.a), which could be owed to the consumption of nitrate by denitrification processes, besides possible NOB hindering by salt concentrations, as previously explained. The maximum contribution of the HD to the removal of nitrogen was estimated to be 21 ± 2% (Figure 3.4.b).

In Stage II, due to the higher proportion of AD supernatant applied to the feeding (86% vs. 50%, Table 3.1), the concentration of TOC (up to 120 mg TOC/L, Figure 3.4.a) and the TOC/N ratio (up to 0.6 g TOC/g N, Figure 3.4.b) were higher, and later decreased and remained at a lower range (43 ± 10 mg TOC/L) with no further changes in the feeding proportions. The removal of TOC increased at the beginning of Stage II up to 81% (days 96 - 112, Figure 3.4.a), coinciding with the higher TOC concentrations in the feeding and the improvement of the partial nitritation (days 96 - 116, Figure 3.1.a). Consequently, the maximum possible contribution of HD to the removal of nitrogen increased up to 55% by day 96 (Figure 3.4.b), while the nitrate to ammonium ratios remained low (Figure 3.3). On the contrary, both the removal of organic matter and the maximum contribution of HD later decreased to a range of 24 - 60% (Figure 3.4.a) and 12 - 23% (Figure 3.4.b), respectively, from day 119 on.

The purpose of the feeding strategy with extra organic matter concentrations in Stage I (50%) and Stage II (16%) was to use it as a supplementary aid for the nitrogen removal process, as previously

reported in the literature (Giustinianovich et al., 2016; Jenni et al., 2014; Li et al., 2018a). The organic matter concentrations during Stages I and II (between 30 and 120 mg TOC/L, Figure 3.4.a) were below the reported non-inhibitory thresholds for anammox bacteria (normally expressed as the COD content): < 100 mg COD/L for the PN-AMX processes (García-Ruiz et al., 2018b) and < 150 mg COD/L for the AMX bacteria (Li et al., 2016; Miao et al., 2014). However, the long-term exposure to high concentrations of organic matter can affect the anammox activity and the removal of nitrogen (Wang et al., 2019), as was probably the case especially during Stage II with the sporadic increase in the TOC/N ratio due to the variability of the industrial wastewater (and despite the lower proportion in the feeding of high strength wastewater - 16%). Furthermore, a shift between heterotrophic denitrifiers and anammox bacteria competing for substrate may occur, especially when the activity of anammox bacteria is low.

For this reason, no further blending of the supernatant with wastewater before the anaerobic digester was applied in the feeding during Stage III. The removal of organic matter remained in a slightly lower range (6 - 51%, Figure 3.4.a), with similar TOC/N ratios in the feeding (0.17 ± 0.03 g/g) and a lower contribution of denitrification to the removal of nitrogen (15.4 ± 2.4%, Figure 3.4.b) compared to Stage II.

Regarding the inorganic carbon (IC), the average concentration during Stage I (except for the first days) was of 398 ± 15 mg IC/L (Figure 3.5). Since the IC is one of the substrates for the partial nitritation (PN) process, the concentration of IC in the effluent showed a similar trend (Figure 3.5, dotted line) to the ammonium concentration in the effluent (Figure 3.1.a, continuous line). The ratio between the nitrogen and IC concentrations in the feeding define the extent of the PN, which might be hindered by alkalinity limitations: for a N/IC ratio of 1 g N/g C, the available alkalinity allows to oxidize only 50% of the ammonium (if enough DO is provided), while higher N/IC ratios restrict the ammonium oxidation below this 50% threshold (Pedrouso et al., 2017). Durán et al. (2014) reported an optimal N/IC ratio of 0.82 g N/g IC. The N/IC ratio was below this threshold value during Stage I (Figure 3.5), and thus no alkalinity limitation for achieving at least 50% of ammonium oxidation was expected.

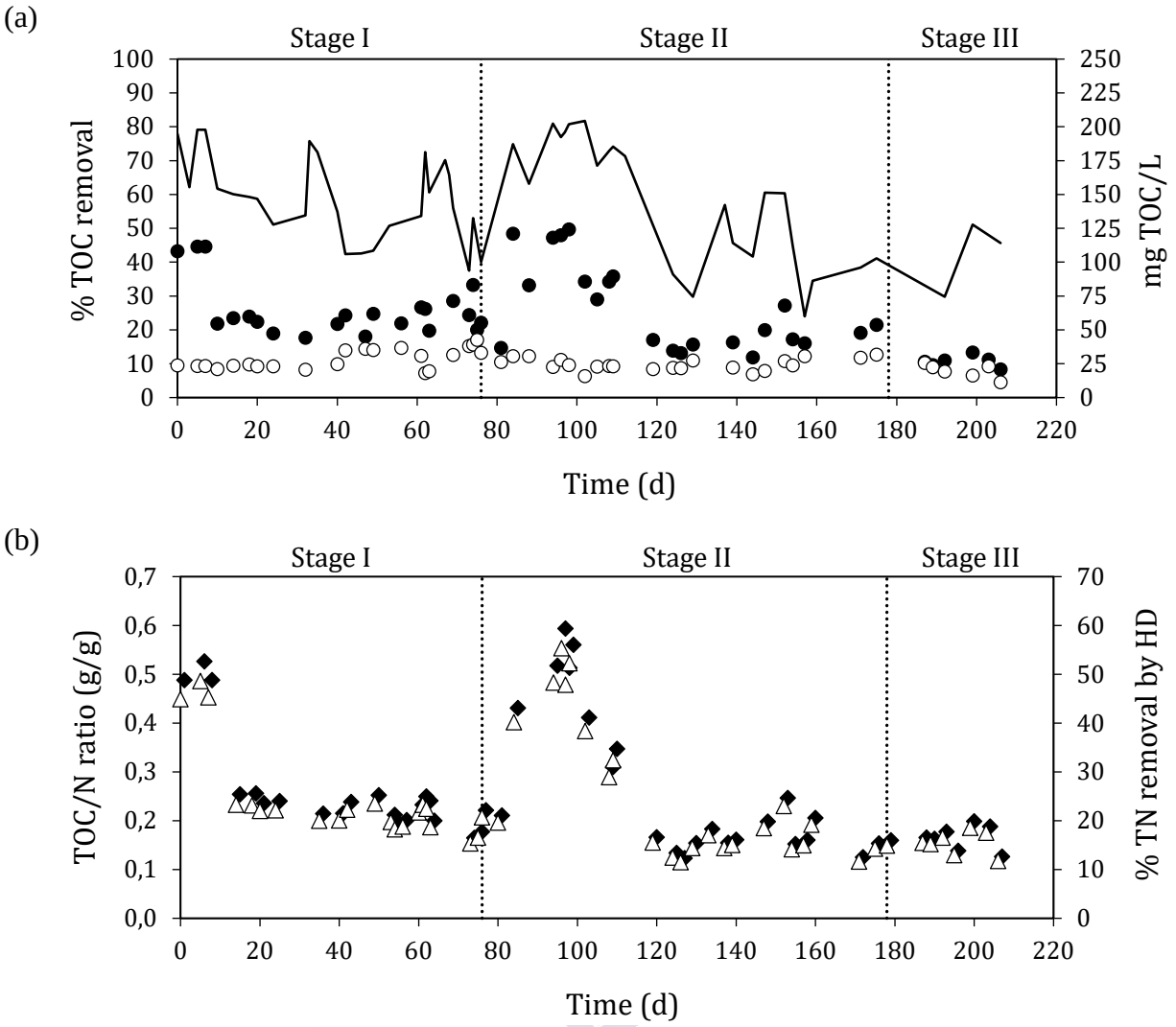


Figure 3.4. Data from the operation of the SBR reactor: (a) concentration of total organic carbon (TOC) in the influent (●) and effluent (○) (mg TOC/L), and % of TOC removal (—); (b) ratio TOC/N in the influent (g TOC/g N, ◆) and maximum % of TN removal by possible heterotrophic denitrification (HD, △).

Although the proportion of digested fish-canning wastewater was higher during Stage II (86%), the concentration of IC fed to the reactor decreased to an average of 277 ± 16 mg IC/L (Figure 3.5), probably due to the varying characteristics of different industrial wastewater batches. The N/IC ratio in the feeding progressively increased up to 1.3 g/g during Stage II, which restricted the oxidation of ammonium by AOB below 50% (Figure 3.1.b, dotted line). Considering the low activity of anammox bacteria, the hindering of the PN probably guided the decreasing efficiency removal of TN, which concurs with the similar trends between TN removal and ammonium removal by AOB in Figure 3.1.b.

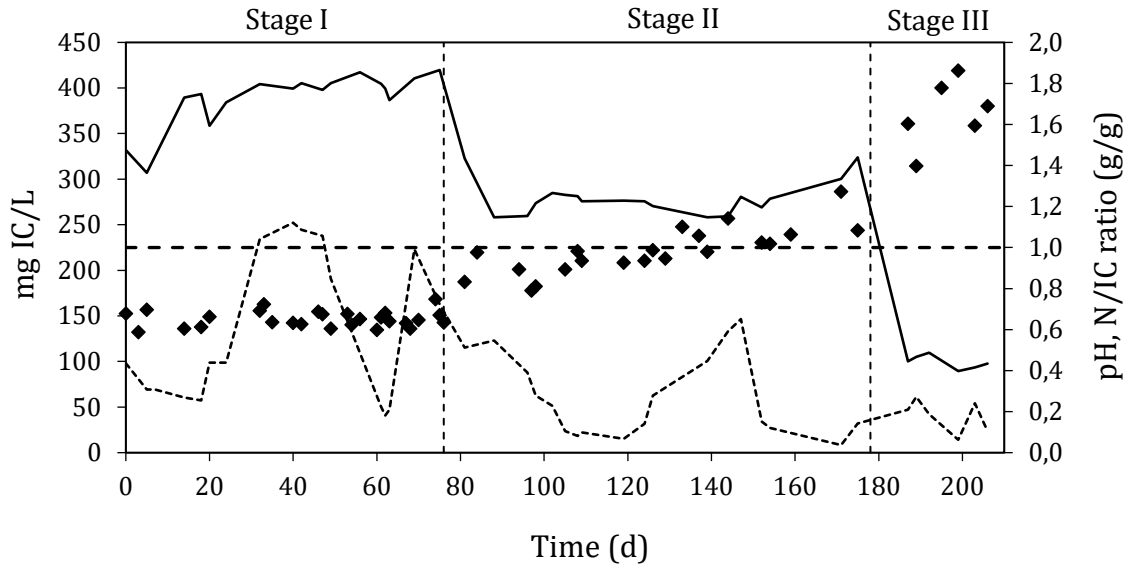


Figure 3.5. Concentration of inorganic carbon (IC) in the influent (—) and effluent (– –) (mg IC/L) of the SBR, ratio N/IC in the influent (g N/g IC, ◆) and N/IC ratio threshold (1 g N/g IC) below which 50% or more of the ammonium can be oxidized (- - -).

The increased N/IC ratio during Stage III (up to 1.9 g/g, Figure 3.5) for the 1:3 diluted feeding limited the oxidation of ammonium below 50%. Still, both the ammonium oxidation by PN and the contribution of denitrification to the removal of nitrogen were in a similar range compared to the end of Stage II (Figure 3.1.b and Figure 3.4.b, respectively). Thus, the slight recovery in the removal of TN up to 20% suggests in turn a certain improvement of the anammox activity, even for the higher N/IC ratios during Stage III. As a conclusion, the acknowledgement of the N/IC ratio in the feeding is crucial to predict the feasibility of the first step of the PN-AMX processes, which can end up driving the removal of total nitrogen.

3.4.5. Granular sludge characteristics

The concentration of solids in the feeding (except for the first days) remained in the range of 33 ± 9 mg VSS/L during Stage I (Figure 3.6), while the concentration in the effluent varied greatly in the range 16 - 178 mg VSS/L. Similarly, during Stage II, the concentrations of the influent and effluent were in the range of 34 ± 8 mg VSS/L and 27 - 135 mg VSS/L, respectively. The reason for these variations in the effluent may be due to operational issues with the withdrawal of the wastewater after each cycle. On the other hand, occasional operational changes like the increase of the settling time in the cycle from 3 min to 10 min by day 129 may explain the decrease in the effluent solids at this point (from 102 mg VSS/L to 40 mg VSS/L, Figure 3.6).

The biomass concentration inside the reactor was not constant, increasing from Stage I (7.3 g VSS/L, day 3) to 9.0 g VSS/L by day 88 (Figure 3.6). This higher biomass concentration observed in the reactor, which occurred at the beginning of Stage II, coincides with the concurrent peak of organic matter removal (Figure 3.4.a) and the higher contribution of HD to the removal of nitrogen (Figure 3.4.b). This, together with the visual perception of higher proportions of flocculent biomass inside the reactor in the first half of Stage II, suggest higher growth of either heterotrophic aerobic and anoxic (denitrifying) bacteria, which masks the actual concentration of granular biomass. Towards the end of Stage II and during Stage III (with no further organic matter in the feeding), the concentration of biomass decreased to final values of 5.8 g VSS/L, in agreement with the lower nitrogen removal efficiencies.

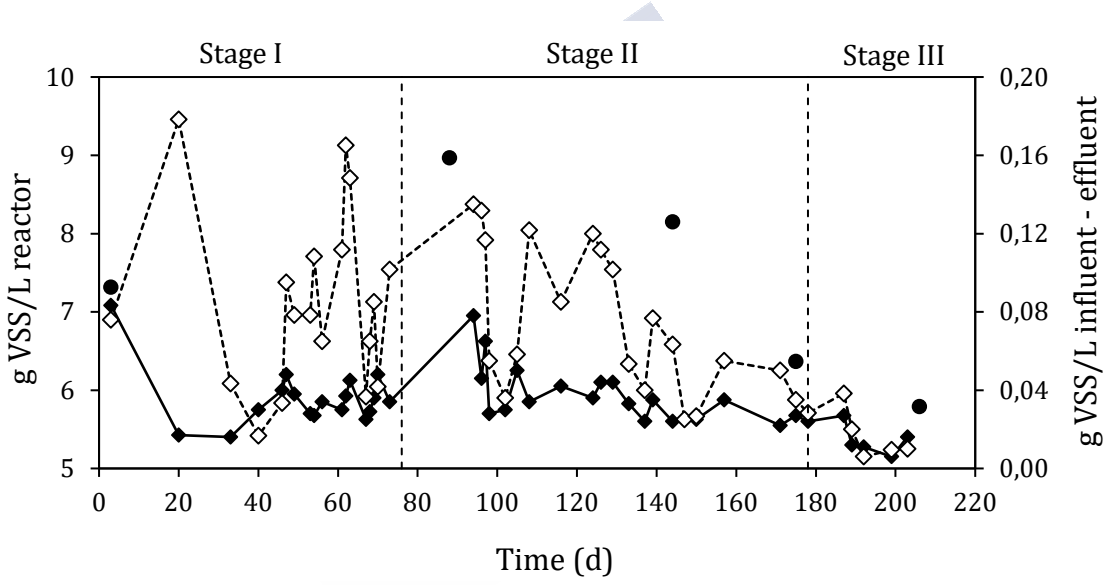
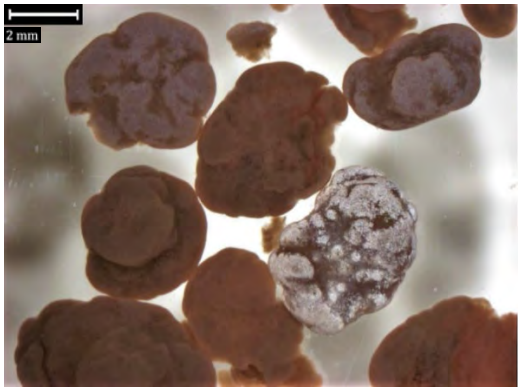


Figure 3.6. Concentration of solids (g VSS/L) in the influent (◆), effluent (◇) and inside the reactor (●).

During Stage I and part of Stage II, the appearance of a whitish layer of precipitates on the surface of some granules took place, a fact that was also found in the precedent operation of the reactor (Val del Rio et al., 2018). The whitish precipitates were intermittently observed between days 10 and 94 of operation, going from a soft layer partially covering the surface of the granules to a more defined, thick layer surrounding almost the entire granule (Figure 3.7.a).

(a) 

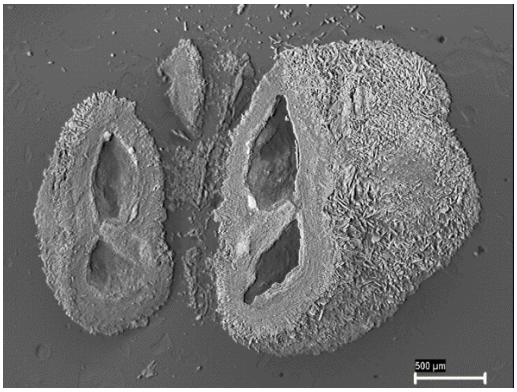
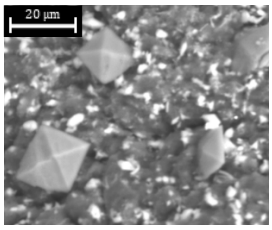
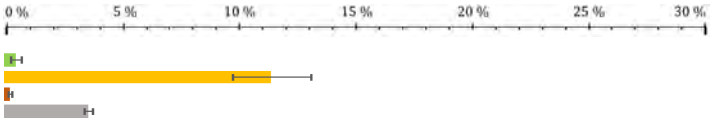
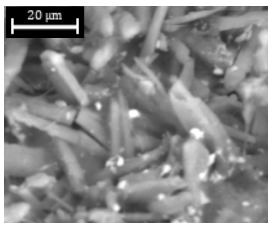
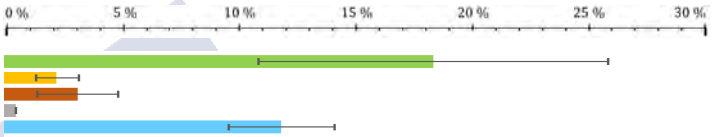
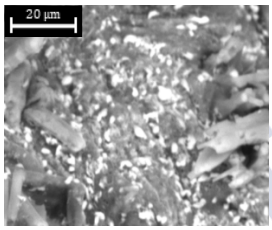
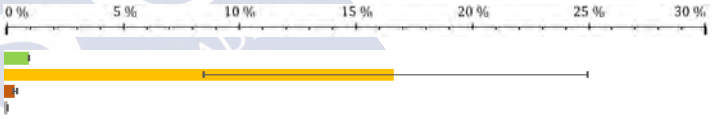
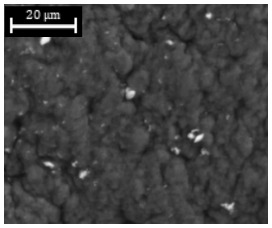
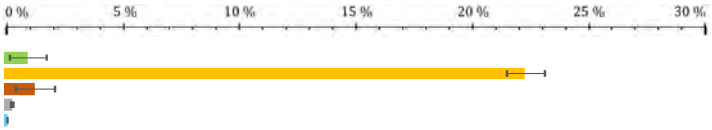
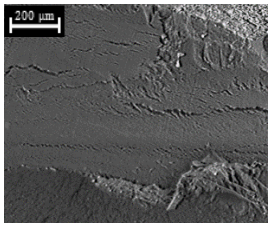
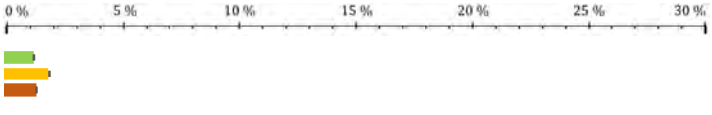
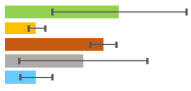
(b) 

Figure 3.7. (a) Picture of PN-AMX granular sludge (day 58) that show the presence of white precipitates in the surface of the granular biomass, (b) SEM image for a sample of granular biomass (day 74).

In order to shed some light over the nature of such precipitates, a granular sample from day 74 was analyzed with the SEM-EDX technique, which provides a magnified image of the sample (Figure 3.7.b, SEM), as well as the spectra and semi-quantitative relation of the detected elements on the surface of the analyzed sample (EDX). Table 3.2 summarizes the relative percentage (in weight) of the main detected elements (except for C and O, which are directly related to the composition of the active biomass), indicating whether a washing or drying step was followed during the preparation of the granular samples for SEM-EDX analysis (see Chapter 2, Section 2.3.3). Several zones were selected to characterize the main elements with the EDX technique: both the precipitates found on the surface (Table 3.2.a-c) and the granular surface itself (Table 3.2.d), as well as the core (or inner surface) of the granule after cutting it (Table 3.2.e, Figure 3.7.b).

As can be observed in Table 3.2, the whitish and rhomboid crystal-shaped precipitates are mainly composed of elemental sulphur (16 - 29% and 11%, Table 3.2.c/a respectively), as well as other zones analyzed in the surface of the granules (22 - 25%, Table 3.2.d), while all the elements other than C and O were below 5% inside the granule (Table 3.2.e). Thus, the main difference between the inner and outer layers of the granules was the elemental sulphur. This fact seems to agree with the higher concentrations of sulphate observed in the effluent during Stage I (203 mg SO_4^{2-}/L, day 58) and Stage II (194 mg SO_4^{2-}/L, day 76) until day 90 approximately (see Figure 3.2.c), with concurrent salt concentrations of 7.4 ± 0.2 g NaCl/L.

Table 3.2. Relative weight percentage (%) of the main elements (other than C and O) identified with the semi-quantitative SEM analysis for different zones of the granular biomass at operational day 74: phosphorus ( ), sulphur ( ), potassium ( ), calcium ( ) and magnesium ( ). "Washed" and "dried" refer to the preparation procedure for the granular biomass before the SEM - EDX analysis.

	SEM pictures (day 74)	Description of the scanned area	Semi-quantitative composition (% weight)
(a)		big rhomboid-shaped grey precipitates on the granular surface, normally found next to (c)	*(washed)* 
(b)		big ridge-shaped grey precipitates on the granular surface, normally found next to (c)	*(washed)* 
(c)		small round-shaped white precipitates, often found spread on the granular surface (see (a), (b) and (d))	*(washed)*  *(dried)* 
(d)		outer surface of the granule	*(washed)*  *(dried)* 
(e)		core of the granule (inner surface after cutting the granule)	*(washed)*  *(dried)* 

However, no straightforward explanation could be found relating the high sulphate concentrations in the reactor and the high relative abundance of elemental sulphur on the granular surface, since anaerobic conditions are required in order to transform SO_4^{2-} into further S^0 (Liu et al., 2012; Wang et al., 2018; Yuan et al., 2015), and the granular surface is more likely to be in aerobic conditions. In any case, these precipitates were already found in the precedent reactor operation, for sulphate concentrations in the effluent up to 300 mg SO_4^{2-}/L (Val del Rio et al., 2018) and similar salt concentrations of 7.9 ± 0.9 g NaCl/L. The formation of such precipitates may suggest a partial explanation to the observed loss in the anammox activity, due to the appearance of substrate transfer limitations between the liquid media and the inner layers of the granules, where anammox are located.

On the other hand, elemental calcium and magnesium had in general a relative percentage below 5% (Table 3.2). Dapena-Mora et al. (2010) observed the formation of precipitates over granular anammox biomass when treating high saline wastewater up to 30 g NaCl/L with $Ca_3(PO_4)_2$ as the main compound justifying these precipitates. Trigo et al. (2006) and Li et al. (2011) observed the precipitation of calcium phosphate salts on anammox granular biomass for synthetic media, which was avoided by proper decrease of the Ca and P concentrations in the feeding. In the present study, the appearance of calcium and phosphorus as the main elements of formed precipitates was mostly observed for the ridge-shaped precipitates (see Table 3.2.b).

3.4.6. Bacterial community of the PN-AMX reactor

To investigate the bacterial diversity in the PN-AMX processes, 16S rRNA bacterial gene libraries were constructed and sequenced for samples from days 20 and 76 of Stage I. More than 1.3 M high-quality sequences (148,905 ± 19,039 per sample) were obtained and clustered into OTUs at 3% cutoff (Table 3.3.a). The bacterial diversity and richness was stable during Stage I (Table 3.3.b), indicating the presence of a community adapted to the operational conditions in the reactor. The microbial community of the PN-AMX reactor is very complex (estimated species for Stage I: 1081 ± 9; Simpson diversity, the higher index the more diverse community: 0.974 ± 0.001). However, the community is markedly uneven (Simpson evenness, the higher the index the more even community: 0.035 ± 0.001) indicating the low evenness of the community, that was dominated by few organisms and maintained numerous low abundant bacteria.

Table 3.3. (a) Distribution of high quality bacterial V4 16S rRNA gene sequences, OTUs and the taxonomic groups at different levels, (b) bacterial alpha diversity, indices of richness, diversity and evenness.

(a)

Sample	No. seqs	OTUs	Phylum	Class	Order	Family	Genera
St.I.d20	181,018	1,213	25	57	120	269	661
St.I.d76	159,804	1,156	24	58	117	261	639

(b)

Sample	Observed species	Simpson diversity	Shannon	Chao1	Evenness
St.I.d20	1097	0.974	6.543	1370	0.035
St.I.d76	1074	0.973	6.440	1369	0.034

The bacteria taxonomic diversity for both samples from Stage I was unequally distributed across 26 phyla, where the 10 most abundant taxa represented over the 90% of the bacterial community (Figure 3.8). The microbial community was dominated by organisms of the *Proteobacteria* phylum (average relative abundance: 65.4 ± 1.8%), especially from the *Beta* (35.4 ± 6.9%), *Alpha* (14.2 ± 0.6%), and *Gammaproteobacteria* (11.1 ± 5.7%). Other major phyla included *Bacteroidetes* (8.5 ± 4.1%), *Firmicutes* (5.7 ± 0.9%), *Actinobacteria* (3.3 ± 1.1%), *Chlorobi* (3.0 ± 0.9%) and *Planctomyces* (2.1 ± 0.1%). These taxa are commonly found in partial nitritation and/or anammox reactors (Agrawal et al., 2017; Dosta et al., 2015; Gonzalez-Martinez et al., 2015; Wang et al., 2017b). The low relative abundance of *Planctomycetes* is in accordance with the low observed anammox activity, although previous reports found the same situation for higher anammox activities (Dosta et al., 2015; González-Martínez et al., 2015). *Chloroflexi*, known to co-exist with anammox bacteria (Yamagishi et al., 2013; Yamamoto et al., 2008) is thought to scavenge organic matter from the dead biomass (Kindaichi et al., 2012) and support granules formation due to its filamentous characteristics (Ni et al., 2011).

The largest fraction of the community was characterized by heterotrophic bacteria. Many of the predominant families (Figure 3.8) were heterotrophic bacteria such as *Rhodocyclaceae*, *Rhodobacteraceae*, *Comamonadaceae*, *Xanthomonadaceae*, *Burkholderiaceae*, *Ignavibacteriaceae*, *Hyphomicrobiaceae* or *Flavobacteriaceae*.

Previous analysis of anammox reactors have already shown that heterotrophic bacteria represent a large fraction of the community (Agrawal et al., 2017; Costa et al., 2014; Dosta et al., 2015; Garcia Costas et al., 2012; Kindaichi et al., 2007; Langone et al., 2014; Laureni et al., 2015; Ni et al., 2012; Persson et al., 2017). Speth et al. (2016) hypothesized that the existence of variable macro- and micro-environments allows the coexistence of such large diversity of heterotrophic bacteria in the reactors. Many of these families also contain known complete or partial denitrifiers and denitrification intermediate reducers (Liu et al., 2012; Ni et al., 2011).

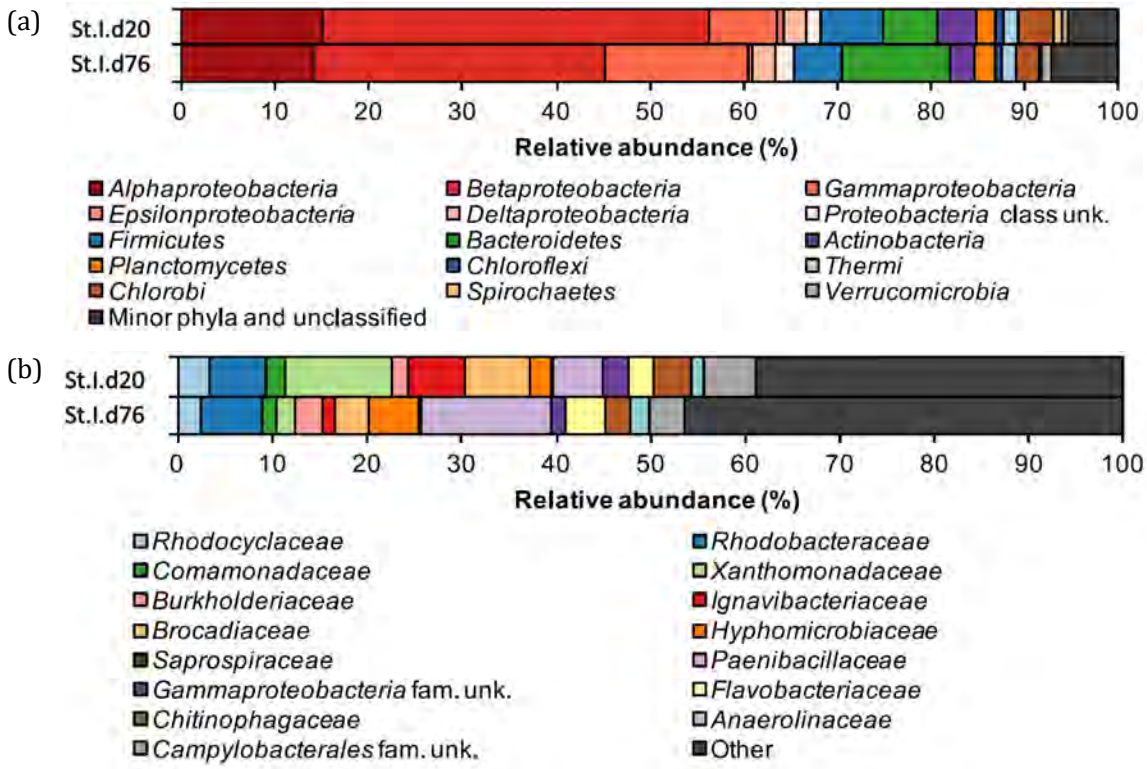


Figure 3.8. Taxonomic profile of the PN-AMX reactor bacterial community for days 20 and 76 of Stage I. (a) Most abundant phyla. For *Proteobacteria* phylum, the different classes are shown; (b) most abundant families. Sample labels indicate the reactor Stage (St.I) followed by the operational day.

The relative abundance for the five most abundant genera of anammox, denitrifying and nitrite oxidizing bacteria are indicated in Figure 3.9 for samples from days 20 and 76. Two anammox bacteria were found within the major genus: Candidatus *Brocadia* and Candidatus *Scalindua*, both with similar relative abundances for days 20 and 76. Previous studies determined that C. *Brocadia* predominate in reactor communities under high nitrite concentrations (see Figure 3.1.a) and COD/N ratios (see Figure 3.4.a) (Jenni et al., 2014; Laureni et al., 2015). Li et al. (2018b) stated that C. *Scalindua* should be the dominant anammox bacteria in saline surroundings, although they might be outcompeted by other anammox species due to the longer doubling times (up to 14 d), as it seems to be the case during Stage I (Figure 3.9).

The relative abundance of the most abundant Candidatus genus (*Brocadia*) was below 2% during Stage I, according to the progressive drop in the NRR below 0.1 g N/(L·d) (Figure 3.1.b). In the precedent reactor operation, the relative abundance of *Brocadia* was between 4 - 6% for a NRR around 0.2 g N/(L·d) (Val del Rio et al., 2018). On the other hand, the relative abundance of denitrifying bacteria was noticeably higher during Stage I (8 - 16% for *Thauera*, 4 - 5% for *Paracoccus*, Figure 3.9), showing a shift between anammox and denitrifying bacteria. The progressive increase of the relative

abundance of denitrifying bacteria, prelude of this bacterial shift, was already observed in Val del Rio et al. (2018), where *Paracoccus* increased from 1.8% to 3.1% and *Thauera* from 0.2% to 1.1%. Heterotrophic bacteria can displace anammox competing for nitrite due to their high growth rates (Jenni et al., 2014; Liang et al., 2014; Zhang et al., 2012). The high maximum contribution of denitrification to the removal of nitrogen during the operation (between 10 - 25% on average, and up to 55% around day 96, Figure 3.4.b) support the idea of the denitrifying microorganisms carrying the main mechanism of nitrogen removal, rather than the anammox bacteria.

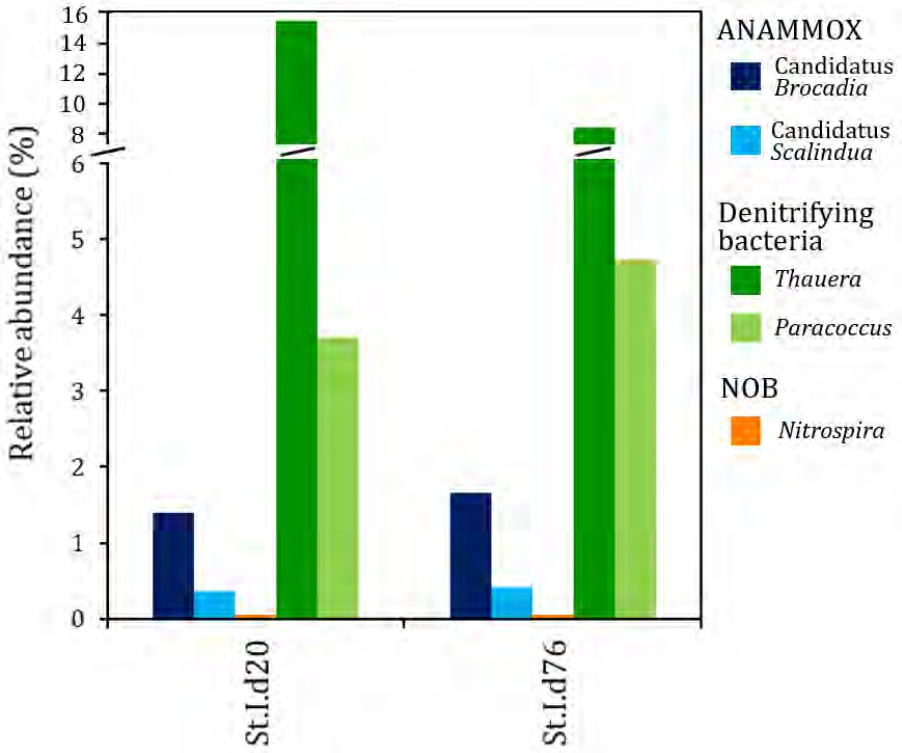


Figure 3.9. Relative abundance of the five most abundant genera of anammox, denitrifying and nitrite oxidizing bacteria. Sample labels indicate the reactor Stage (St.I) followed by the operational day.

Nitrospira was the most abundant NOB detected in the precedent operation, with average relative abundances lower than 0.6% (Val del Rio et al., 2018). This is accordance with the low potential activity of NOB during Stage I due to FA inhibition, and probably owed to long-term concentrations of salt of the precedent operation (Val del Rio et al., 2018), which were maintained during Stage I (7.4 ± 0.2 g NaCl/L, Figure 3.2.a). *Nitrospira* has been previously reported as the main NOB of a PN-AMX system under high salinity conditions (Wang et al., 2017b). When the salinity was kept over 6 g NaCl/L, *Nitrospira* dropped in abundance (agreeing with Val del Rio et al., 2018) and remained as rare bacteria as other NOB (*Nitrobacter*).

Some AOB (*Nitrosospira*, *Nitrosovibrio* and *Nitrosococcus*) were also detected within the rare bacteria (< 0.01%). Previous studies have reported low fractions of AOB and NOB in PN-AMX communities (Agrawal et al., 2017; Almstrand et al., 2014). For days 20 and 76 (Stage I), however, no AOB were detected among the abundant bacteria, despite the better performance of the partial nitritation on day 20 for example (see Figure 3.1.b). One plausible explanation could be the presence of Ammonia Oxidizing *Archaea* (AOA). Unfortunately, Archaea was not explored in this study. AOA group has been detected as the dominant nitrifying community in a marine aquarium biofilter and appears to be widespread in WWTPs (Junier et al., 2010) and that AOA can outcompete AOB as they are less sensitive to salinity (Wang et al., 2017a).

3.5. Conclusions

The study of the PN-AMX processes with industrial saline wastewater indicated that the PN-AMX processes suffered from a concatenation of synergic factors affecting its activity and the removal of nitrogen. On one hand, the conductivity and salinity in the range 12 - 15 mS/cm and 7.3 - 8.2 g NaCl/L, respectively, may have contributed to the hindering of the nitrogen removal, despite the previous long-term exposition of the biomass to similar salt concentrations. The organic matter concentrations were in the range 40 - 120 mg TOC/L, favoured by the fluctuations in the blended feeding, while the nitrogen to inorganic carbon (N/IC) ratio had an average value of 1.55 ± 0.11 g/g at the end of the experiment. The long-term exposition to these organic matter concentrations probably affected both the anammox activity and the removal of nitrogen, which decreased from 50% (NRR of 0.10 g N/(L·d)) to 10% (NRR of 0.02 g N/(L·d)), while the N/IC ratio above the limiting threshold (1 g/g) hindered the removal of ammonium in the partial nitritation due to alkalinity limitations. Furthermore, the presence of high concentrations of phosphate and sulphate led to the appearance of whitish precipitates over the granular PN-AMX biomass, composed of mainly sulphur and phosphorus, which may have diminished the substrate transfer efficiency from the liquid medium.

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Chapter 4

Treatment of pig slurry wastewater with the PN-AMX processes

Summary

Pig manure treatment takes place frequently by anaerobic digestion to diminish the organic matter content and produce biogas. The resulting digestate requires subsequent treatment for the removal of nitrogenous compounds. The partial nitritation-anammox (PN-AMX) processes constitute an alternative for this purpose. In the present study, three different short experiments were performed to study the influence of nitrite oxidizing bacteria (NOB) present in the inoculum and the pig slurry composition over the start-up of the PN-AMX processes. The absence of NOB in the inoculum showed to be more crucial than the available anammox activity for a good performance of the PN-AMX processes. Batch activity experiments showed a reduction of at least 44.4% in the maximum specific anammox activity due to the pig slurry, probably owed to its conductivity (between 6 - 8 mS/cm). In the subsequent long-term operation of the PN-AMX processes with non-diluted pre-treated pig slurry, the NOB activity was successfully limited for DO concentrations of 0.1 mg O_2/L. A nitrogen removal rate (NRR) of 0.1 g N/(L·d) was achieved despite the presence of significant NOB activity in the start-up. A strict control of the DO concentration, with an optimal range of 0.07 - 0.10 mg O_2/L, was fundamental to balance the removal of nitrogen by PN-AMX and prevent NOB activity. The presence of organic matter, with a ratio sCOD/N in the influent between 0.18 and 1.14 g/g, did not hinder the PN-AMX processes performance, and the contribution of heterotrophic denitrification to the removal of nitrogen was less than 10%.

OUTLINE

4.1. Introduction

The increasing pig production in EU and the current trend to concentrate it in small and localized areas to reduce the production costs have led to increased livestock wastes generation (Bernet and Béline, 2009). The abusive use of manure as fertilizer causes a rise in nutrient concentration in soils (mainly N and P), groundwater and surface water (Riaño and García-González, 2015). This growing situation forced pig farms to confront with an increasing number of environmental regulations, concerning the application of manure as direct fertilizer on agricultural land. Thus, sustainable solutions for pig manure treatment regarding nitrogen removal need to be implemented, considering that swine liquid effluents are among the most problematic livestock waste streams (Molinuevo et al., 2009; Waki et al., 2010).

One of the most attractive treatment alternatives to land application is the previous anaerobic digestion (AD) for the removal of organic matter and the production of biogas as a valuable product (see Table 1.14.a), whereas biological removal is the most widely adopted approach regarding the removal of nitrogenous compounds (see Table 1.14.b). With respect to the latter one and far from conventional systems like nitrification-denitrification, new schemes have been investigated based on the anammox (anaerobic ammonium oxidation) process, and this autotrophic nitrogen removal has mainly drawn the attention of researchers (Bernet and Béline, 2009).

In particular, partial nitritation coupled to the anammox process (PN-AMX) is regarded as one of the best suitable options for the removal of nitrogen following anaerobic digestion (Yamamoto et al., 2008). In this combined process, ammonium oxidizing bacteria (AOB) oxidize the ammonium present in the wastewater to nitrite during partial nitritation, and in a second step the autotrophic anammox bacteria use this nitrite as electron acceptor to oxidize the ammonium to nitrogen gas, while nitrate formation is minimized. The interest for this processes focuses on the lower operational costs in comparison with conventional processes (e.g. nitrification-denitrification): less energy and aeration requirements, negligible sludge production and no necessity of external carbon (organic matter) addition (Bernet and Béline, 2009; Yamamoto et al., 2008).

Regarding the application of the PN-AMX processes to pig manure effluents after anaerobic digestion (pig slurry), some compounds of the digested slurry may inhibit the bacterial activities, like for example salts (Scaglione et al., 2017), and certain concentrations of biodegradable organic matter (Molinuevo et al., 2009). Furthermore, the presence of an excessive concentration of biodegradable organic matter may provoke the competition for the nitrite between heterotrophic bacteria and autotrophic anammox bacteria, rather than the competition with AOB for the oxygen (Staunton and Aitken, 2015). However, it is the presence of nitrite oxidizing bacteria (NOB) activity one of the most reported problems at full scale (Lackner et al., 2014), that need to be controlled to avoid the complete

oxidation of ammonium to nitrate, and also to avoid the competition with AOB for oxygen and with anammox bacteria for nitrite (Magrí et al., 2012). Microaerobic conditions, for example, can favor the partial nitritation to nitrite instead of the total nitrification to nitrate (Staunton and Aitken, 2015), as well as sufficiently high concentrations of free ammonia (FA) in the system (Pedrouso et al., 2017).

More than 100 PN-AMX installations have been implemented at full-scale over the last decade, the majority of them applied the one-stage configuration for the treatment of the supernatant from sludge anaerobic digester in municipal WWTP (Lackner et al., 2014; Pedrouso et al., 2018). However, the full-scale application of the PN-AMX processes for the treatment of pig slurry using the aforementioned alternative is still scarce (Magrí et al., 2013) and more research is necessary, principally to evaluate the presence of NOB activity in the available inoculum and the possible inhibitory effects of slurry compounds over the anammox activity.

4.2. Objectives

The research work presented aims at evaluating the operation of the PN-AMX processes in a single unit for the treatment of pig slurry with significant NOB activity in the inoculum. Special attention was paid to the loss of anammox activity due to the conductivity and the organic matter content of the pre-digested slurry is discussed. The gathered knowledge was meant to address potential issues during the start-up of the process for its application at full-scale.

4.3. Materials and methods

4.3.1. Experimental set-up

Two laboratory sequencing batch reactors (SBRs) with working volumes of 0.65 and 1.4 L were used for three short experiments (SE) and a continuous operation period, respectively (Table 4.1). The aeration system for each of the reactors consisted of a diaphragm pump (Laboport N86, KNF) which supplied air through a diffuser located at the bottom of the reactor, promoting the aeration with the formation of small bubbles. The aeration inside the reactor provided both a good mixture and the dissolved oxygen (DO) necessary to carry out the biological aerobic processes. The DO concentration was measured periodically with a DO probe (Hach Lange LD01) and was manually regulated by changing the opening degree of an air valve located in the gas inlet conduction.

Table 4.1. Characterization of the pre-digested pig slurry feeding and operational conditions in the SBRs during the short and continuous experiments.

	Short experiments			Continuous experiment	
	SE-1	SE-2	SE-3	Stage I	Stage II
Days	0 - 42	0 - 42	0 - 43	0 - 78	79 - 225
Feeding (pre-digested pig slurry)					
Dilution ratio	ND - 1:3 *	1:2	1:2	ND	ND
pH	7.8 ± 0.1	7.8 ± 0.1	7.8 ± 0.2	7.9 ± 0.1	8.2 ± 0.2
NH_4^+ (mg N/L)	111 - 531	230 ± 50	137 - 201 **	429 ± 31	513 ± 23
sCOD (mg/L)	94 - 230 **	151 ± 23	130 ± 39	232 ± 65	182 ± 58
Ratio sCOD/NH_4^+-N (g/g)	0.5 - 0.9 **	0.62 ±	0.20 - 1.14	0.18 - 0.74	0.21 - 0.65
IC (mg C/L)	46 - 634 **	289 ± 20	185 ± 17	487 ± 34	524 ± 37
NaCl (g/L)	0.33 - 1.01	0.23 ±	0.42 ± 0.02	0.97 ±	1.01 ± 0.05
Conductivity (mS/cm)	2.2 - 7.5 **	3.3 ± 0.8	2.68 ± 0.46	5.8 ± 0.8	8.6 ± 2.6
Operational Conditions					
DO (mg O_2/L)	0.02 - 0.67	0.01 - 0.70	0.01 - 0.15	0.04 - 0.18	0.02 - 0.15
Temperature (°C)	33.3 ± 1.0	33.2 ± 1.5	34.5 ± 2.0	33.1 ± 1.1	32.2 ± 1.8
HRT (d)	0.78 ±	0.70 ±	0.82 ± 0.12	1.64 ±	1.65 ± 0.11
Reactor volume (L)	0.65	0.65	0.65	1.40	1.40
VER (%)	64.7 ± 8.4	72.0 ± 3.3	64.2 ± 3.7	30.6 ± 1.2	30.4 ± 1.5
Aeration pulse (ON:OFF, s)	2:8	1:9	1:9	1:9	1:9

DO: dissolved oxygen; HRT: hydraulic retention time; IC: inorganic carbon; ND: no dilution; sCOD: soluble COD; VER: volume exchange ratio.

* No dilution between days 0 – 9; 1:3 dilution between days 10 - 42.

** A range of values (minimum-maximum) is presented, instead of the average value and the standard deviation, when there is a high variation in the values.

The SBRs were operated at a temperature range between 32 - 35 ^oC (Table 4.1) by means of a thermostatic bath, which is close to the expected values for the pig slurry wastewater after mesophilic AD (Yamamoto et al., 2008). The operational cycles were of 12 h distributed in different phases (Table 4.2). Due to the low inlet flow rate, the feeding step was performed discontinuously in shifts of 20 min (10 min ON - 10 min OFF). In order to have the DO concentration necessary only for the partial nitritation process, intermittent aeration was provided during all the aeration periods (Table 4.1).

Table 4.2. Distribution of the operational cycle (min) for the short and continuous experiments.

	Short experiments			Continuous experiment					
	SE-1	SE-2	SE-3	Stage I			Stage II		
Days	0 - 42	0 - 42	0 - 43	0 - 19	20 - 31	32 - 78	79 - 101	102 - 173	174 - 225
Feed + Aeration	660			660	660	80	680	660	645
Aeration (only)	20			20	20	-	-	-	-
Idle phase	-			-	-	-	-	10	10
Anoxic phase[a]	-			-	-	-	-	20	40
Settling	20			20	15	15	15	15	10
Withdrawal	20			20	25	25	25	15	15

[a] During the anoxic phase, the mixing of the liquid into the reactor was provided through internal recirculation

The pre-treated pig slurry was periodically collected and stored at 4 ^{o}C from a pig farm (Chiclana, Cádiz). In the farm, the solids were previously separated from the slurry, in a mechanical grid, before the organic matter removal in an anaerobic expanded granular sludge bed (EGSB) reactor at pilot scale (4.7 m^3).

4.3.2. Operational conditions

Due to the low availability of PN-AMX inoculum with negligible NOB activity for the start-up of a future pilot plant treating pig slurry, different short experiments (SE-1, SE-2, SE-3) were previously performed at laboratory scale to compare the start-up of the reactor with distinct inocula regarding the specific anammox activity (SAA) and the maximum nitrogen removal rates (NRR_{max}, Table 4.3). In these short experiments the pre-treated pig slurry fed was diluted (Table 4.1). Furthermore, the influence of the pre-treated pig slurry over the start-up of the PN-AMX processes was assessed in SE-3 by a SAA batch test.

After the short experiments, the long-term operation of the PN-AMX processes with no diluted pre-treated pig slurry was evaluated. The start-up was with PN-AMX biomass with a specific anammox activity (SAA) of 0.448 g N/(g VSS·d) (Table 4.3). The feeding had nitrogen and organic matter concentrations of 429 - 513 mg NH_4^+-N/L and 182 - 232 mg sCOD/L, respectively (Table 4.1). The operation lasted 225 days and was divided in two stages: Stage I (days 0 – 78, active presence of NOB) and Stage II (days 79 – 225, limitation of NOB activity). An anoxic phase was implemented in Stage II on day 102 (Table 4.2), in which the supernatant was recirculated (1:8 recirculation ratio) to the bottom of the reactor (internal conduction and peristaltic pump) to provide a gentle mixture of the settled biomass.

Table 4.3. Characteristics of the mixed biomass inoculated in the different experimental periods.

	Short experiments			Continuous experiment	
	SE-1	SE-2	SE-3	Stage I	Stage II
Source of mixed inoculum	PN-AMX (PP, urban WW)	AMX (lab, synthetic) + SE-1	AMX (lab, synthetic) + SE-2	PN-AMX (PP, pig slurry [a]) + AMX (lab, synthetic) + SE-3	Stage I
Solids (g VSS/L)	8.81	7.12	5.50	6.45	4.41
SAA_{PBS} (g N/(g VSS·d))	0.071 ±	0.049 ±	0.322 ±	0.448 ± 0.004	0.102 ±
SAA_{slurry} (g N/(g VSS·d))	-	-	0.179 ±	-	0.082 ±
NRR_{max} (g N/(L·d))	0.63	0.35	1.77	2.89	0.45

[a] Anaerobically pre-digested pig slurry

NRR_{max}: maximum nitrogen removal rate capacity; PN-AMX: partial nitritation-anammox; PP: pilot plant; SAA_{PBS}: specific anammox activity with phosphate buffer solution (PBS) as medium; SAA_{slurry}: specific anammox activity with sewage slurry as medium.

3.3.3. Analytical methods

Analytical determination of ammonium (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N), chemical oxygen demand (COD), conductivity, pH, total suspended solids (TSS) and volatile suspended solids (VSS), was carried out according to the Standard Methods (APHA/AWWA/WEF, 2012). The inorganic carbon (IC) concentration was determined by a Shimadzu analyzer (TOC-L_{CSN}, automatic sample injector Shimadzu ASI-L). Cation and anion concentrations were determined by ion chromatography with an Advanced Compact IC system (861, Metrohm), equipped with a CO_2 suppressor (MCS 853, Metrohm) and a sample processor/injector (838, Metrohm). For all these previous measurements the samples were filtered by 0.45 µm, except for VSS. Full description of the analytical methods is provided in Chapter 2 (Section 2.1).

The SAA, expressed as g N/(g VSS·d), was assessed in batch tests at 30 °C by periodically measuring the headspace overpressure due to the production of nitrogen gas by anammox bacteria with a differential pressure transducer (Centerpoint Electronics). The methodology described by Dapena-Mora et al. (2007) was followed, which is fully described in Chapter 2, Section 2.3.2.1. The SAA tests were performed for the short and continuous experiments using two different liquid medium (Table 4.3): phosphate buffer solution (PBS) to determine the maximum activity (SAA_{PBS}), which serves as control, and AD pre-treated pig slurry to determine the reduction in the SAA in comparison with the control (SAA_{slurry}) for SE-3 and Stage II. In all these tests, both ammonium (as NH_4Cl) and nitrite (as $NaNO_2$) were added as substrates, with the latter as the sole substrate when the slurry (instead of phosphate buffer solution – PBS) was used as liquid medium due to the ammonium excess in the slurry.

4.3.4. Molecular techniques and sequence analysis

The identification of active bacterial populations was carried out by the Fluorescence *in situ* Hybridization (FISH) technique for biomass samples from SE-1 (inoculum and day 33), SE-3 (inoculum and day 43), Stage I (days 19, 64) and Stage II (days 176, 225). The fixation, hybridization and capture of fluorescence signals of the biomass samples were performed according to Amann et al. (1990). Bacterial cells hybridized with FISH probes $EUB338_{mix}$ for the *Bacteria* domain and Ntspa712 for phylum *Nitrospirae*, and all probes were 5' labelled by fluorochromes FITC (Fluorescein-5-isocyanate) or Cy3 (Carbocyanine 3). DAPI (4, 6-diamidino-2-phenylindole) was used as universal dye for detection of all DNA in the samples. Total genomic DNA was extracted and sequenced according to Alonso-Gutiérrez et al. (2009). The methodologies used for FISH and DNA extraction and sequencing are fully described in Chapter 2 (Sections 2.4.1 and 2.4.2, respectively).

4.3.5. Calculations

The ammonia and nitrite oxidation rates due to AOB and NOB activities (AOR and NOR, respectively), as well as the nitrogen loading and removal rates (NLR and NRR, respectively) and the total nitrogen (TN) removal efficiency were estimated based on nitrogen balances and the anammox process stoichiometry, and expressed as g N/(L·d), following eq. 2.9, eq. 2.10, eq. 2.5, eq. 2.7 and eq. 2.12, respectively (Chapter 2, Section 2.5.1.1 and 2.5.1.2). The NRR_{max} (as g N/(L·d)) of the biomass was determined by multiplying the SAA_{PBS} (eq. 2.27, Section 2.5.4.2) by the solids concentration inside the reactor. The maximum nitrogen removal percentage by a possible heterotrophic denitrification (HD) process was determined based on the mass balance of the soluble chemical oxygen demand (sCOD), following eq. 2.14 (Section 2.5.1.2). Since part of the organic matter can be consumed for growth and/or aerobic oxidation due to the presence of oxygen, this maximum TN removal percentage considers only the maximum "potential" or contribution for the HD, not the actual.

The concentration of free ammonia (FA, NH_3) and free nitrous acid (FNA, HNO_2) were calculated at the operational temperature based on both the NH_4^+ and NO_2^- concentrations, respectively, and the pH in the bulk liquid (i.e., in the effluent of the reactor) according to Anthonisen et al. (1976) (eq. 2.15 and eq. 2.16, Section 2.5.1.3, Chapter 2).

4.4. Results and discussion

4.4.1. Short experiments to evaluate the start-up of the PN-AMX processes for pig slurry treatment

In the first short experiment (SE-1, Figure 4.1) the inoculum used was collected from a PN-AMX pilot plant treating the supernatant of an anaerobic sludge digester in a municipal WWTP. The inoculated biomass had a SAA_{PBS} of 0.071 g N/(g VSS·d) and the concentration of solids was of 8.81 g VSS/L (Table 4.3). This means a NRR_{max} of approximately 0.63 g N/(L·d), which was lower than the applied NLR in the first 9 operational days (between 0.7 - 0.8 g N/(L·d), Figure 4.1.a). Despite the later decrease in the applied NLR to 0.2 - 0.3 g N/(L·d) by the dilution of the pig slurry (1:3), the anammox activity was not recovered and it was estimated to be approximately of 0.011 g N/(g VSS·d) inside the reactor at the end of SE-1, which means a decrease of 84% regarding the maximum SAA_{PBS} of the inoculum in batch tests. During SE-1 the concentration of nitrite in the effluent was negligible, while the concentration of nitrate was up to 170 mg NO_3^--N/L (Figure 4.1.b). The ratio between nitrate produced and ammonium oxidized was of 0.3 - 1.0 g N/g N (Figure 4.1.c), which was higher than the theoretical value for the PN-AMX processes (0.11 g N/g N), suggesting the active presence of NOB which may compete with the anammox bacteria for the nitrite. The ammonium removal percentage was over 80% from day 25 on and, consequently, the concentration of FA was below 10 mg NH_3-N/L (Figure 4.1.c), which did not have an inhibitory effect over the NOB. Due to the activity of NOB, the removal efficiency for total nitrogen decreased from 49 - 55 % to values below 30%, as the NRR dropped from 0.2 below 0.1 g N/(L·d) (Figure 4.1.a).

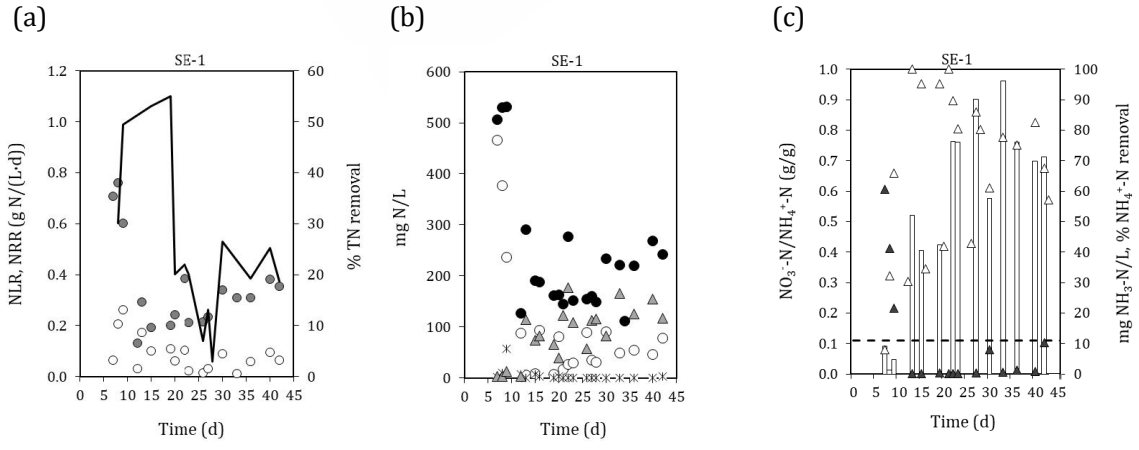

Figure 4.1. Data from the short experiment **SE-1**: (a) Nitrogen Loading Rate, NLR (g N/(L·d), ●), Nitrogen Removal Rate, NRR (g N/(L·d), ○) and % of total nitrogen (TN) removal (—); (b) concentration of NH_4^+-N in the influent (●), and NH_4^+-N (○), NO_2^--N (∗) and NO_3^--N (▲) in the effluent (mg N/L); (c) nitrate produced to ammonium oxidized ratio (g NO_3^--N/g NH_4^+-N, ▯), concentration of free ammonia (mg NH_3-N, ▲). % of NH_4^+-N removal (△) and stoichiometric nitrate produced to ammonium consumed ratio for the PN-AMX processes (0.11 g N/g N) (**- - -**).

In the second short experiment (SE-2) the inoculum consisted of a mixture of biomass from previous SE-1 and biomass from a laboratory reactor performing only the anammox process (fed with synthetic medium and operated at 30 °C). The mixed sludge used as inoculum had a NRR_{max} capacity of 0.35 g N/(L·d) (Table 4.3) and the applied NLR (Figure 4.2.a) was similar to that in SE-1. Furthermore, in SE-2 the air pulse was reduced from 2:8 to 1:9 (ON:OFF, in seconds) to limit the NOB activity, with a similar range of operational DO concentration (0.01 - 0.70 mg O_2/L, Table 4.1) compared to SE-1. In SE-2 the nitrate was completely consumed by day 19 (Figure 4.2.b), while both the NRR and the TN removal percentage slightly increased to values close to the end of SE-1 (Figure 4.2.a). The concentration of ammonium in the effluent above 200 mg NH_4^+-N/L from day 19 to 25 (Figure 4.2.b), owed to the limiting DO concentration conditions, provoked concentrations of FA up to 30 mg NH_3-N/L (Figure 4.2.c). This low oxidation efficiency of ammonium by AOB (below 30%, Figure 4.2.c) provoked a deficiency of nitrite. Therefore, the low oxidation of ammonium rather than the FA concentration was probably the main reason for NOB activity limitation in this case.

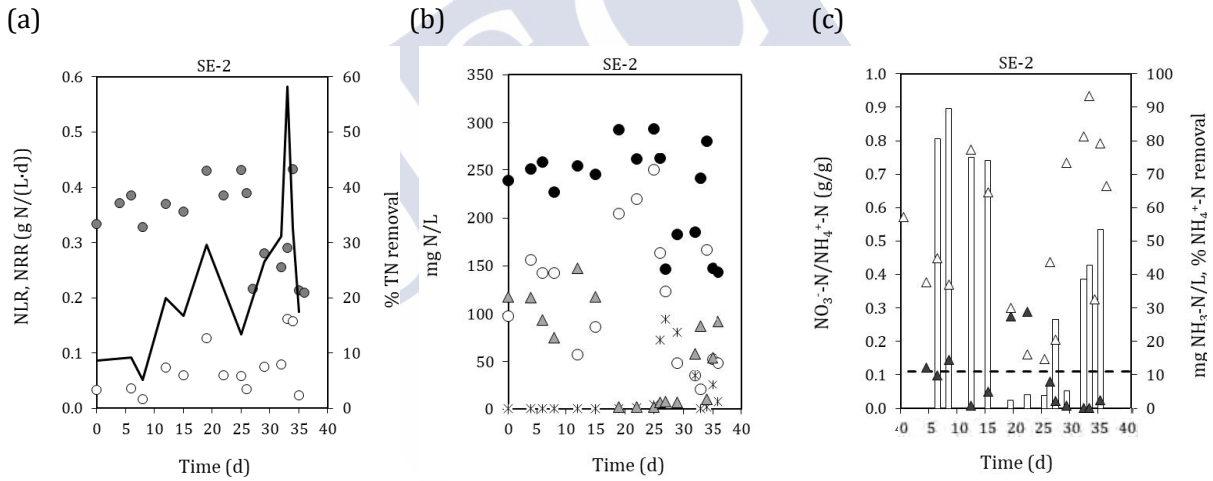

Figure 4.2. Data from the short experiment **SE-2**: (a) Nitrogen Loading Rate, NLR (g N/(L·d), ●), Nitrogen Removal Rate, NRR (g N/(L·d), ○) and % of total nitrogen (TN) removal (—); (b) concentration of NH_4^+-N in the influent (●), and NH_4^+-N (○), NO_2^--N (∗) and NO_3^--N (▲) in the effluent (mg N/L); (c) nitrate produced to ammonium oxidized ratio (g NO_3^--N/g NH_4^+-N, □), concentration of free ammonia (mg NH_3-N, ▲), % of NH_4^+-N removal (△) and stoichiometric nitrate produced to ammonium consumed ratio for the PN-AMX processes (0.11 g N/g N) (**- - -**).

In the third short experiment (SE-3), the inoculum consisted of a mixture of biomass from previous SE-2 and the biomass from a laboratory reactor performing only the anammox process. In this case, the inoculum had a NRR_{max} capacity of 1.77 g N/(L·d) (Table 4.3). This NRR_{max} was high in comparison with the applied NLR (0.25 g N/(L·d), Figure 4.3.a) and with previous experiments SE-1 and SE-2. Furthermore, in SE-3 a lower DO concentration (only up to 0.20 mg O_2/L) than in SE-1 and

SE-2 was used (Table 4.1). Therefore, in the first days the nitrogen removal was favored with a NRR of 0.20 g N/(L·d) and TN removal efficiencies of approximately 80% (Figure 4.3.a). However, the concentration of ammonium in the effluent progressively increased and reached values close to those in the influent (Figure 4.3.b), decreasing the NRR to values below 0.05 g N/(L·d). Thus, the removal of nitrogen was clearly hindered in SE-3 in the partial nitritation step, as seen in the percentage of ammonium removal depletion from 96% to below 20% (Figure 4.3.c). This was probably owed to the DO-limiting conditions to avoid NOB activity. The ratio between nitrate produced and ammonium oxidized was lower than in SE-1 and SE-2, but it was still higher than stoichiometric one (Figure 4.3.c). This fact suggests that the activity of NOB was not completely inhibited despite the lower DO concentration used (Table 4.1) and the limited AOB activity.

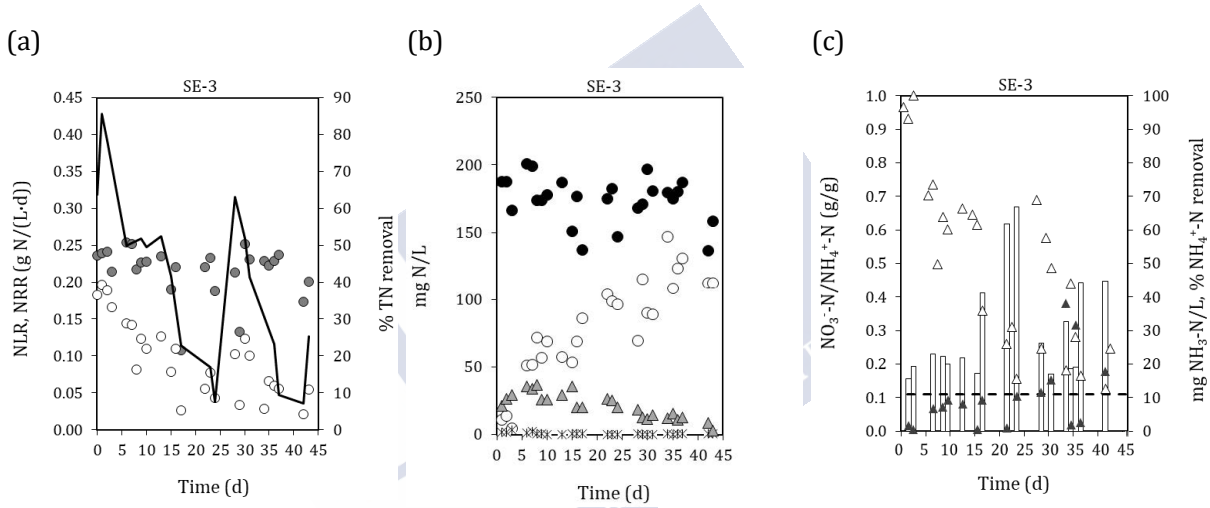

Figure 4.3. Data from the short experiment **SE-3**: (a) Nitrogen Loading Rate, NLR (g N/(L·d), ●), Nitrogen Removal Rate, NRR (g N/(L·d), ○) and % of total nitrogen (TN) removal (—); (b) concentration of NH_4^+-N in the influent (●), and NH_4^+-N (○), NO_2^--N (∗) and NO_3^--N (▲) in the effluent (mg N/L); (c) nitrate produced to ammonium oxidized ratio (g NO_3^--N/g NH_4^+-N, ▯), concentration of free ammonia (mg NH_3-N, ▲), % of NH_4^+-N removal (△) and stoichiometric nitrate produced to ammonium consumed ratio for the PN-AMX processes (0.11 g N/g N) (**- - -**).

Therefore, the start-up of the PN-AMX processes was highly influenced by the presence of NOB activity in the inoculum, independently of using low (SE-1 and SE-2) or high/extra (SE-3) anammox activity, based on SAA measurements.

Apart from the presence of NOB activity, another aspect to consider is that the composition of the pre-treated pig slurry may have an impact over the anammox activity. In order to estimate its influence, the SAA_{PBS} and SAA_{slurry} were determined for the inoculum of SE-3. The values obtained of SAA_{PBS} = 0.322 g N/(g VSS·d) and SAA_{slurry} = 0.179 g N/(g VSS·d) (Table 4.3) indicated a reduction in

the anammox activity of 44.4% with the pig slurry. Therefore, it is probable that this decrease in the anammox activity will favor the nitrite availability for NOB inside the PN-AMX system, promoting their development, and as a circular effect, outcompeting the anammox bacteria.

4.4.2. Long-term performance of the PN-AMX processes

In the long-term operation of the PN-AMX processes treating pig slurry (without dilution) the inoculum used was a mixture of sludge from SE-3 and sludge from a PN-AMX pilot plant treating the same pig slurry. This inoculum had a NRR_{max} capacity of 2.89 g N/(L·d) (Table 4.3), which was high in comparison with the applied NLR (0.25 - 0.35 g N/(L·d), Figure 4.4.a) and also in comparison with the previous short experiments. The NRR at the beginning of Stage I was between 0.15 - 0.20 g N/(L·d) with TN removal efficiencies of 60 - 70% (Figure 4.4.a). However, the concentration of inorganic carbon (IC) in the effluent between days 50 to 64 (below 25 mg IC/L) was much lower compared to previous values during Stage I (172 ± 70 mg IC/L). Since the percentage of ammonia removal (94 ± 5%, Figure 4.4.c), the ratio between nitrate produced and ammonium oxidized (0.7 ± 0.1 g N/g N, Figure 4.4.c) and the nitrate concentration in the effluent (269 ± 51 mg NO_3^--N/L, Figure 4.4.b) indicate a high activity for both AOB and NOB between days 50 to 64, the IC depletion was probably owed to its consumption during the nitrification. Consequently, the pH in the reactor dropped to 5.0 (days 50 - 64), very low in comparison with the previous pH values during Stage I until day 50 (8.0 ± 0.2). Subsequently, the removal of nitrogen decreased to 30%, with a NRR of approximately 0.05 g N/(L·d) (Figure 4.4.a).

The physiological pH range for anammox bacteria is 6.7 - 8.3 (Strous et al., 1999), while Lackner et al. (2014) found that pH values below 6.8 entail loss of anammox activity. Therefore, the low pH values attained until day 50 probably affected the anammox activity. On the other hand, the 2-fold increase in the nitrate concentration between days 58 to 63 (up to 300 mg NO_3^--N/L, Figure 4.4.b) strongly suggests NOB activity and probably the outcompetition of the anammox bacteria in these conditions, as it will be later demonstrated from the microbiology results.

In Stage I, no limitation in the activity of NOB was observed when more than 80% of ammonium was removed and the FA concentration was below 12 mg NH_3-N/L (for example, days 41 – 63, Figure 4.4.c). Afterwards, the oxidation of ammonium decreased from 95% to 30% due to the decrease in the DO concentration from 0.10 to 0.05 mg O_2/L, and the nitrate diminished from 300 to 33 mg NO_3^--N/L within 25 days (Figure 4.4.b), indicating the hindering of NOB activity due to the low oxidation of ammonium to nitrite. This observation is in accordance with the decreasing NRR and removal percentage of TN during Stage I (Figure 4.4.a), as well as the lower ratio between nitrate produced and ammonium oxidized between days 68 - 80 (from 0.7 to 0.2 g N/g N, Figure 4.4.c).

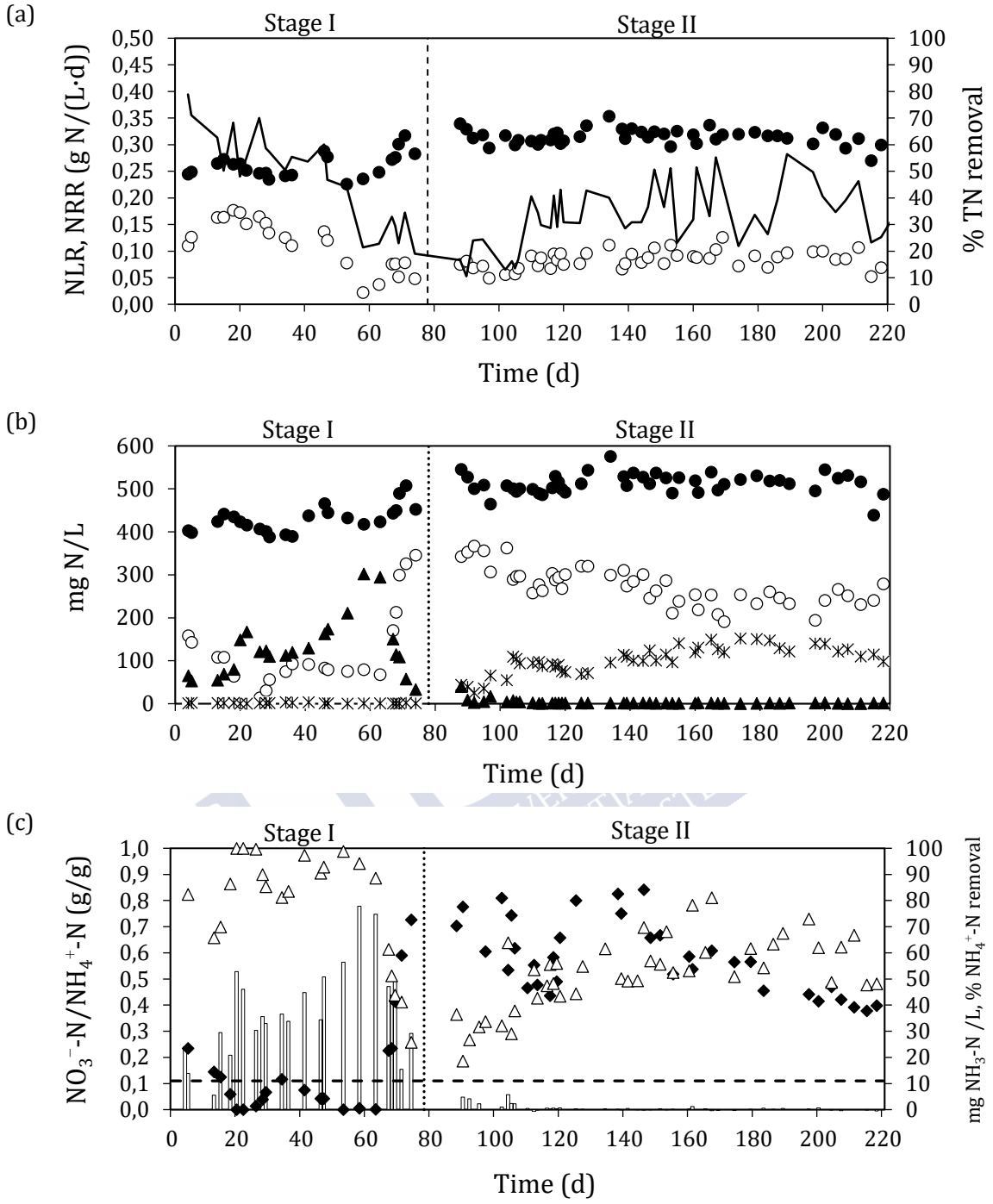

Figure 4.4. Data from the continuous experiment (Stages I-II): (a) Nitrogen Loading Rate, NLR (g N/(L·d), ●), Nitrogen Removal Rate, NRR (g N/(L·d), ○) and % of total nitrogen (TN) removal (—); (b) concentration of NH_4^+-N in the influent (●), and NH_4^+-N (○), NO_2^--N (∗) and NO_3^--N (▲) in the effluent (mg N/L); (c) nitrate produced to ammonium oxidized ratio (g NO_3^--N/g NH_4^+, ▯), concentration of free ammonia (mg NH_3-N, ◆), % of NH_4^+-N removal (△) and stoichiometric nitrate produced to ammonium consumed ratio for the PN-AMX processes (0.11 g N/g N) (- - -).

As a consequence of the NOB activity limitation and the previous inhibition of the anammox biomass, the nitrite was accumulated and increased in Stage II up to 170 mg NO_2^--N/L (Figure 4.4.b). The accumulation of nitrite is one of the main problems regarding the deterioration of anammox systems (Li et al., 2018). However, there is no clear nitrite inhibition threshold reported in the literature: Strous et al. (1999) consider a safe range below 100 mg NO_2^--N/L, while other authors report no inhibition of the anammox process below 250 mg NO_2^--N/L (Dapena-Mora et al., 2006; Fernández et al., 2012; Jaroszynski et al., 2011) and for nitrite concentrations as high as 400 mg NO_2^--N/L (Kimura et al., 2010). On the other hand, Jetten et al. (1998) indicated low performance of the anammox bacteria above 140 mg NO_2^--N/L and complete inhibition above 280 mg NO_2^--N/L, similarly to Strous et al. (1999), and Isaka et al. (2007) confirmed 280 mg NO_2^--N/L as the inhibition threshold for anammox bacteria. In summary, the high nitrite concentrations observed during Stage II (111 ± 22 on average, Figure 4.4.b) are closer to the safe (< 100 mg NO_2^--N/L) rather than to the warning (280 mg NO_2^--N/L) zones suggested by Jin et al. (2012). Still, the long-term exposure to high nitrite concentrations (from day 104 on) may act as another synergistic inhibiting factor that prevented the anammox bacteria from recovering.

In order to stimulate activity of anammox bacteria through the consumption of this nitrite, an anoxic phase was implemented in the cycle of the SBR during Stage II (day 102, Table 4.2). The strict control of the DO concentration allowed the ammonium removal to reach the 60% on average (Figure 4.4.c), with ammonium concentrations in the effluent between 200 and 300 mg NH_4^+-N/L (Figure 4.4.b), and the removal of nitrogen was able to recover up to a 36 ± 9% on average. The concentrations of soluble COD (sCOD) in the influent and effluent had average values of 213 and 182 mg sCOD/L, respectively, which means a potential 3% of nitrogen removal due to the HD. The low sCOD/N ratio in the feeding (0.4 g sCOD/g N for Stage II) was probably not enough for the denitrifiers to act as a supplementary aid for the removal of nitrogen.

As opposed to the short experiments, where the NOB activity suppression was not maintained with time and was only achieved sporadically when the oxidation of ammonium to nitrite was limited, in Stage II it was maintained stable for more than 120 days favored by the high concentrations of FA in the reactor (between 40 - 85 mg NH_3-N/L, Figure 4.4.c).

4.4.3. Bacterial community of short and long-term experiments

In order to confirm the presence of the nitrite oxidizing bacteria (NOB), the community of *Nitrospira* bacteria was identified by FISH analysis. Positive results were obtained by the application of the general Bacteria domain probe ($EUB338_{mix}$) and the specific *Nitrospira* probe (Ntspa712), as an

indication of the active presence of the NOB genus. As expected, the NOB were clearly detected for the inoculum of SE-1 and SE-3 (Figure 4.5.a and Figure 4.5.c, respectively), as well as during the short-experiments operation (day 33 of SE-1, Figure 4.5.b and day 43 of SE-3, Figure 4.5.d), indicating that the NOBs remained active in the PN-AMX reactor during the short experiments (no samples for FISH analysis were available for short experiment SE-2).

On the other hand, there was a clear presence of NOB during Stage I (day 19, Figure 4.5.e and day 64, Figure 4.5.f), according to the rise in the NOB activity suggested both from the high concentration of nitrate in the effluent for these days (Figure 4.4.b) and the high ratio between nitrate produced and ammonium oxidized (Figure 4.4.c). Then, the observed active NOB were progressively decreasing in Stage II for day 176 (Figure 4.5.g) and day 225 (Figure 4.5.h), which agrees with the NOB activity limitation due to the low DO concentration (< 0.05 mg O_2/L) and the high concentrations of free ammonia (Figure 4.4.c).

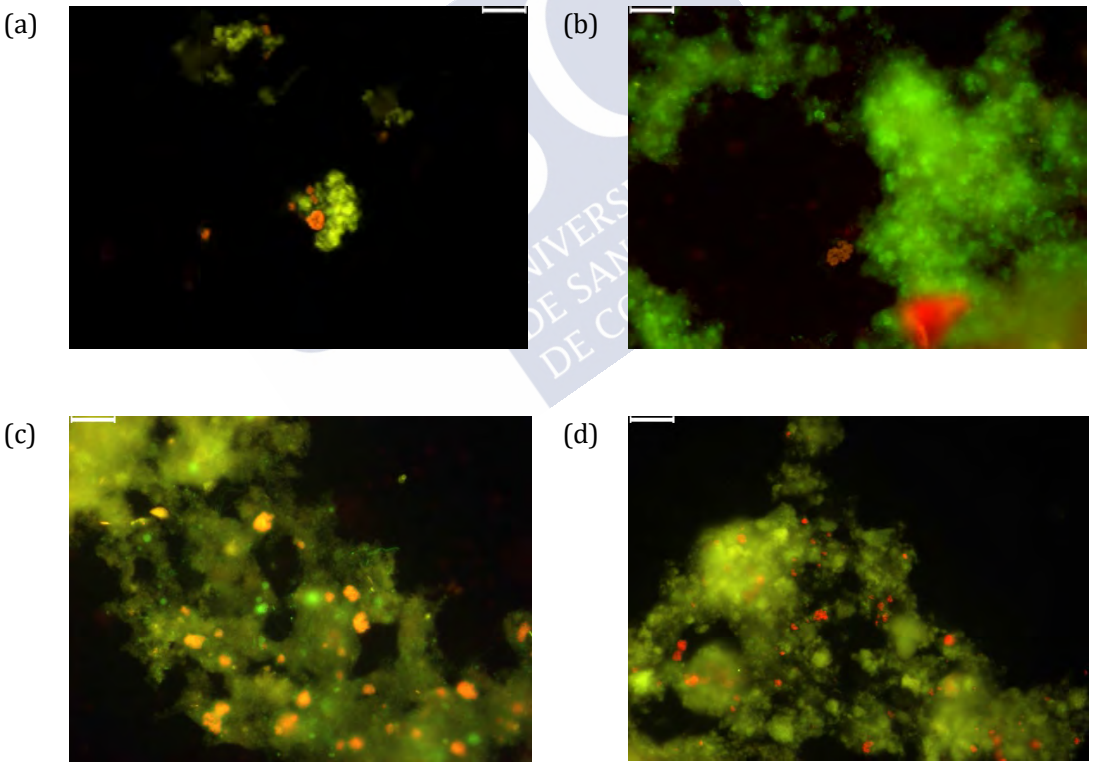

Figure 4.5. FISH images. Phylum *Nitrospirae* (NTSPA712; Cy3; red) and all bacteria (EUB338mix; FTIC; green) for days (a) 0 (SE-1), (b) 33 (SE-1), (c) 0 (SE-3), (d) 43 (SE-3), (e) 19 (Stage I), (f) 64 (Stage I), (g) 176 (Stage II) and (h) 225 (Stage II). The bar represents 40 μm.

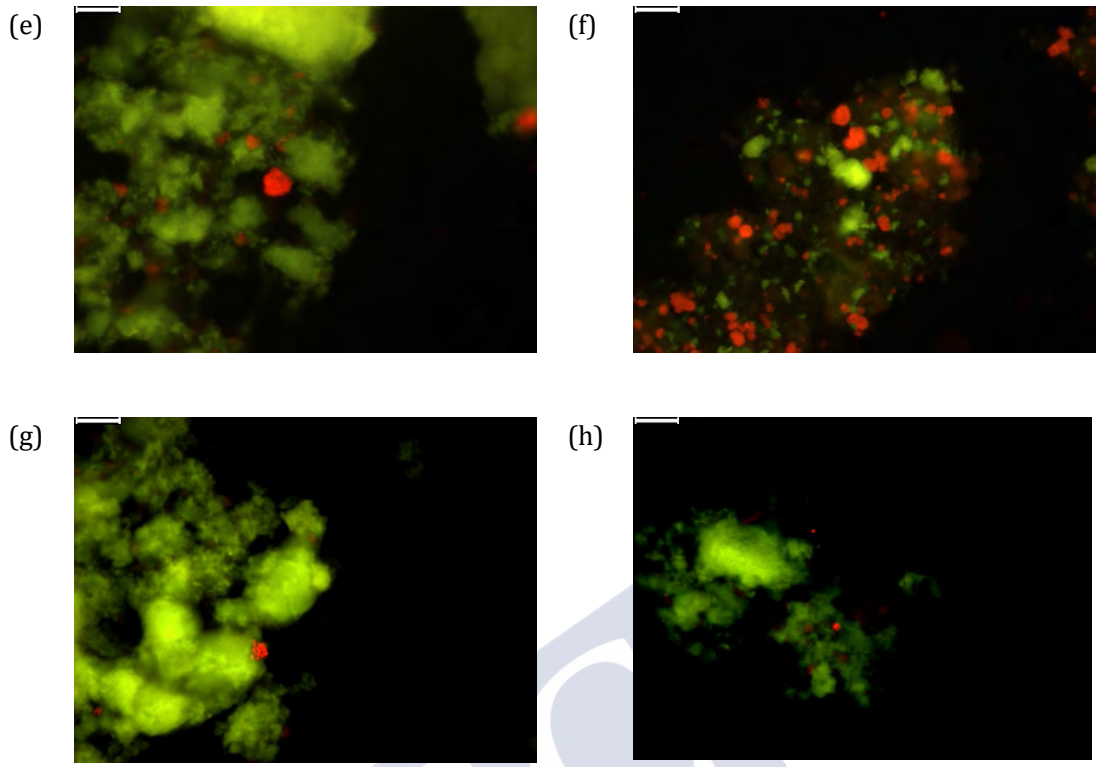

Figure 4.5 (continued). FISH images. Phylum *Nitrospirae* (NTSPA712; Cy3; red) and all bacteria (EUB338mix; FTIC; green) for days (a) 0 (SE-1), (b) 33 (SE-1), (c) 0 (SE-3), (d) 43 (SE-3), (e) 19 (Stage I), (f) 64 (Stage I), (g) 176 (Stage II) and (h) 225 (Stage II). The bar represents 40 μm.

Furthermore, 16S rRNA bacterial gene libraries were constructed and sequenced at different times of short and long-term experiments to investigate the bacterial diversity in the PN-AMX reactor. The bacteria taxonomic diversity was unequally distributed across 26 phyla, where the 12 most abundant taxa represented over the 90% of the bacterial community (Figure 4.6.a). The microbial community was dominated by organisms of the *Proteobacteria* phylum (average relative abundance: 40.1 ± 6.2%), especially from the *Beta* (19.1 ± 5.6%), *Gamma* (12.5 ± 1.8%) and *Alphaproteobacteria* (5.6 ± 1.2%) classes. Other major phyla included *Bacteroidetes* (3.1 - 23.5%), *Chlorobi* (4.2 - 14.9%), *Chloroflexi* (1.6 - 24.3%) and *Planctomycetes* (1.4 - 10.6%). These taxa are commonly found in partial nitritation and/or anammox reactors (Val del Rio et al., 2018). On the other hand, the heterotrophic bacteria (families *Ignavibacteriacea*, *Comamonadaceae*) and anammox bacteria (family *Brocadiaceae*) were found among the largest fraction of the community (Figure 4.6.b).

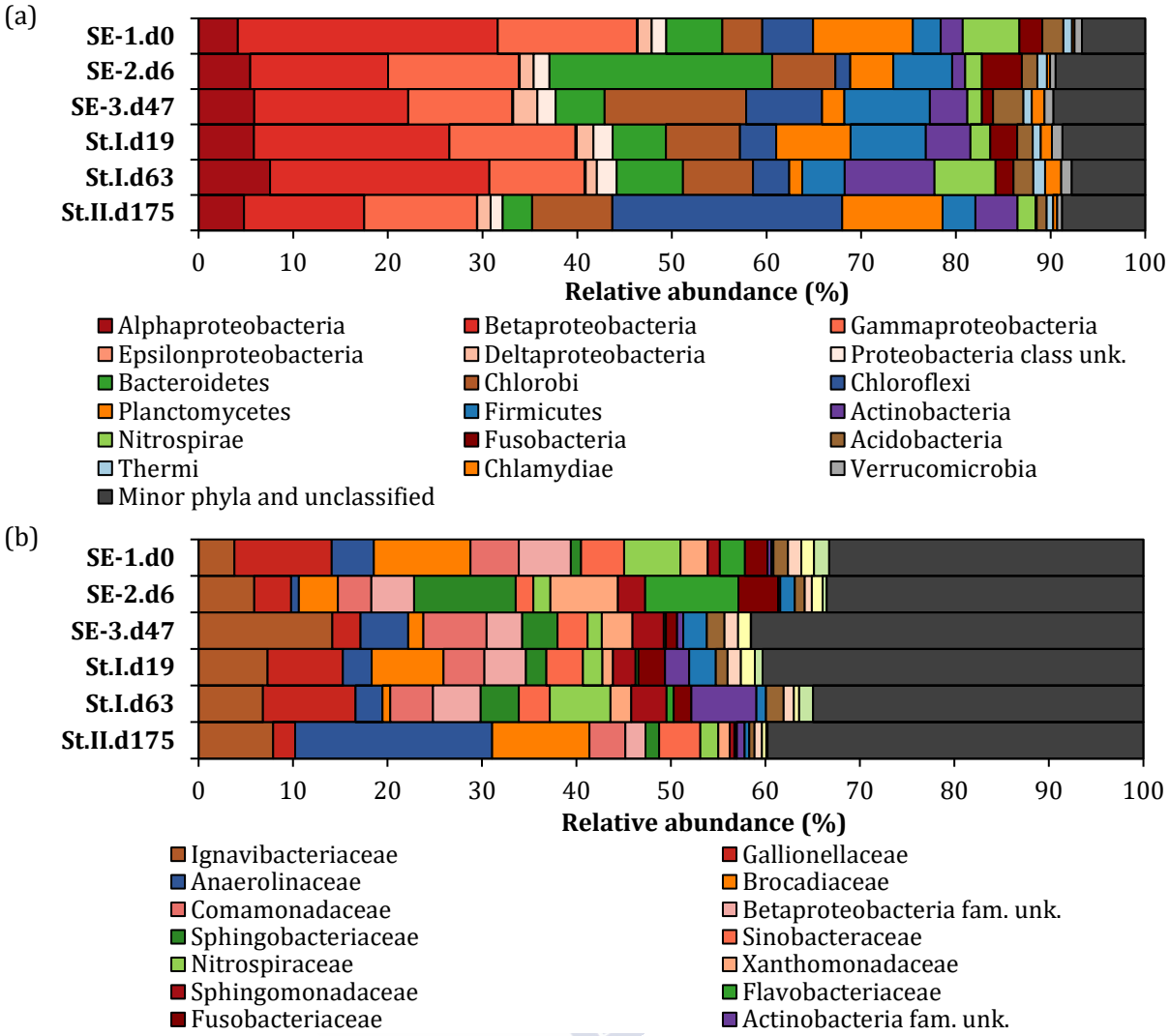

Figure 4.6. Taxonomic profile of the inoculum for SE-1 and the PN-AMX reactor bacterial community during the short experiments (SE-2, SE-3) and the long-term experiment (Stages I-II). (a) Most abundant phyla. For *Proteobacteria* phylum, the different classes are shown. (b) Most abundant families. Sample labels indicate the short experiments (SE-1 to SE-3) or the reactor Stages (St.I-II) followed by the operational day. The colour of the bars correlates each family (b) with the corresponding phylum (a).

Regarding the genera with higher relative abundance (%), two anammox bacteria were found: Candidatus *Brocadia* (0.7 - 8.8%) and Candidatus *Scalindua* (0.1 - 1.6%), both with similar temporal tendencies (Figure 4.7). *Nitrospira*, as inferred from the FISH analysis, was the most abundant NOB detected (1.51 - 6.42%, Figure 4.7), whereas some AOB like *Nitrosospira* (0.02 - 1.46%), *Nitrosovibrio* (0 - 1.29%) and *Nitrosococcus* (0 - 0.29%) were also detected. The two major genera observed for denitrifying bacteria were *Pedobacter* (0.9 - 8.1%) and *Steroidobacter* (1.1 - 3.5%) (Figure 4.7). In agreement with the FISH images, the observed tendencies for the *Nitrospira* genus show a significant active presence of the NOB in the inoculum of SE-1, which included biomass from the pilot plant (SE-1

and Stage I, see Table 4.3), and when the ratio between nitrate produced and ammonium oxidized was high, as on day 63 of Stage I (Figure 4.4.c).

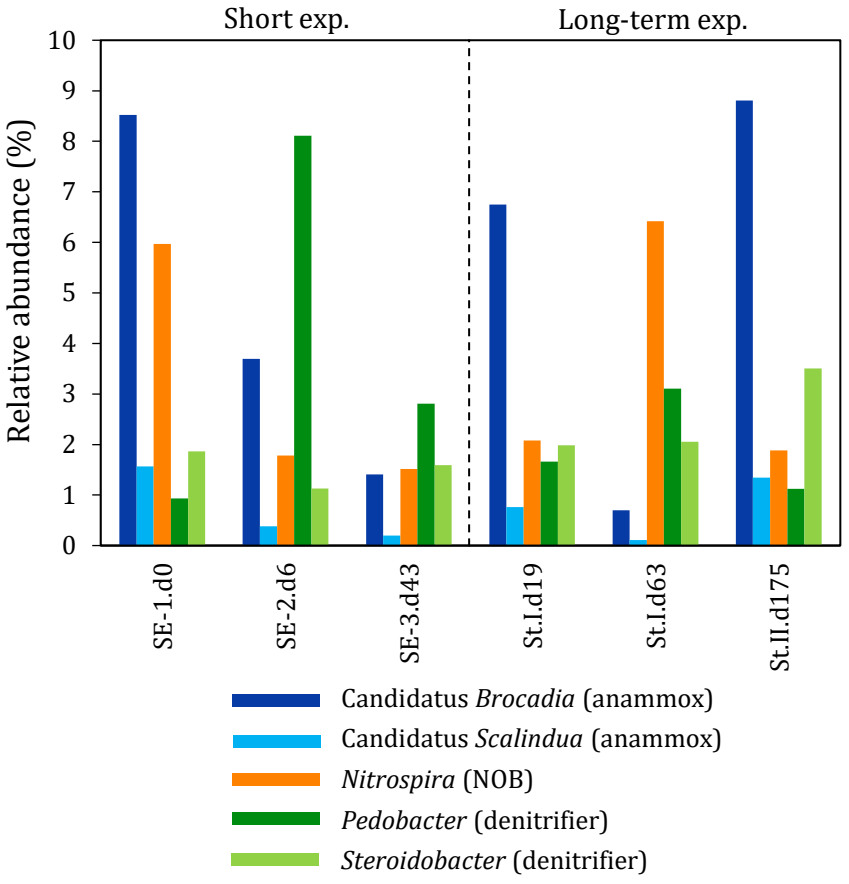

Figure 4.7. Temporal changes of the major anammox bacteria (genera Candidatus *Brocadia* and Candidatus *Scalindua*), NOB (genera *Nitrospira*) and denitrifiers (genera *Pedobacter* and *Steroidobacter*). Sample labels indicate the short experiments (SE-1 to SE-3) or the reactor Stages (St.I-II) followed by the operational day.

4.4.4. Salinity and conductivity

The pre-treated pig slurry used as feeding in this research work contained different ions, being Na^+, K^+ and Cl^- the ones with the highest concentrations (Table 4.4). The salinity as NaCl was in the range of 0.2 - 1.0 g NaCl/L for the short experiments (diluted) and 0.99 ± 0.05 g NaCl/L for the continuous operation (without dilution), while the salinity as KCl was in the range 0.20 - 1.41 g KCl/L for the short experiments and 1.00 ± 0.16 g KCl/L for the continuous operation (Table 4.4). Considering the NaCl IC_{50} values (11.7 g NaCl/L for AOB (Hunik et al., 1992), 13.5 g NaCl/L for anammox bacteria (Dapena-Mora et al., 2007)) and KCl IC_{50} values (22.3 g KCl/L for AOB (Hunik et al., 1992), 14.9 g KCl/L

for anammox bacteria (Dapena-Mora et al., 2007)), the observed salt concentrations during the short and continuous experiments were too low to induce inhibition over the PN-AMX processes.

Table 4.4. Characterization of the pre-digested pig slurry wastewater fed to the PN-AMX SBR during the short and continuous experiments, regarding the salinity (expressed as NaCl and as KCl) and the concentration of anions and cations.

	Short experiments			Continuous experiment	
	SE-1	SE-2	SE-3	Stage I	Stage II
Days	42	42	43	0 - 78	79 - 225
Cations (mg/L)					
Na^+	218 ± 75	127 ± 24	146 ± 5	331 ± 14	341 ± 18
K^+	195 ± 68	125 ± 23	112 ± 11	297 ± 13	349 ± 22
Ca^{2+}	30 - 162	-	27 - 80	46 - 345	15 - 165
Mg^{2+}	25 - 77	13 ± 1	32 ± 3	21 - 91	37 - 111
Anions (mg/L)					
Cl^-	214 - 705	-	276 ± 15	632 ± 67	658 ± 29
PO_4^{3-}	12 - 66	-	23 ± 9	27 ± 7	23 - 121
SO_4^{2-}	29 - 78	-	63 - 134	100 ± 11	143 ± 39
Br^-	1.4 - 7.6	-	6.8 ± 0.1	7.6 ± 0.1	7.2 - 14.2
NaCl (g/L)	0.33 - 1.01	0.27 ± 0.09	0.42 ± 0.02	0.97 ± 0.06	1.01 ± 0.05
KCl (g/L)	0.36 - 1.49	0.20 ± 0.07	0.38 ± 0.07	0.93 ± 0.12	1.04 ± 0.16

Regarding the presence of other salts like the potassium ones, Kartal et al. (2006) observed complete loss of anammox activity for 4.5 g KCl/L (2.4 g K^+/L) and inactivation at 9 g KCl/L (4.7 g K^+/L). Dapena-Mora et al. (2007) showed an inhibitory effect of potassium (as KCl) over the anammox bacteria activity for concentrations over 7.45 g KCl/L (3.9 g K^+/L), whereas Chen et al. (2014) observed a 44.9% reduction of the maximum specific anammox activity for 18.6 g KCl/L (9.7 g K^+/L). The concentrations of potassium in the treated pig slurry, both for the short experiments (100 - 280 mg K^+/L) and during Stages I and II (270 - 390 mg K^+/L) (Table 4.4 and Figure 4.8), were thus low enough to avoid affecting the anammox activity.

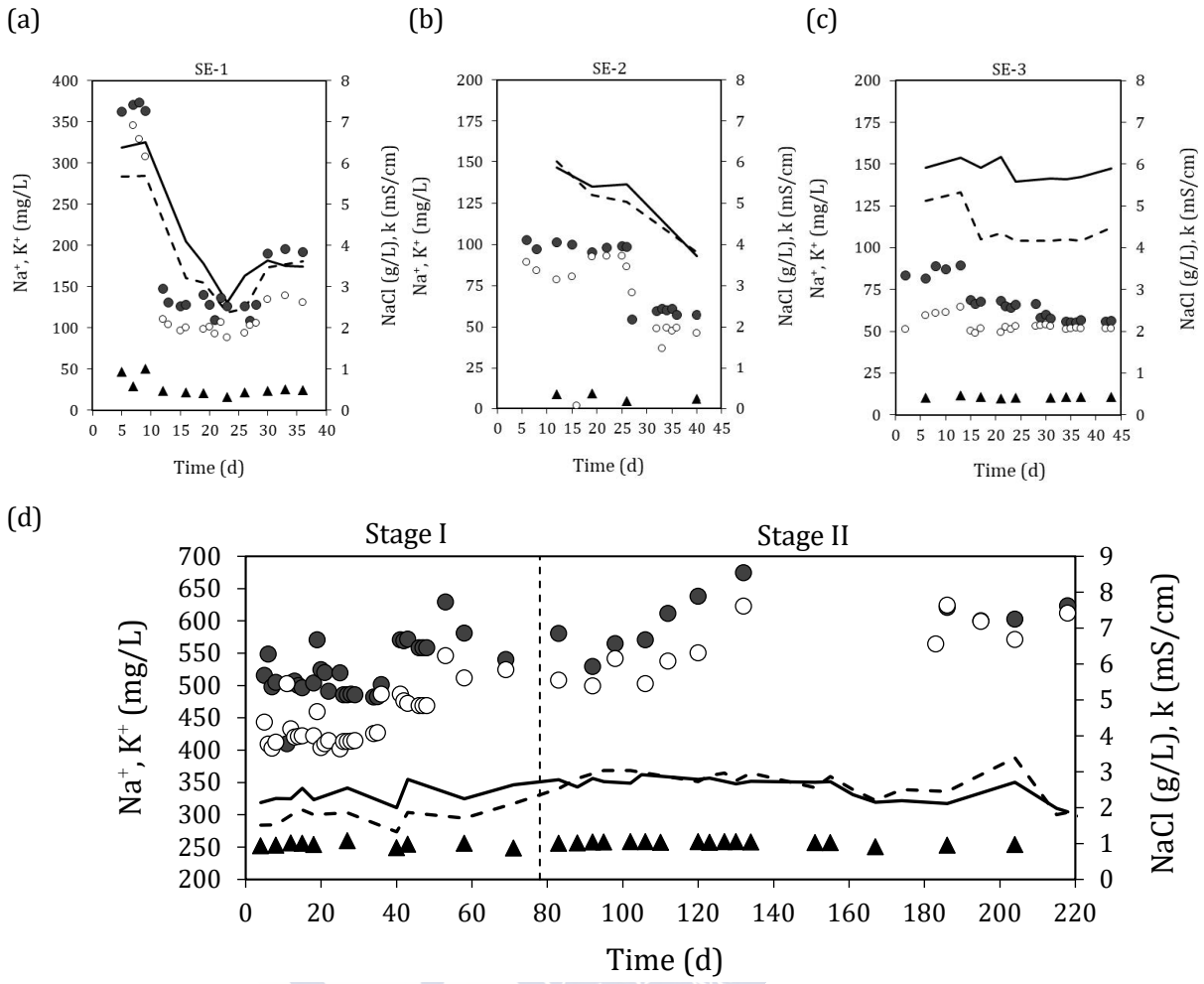

Figure 4.8. Concentrations in the influent of Na^+ (g/L, —), K^+ (g/L, – –), NaCl (g/L, ▲), and conductivity, k (mS/cm) in the influent (●) and effluent (○) for the short experiments SE-1 (a), SE-2 (b), SE-3 (c) and continuous operation, Stages I-II (d).

On the other hand, the conductivity instead of the salinity can become a problem in the correct performance of the anammox process, as reported by Scaglione et al. (2017). These authors found a half maximal inhibitory concentration (IC_{50}) value of 6.1 mS/cm for anammox activity, stating that the conductivity rather than the saline concentrations may be used to quantify the strength of the potential inhibition over the anammox activity. In the present research study, slightly higher values than 6.1 mS/cm for conductivity in the influent were present in the pig slurry without dilution, i.e. at the beginning of SE-1 (7.0 - 7.5 mS/cm, Figure 4.8.a) and in the continuous operation of the reactor (5.2 - 8.5 mS/cm, Figure 4.8.d). Despite the ammonium consumption, the conductivity in the effluent was still above the reported IC_{50} value of 6.1 mS/cm at the beginning of SE-1 (6 - 7 mS/cm) and during Stage II (5.4 - 7.6 mS/cm), and close to the values in the influent. Therefore, no strong inhibition of the anammox bacteria should be expected from the conductivity values, which may have contributed to its hindering only on the long-term synergistically with other inhibiting factors.

4.4.5. Organic matter concentration and sCOD/N ratio

The organic matter content of the fed pig slurry varied in the different operational periods between 94 - 230 mg sCOD/L (Table 4.1), and consequently the removal of soluble COD (sCOD) between the influent and the effluent of the PN-AMX system varied in the range 10 - 51% for the short experiments, and in the range 17 - 65% for the continuous operation. Although the removal of organic matter can be performed both by aerobic oxidation and/or heterotrophic denitrification (Giustinianovich et al., 2016), only the latter pathway was considered in order to estimate the maximum potential contribution of the HD to the removal of nitrogen (see Section 2.5.1.2 in Chapter 1). In this case, the HD contributed on average to removal of 10% of the total nitrogen for the short experiments and the long-term operation. This low percentage is probably due to the low sCOD concentrations and sCOD/N ratio in the feeding (Table 4.1), the latter of which was below 1.14 g sCOD/g NH_4^+-N, while at least 1.71 g sCOD/g NH_4^+-N are required to favour the denitrification via nitrite (or denitritation) pathway in the system (Jenni et al., 2014). Furthermore, the implementation of an anoxic phase in the cycle of the reactor from day 102 onwards (Stage II) did not show a significant increase in the nitrogen removal allocated to the denitrification processes.

On the other hand, part of the COD could also be oxidized aerobically because of the mixed aerobic/anoxic conditions of the system, but the observed profiles for the organic matter consumption during the reactor cycles did not show a rapid or initial consumption of sCOD during the aerated phase, otherwise indicating the main role for the aerobic heterotrophic bacteria as suggested by Chamchoi et al. (2008). Chen et al. (2013) considered the denitritation and denitratation as the main contributors for the consumption of the available COD in anammox systems. In the present research work, the ratio between nitrate produced and ammonium oxidized during Stage II was close to zero (Figure 4.4.c), meaning that the nitrate produced by anammox was probably removed by the denitrifying organisms, which helped to improve the TN efficiency removal.

Table 4.5 summarizes reported values for the concentration of organic matter and the COD/N ratio, regarding the performance of the anammox process working both with synthetic wastewater and pre-digested pig slurry. From Table 4.5, no obvious differences seem to exist when comparing the COD/N ratio between synthetic wastewater and pre-digested pig slurry, showing the ability of the partial nitritation and anammox processes to withstand higher ratios in the slurry.

Table 4.5. Reported values of COD/N ratio and COD concentration regarding the coupling of the anammox bacteria and Heterotrophic Denitrifying Bacteria (HDB) processes.

Ref.	COD/N* (g/g)	COD (mg/L)	NLR (g N/(L·d))	Observations
Synthetic wastewater				
[1]	< 2.0	-	0.3 - 3.6	Coexistence of HDB and AMX bacteria
[2]	2.0	300	0.10	Depletion of AMX activity
[3]	< 2.0	-	0.1	Good coupling of AMX bacteria and HDB (97.5% TN removal)
[4]	0.5 - 1.71	-	1.8 - 3.9	Good coupling of AMX bacteria and HDB (95% TN removal)
[5]	2.92[a]	-	0.77 - 2.70	Complete inhibition of AMX growth due to HDB
Pre-digested pig slurry				
[6]	1.25	237 - 290	1.8	Safe range for AMX reaction (98.5% TN removal)
[7]	1.71	419	0.46	Good performance of the AMX process (75% TN removal)
[8]	1.21	1420	0.6 - 0.9	Biodegradable COD can potentially affect the AMX growth
[9]	3.87	1544	0.13	Good coupling of PN and AMX processes below 0.82 g NH_4^+/g
[10]	1.02 - 1.34	242- 460	1.1	Better COD removal up to 0.95 g COD/g TN
[11]	1.10 - 1.51	305 - 998	1.1 - 15.3	COD and heavy metals inhibit PN-AMX (81% TN removal)
[12]	1.93	2355	0.5 - 0.7	AMX improved by residual organics (91% TN removal)
[13]	4.68	7408	1 - 4	Low (8.6%) organic matter oxidation during PN due to slowly-biodegradable organic matter
[14]	6.25	500[b]	0.13	Inhibition of AMX bacteria

* Usually expressed as g COD and g N (as ammonium) in the feeding. [a] Expressed here as g COD/g NO_2^--N. [b] Synthetic feeding + diluted pig slurry.

References: [1] Kumar and Lin (2010), [2] Chamchoi et al. (2008), [3] Du et al. (2014), [4] Jenni et al. (2014), [5] Tang et al. (2010), [6] Molinuevo et al. (2009), [7] Figueroa et al. (2012), [8] Frison et al., (2018), [9] Durán et al. (2014), [10] Meng et al. (2017), [11] Wang et al. (2017), [12] Scaglione et al. (2015), [13] Qiao et al. (2009), [14] Monballiu et al. (2013).

Regarding the reported values for pre-digested pig slurry (Table 4.5), the applied NLR was in an intermediate range (≤ 0.5 g N/(L·d)). On the other hand, the sCOD/N ratio observed during both the short experiments and Stages I and II were in the range 0.18 - 1.14 g C/g N (Table 4.1), which is below the values in Table 4.5 and Table 1.14.b (Chapter 1). Furthermore, this ratio was below the reported thresholds for inhibiting the anammox and PN-AMX processes (2 g/g, Lackner et al. (2008); Ni et al. (2012)) and for complete heterotrophic denitritation (1.71 g/g, Jenni et al. (2014)).

Furthermore, while the relative abundance of anammox bacteria (Candidatus *Brocadia* + *Scalindua*) varied within the range 0.8 - 10.1% (Figure 4.8), the relative abundance for the major denitrifying bacteria remained (*Pedobacter* and *Steroidobacter*) at an average value of 5.4 ± 0.4% for

the short and long-term experiments (except for SE-2, Figure 4.8), thus not showing a significant correlation between both bacteria for resource competition.

4.4.6. NOB activity and suppression

Some of the strategies reported to avoid the NOB activity are the application of low DO concentrations (Bagchi et al., 2010), the alternation between anoxic and aerobic conditions (Sobotka et al., 2015) and the presence of free nitrous acid in high concentrations (Pedrouso et al., 2017). However, the available research studies addressing DO levels to suppress NOB activity mainly focus on synthetic and reject wastewater (Akaboci et al., 2018). In the present study, both the intermittent aeration applied and the low DO concentration provided in the reactor (Table 4.1) only contributed to the kinetic limitation of the NOB activity in sporadic days, but did not suppress it effectively during the short experiments and during Stage I. For example, on day 17 of SE-2 (Figure 4.2.c) or on day 65 of Stage I (Figure 4.4.c), when the NOB activity was completely depleted within 25 days due to the low oxidation of ammonium by AOB. Therefore, the TN removal in the PN-AMX processes was compromised by the DO concentration applied. If the value of DO concentration was high enough (around 0.18 mg O_2/L, Table 4.1) the oxidation of ammonium was good, for example from day 25 onwards (SE-2, Figure 4.2.a), but a concurrent enhancement of the NOB activity occurred (i.e. higher nitrate concentrations in the effluent and higher nitrate produced to ammonium oxidized ratios). Whereas if the value of the DO concentration was too low (0.02 mg O_2/L) the oxidation of ammonium was hindered and so did the removal of total nitrogen.

Bagchi et al. (2010), for example, determined an optimal specific DO concentration (regarding the residual concentration of ammonium in the bulk liquid) below 60 μg O_2/(mg NH_4^+-N·d) to maintain the PN-AMX processes and avoid the activity of NOB with synthetic wastewater. In this case, certain relation was observed regarding this parameter but for lower ranges. During the short experiments, the specific DO concentration was on average below 4 μg O_2/(mg N·d), however NOB activity was observed for sufficiently high concentrations (14 μg O_2/(mg N·d)). On the other hand, this value was between 0.3 - 1.2 μg O_2/(mg N·d) during Stage I, and the NOB activity decreased concurrently when the specific DO concentration decreased below 0.3 μg O_2/(mg N·d) after day 65 (Figure 4.4.c), in accordance with the hindered partial nitritation and the higher FA concentrations (40 - 85 mg NH_3-N). As a conclusion, the control of NOB activity through either lack of substrate (nitrite) or high FA concentrations relies on a tight oxygen control for the oxidation of ammonium. This fact can be observed in Figure 4.9, which depicts the variation of ammonium and TN removals against the DO concentration.

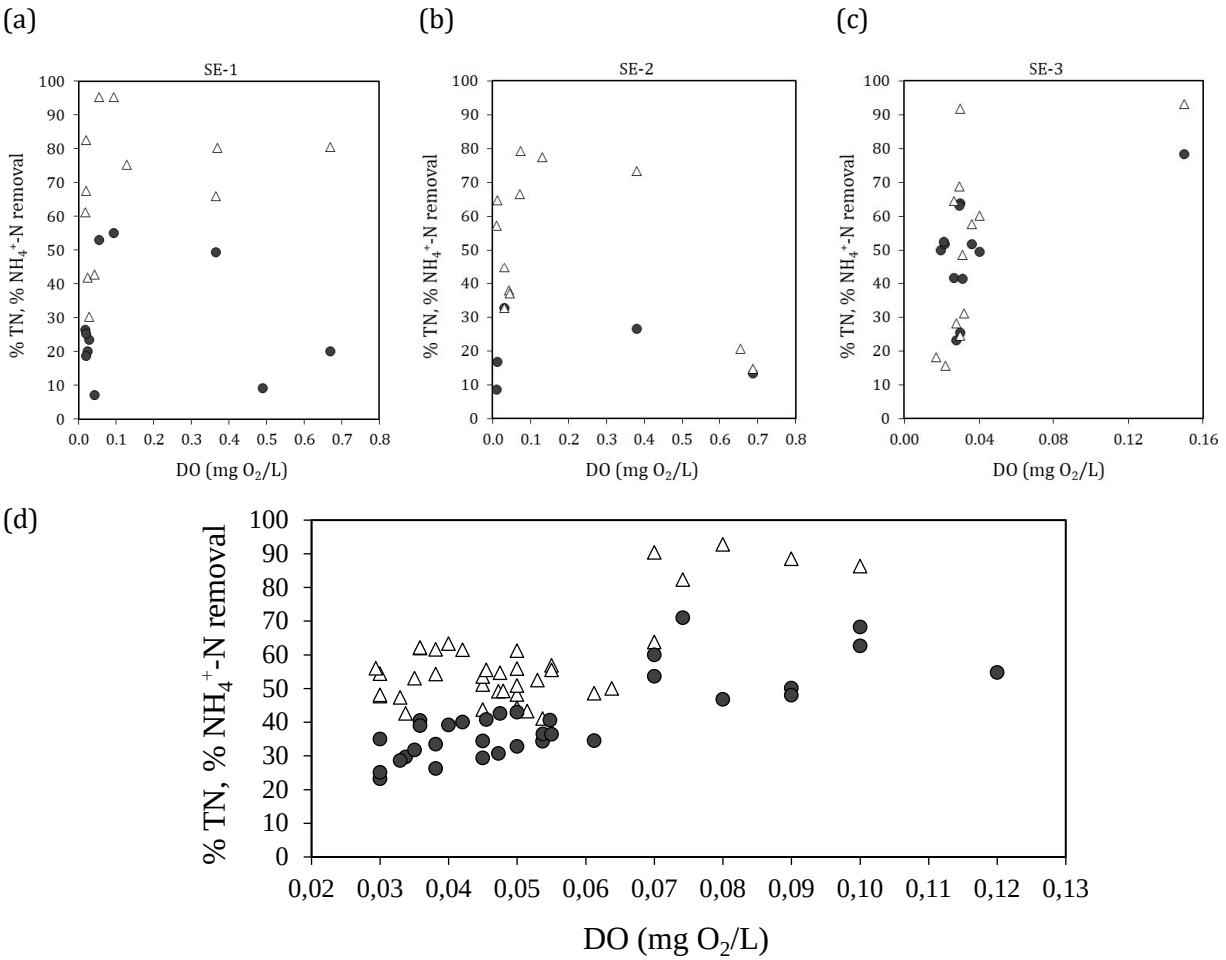

Figure 4.9. Relation between the concentration of dissolved oxygen, DO (mg O_2/L) with the % of total nitrogen (TN) removal (●), and with the % of NH_4^+-N removal (△) for the short experiments SE-1 (a), SE-2 (b), SE-3 (c) and continuous operation, Stages I-II (d).

From Figure 4.9, the optimal values of DO concentration required to have a good TN removal when treating the pig slurry with the PN-AMX processes were summarized in Table 4.6, showing an optimal range of 0.07 - 0.12 mg O_2/L. Given the lower DO half saturation constant of AOB (0.3 mg O_2/L) compared to NOB (1.1 mg O_2/L) between 25 and 30 °C, a low DO range allow the AOB to outcompete the NOB and favour the nitrite accumulation (Ma et al., 2016; Wiesmann, 1994). On the other hand, although DO concentrations below 0.2 mg O_2/L are required for avoiding inhibition of the anammox bacteria (Jung et al., 2007), too low DO levels may affect the partial nitritation within the PN-AMX processes. Furthermore, the optimal range found in Table 4.6 is below the recommended range for the PN-AMX combined process (0.2 - 1.0 mg O_2/L, Liu et al. (2013); Yue et al. (2018)). As a conclusion, the observed tight range for the optimal DO concentration supports the idea of the crucial role of the aeration control when treating pig slurry effluents with the PN-AMX processes.

Table 4.6. Summary of dissolved oxygen (DO) concentration ranges and optimal values regarding the removal of total nitrogen (TN) and ammonium, during the short- and long- experiments.

	Short experiments			Long-term experiment
	SE-1	SE-2	SE-3	Stages I-II
Days	42	42	43	0 - 225
DO range (mg O_2/L)	0.02 - 0.67	0.01 - 0.70	0.01 - 0.20	0.02 - 0.15
DO optimal (mg O_2/L)	0.093	0.074	0.12	0.080
Maximum nitrogen removal for optimal DO				
%TN removal	55.0	37.8	78.3	71.0
% NH_4^+ removal	95.2	79.3	93.2	92.8

On the other hand, the NOB activity was successfully limited for 120 days during Stage II. Despite the accumulation of nitrite in this stage to values as high as 170 mg NO_2^--N/L (Figure 4.4.b), the free nitrous acid (FNA) was very low (< $13 \cdot 10^{-4}$ mg N/L) in comparison with the reported values for NOB inhibition (0.02 mg N/L) (Blackburne et al., 2007). However, the FA concentration in Stage II (between 40 and 80 mg NH_3-N/L, Figure 4.4.c) could have contributed to the inhibition of nitrite oxidizers, according to their lower inhibition range values (0.082 - 22 mg NH_3-N/L) (Anthonisen et al., 1976; Jianlong and Ning, 2004; Kim et al., 2010), in comparison with AOB (8 - 123 mg NH_3-N/L) (Anthonisen et al., 1976). In this sense, the nitrite oxidizing rate (NOR) was depleted to zero for FA concentrations higher than 40 mg NH_3-N/L (Figure 4.10), and the ratio between the nitrate in the effluent and the ammonium consumed was below the stoichiometric values for the PN-AMX combined process during Stage II (Figure 4.4.c). Regarding the adaptation of NOB to high FA concentrations, this was previously reported for 30 - 50 mg NH_3-N/L (*Nitrobacter* spp.) and 0.8 - 22 mg NH_3-N/L (*Nitrospira* spp.) (Kim et al., 2010). During Stage II, for a recovered ammonium oxidation range of 50 - 70% (day 140 on, Figure 4.4.c) and FA concentrations above 38 mg NH_3-N, no signs of NOB adaptation were observed.

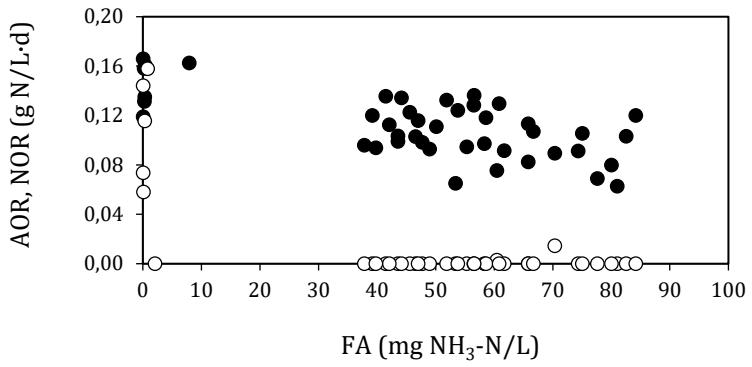

Figure 4.10. Relation between the FA concentration (mg NH_3-N/L) with the ammonium oxidizing rate, AOR (g N/(L·d), ●) and nitrite oxidizing rate, NOR (g N/(L·d), ○) for the short experiments SE-1 (a), SE-2 (b), SE-3 (c) and continuous operation (d).

The inhibition of the NOB during Stage II was confirmed by FISH images, which show the decrease in the presence of active *Nitrospira* between the Stage I (day 64, Figure 4.5.f) and the end of Stage II, where the active NOB are barely observed (day 225, Figure 4.5.h). On the other hand, the DNA data confirmed both a high relative abundance of *Nitrospira* for the inoculum of SE-1 (6.0%) and the continuation of the NOB presence during SE-2 (1.8% on day 6) (Figure 4.7), where the ratio between nitrate produced and ammonium oxidized was 8-fold higher than the stoichiometric value (day 6, Figure 4.2.c), and at the end of SE-3 (1.5% on day 43, Figure 4.7). The *Nitrospira* and anammox bacteria clearly showed opposite trends during Stages I-II (Figure 4.7): the relative abundance of *Nitrospira* grew during Stage I from 2.1% (day 19) to 6.4% (day 63), in agreement with the comparative higher presence of NOB in the FISH images (Figure 4.5.e and Figure 4.5.f, days 19 and 64 respectively). On the other hand, the relative abundance of anammox bacteria (Candidatus *Brocadia* + *Scalindua*) dropped from 7.5% to 0.8% for the same days (Figure 4.7). Later, the *Nitrospira* dropped to 1.9% (day 175) owed to FA inhibition (note the lower presence of NOB in Figure 4.5.g) while the anammox bacteria achieved a relative abundance of 10.1%. These opposite trends show the influence and competition between both bacterial communities, supporting the FA as a suitable tool for NOB inhibition.

Nevertheless, the free ammonia may become a problem for the PN-AMX combined process if sufficient high amounts are formed in the system. On one hand, a wide IC_{50} range for AOB is reported: 0.28 - 4.30 mg NH_3-N/L (Torà et al., 2010), 4.93 - 27.2 mg NH_3-N/L (Park and Bae, 2009), as well as FA inhibitory ranges from 8.2 to 130 mg NH_3-N/L (Anthonisen et al., 1976; Li et al., 2012; Tian et al., 2013). On the other hand, IC_{50} values of 38 mg NH_3-N/L were reported for the anammox bacteria (Fernández et al., 2012), with inhibitory FA concentrations ranging from 13 to 187 mg NH_3-N/L (Daverey et al., 2013; Fernández et al., 2012; Tang et al., 2010a; Waki et al., 2007). Thus, the FA concentrations observed during Stage II (38 - 80 mg NH_3-N/L, Figure 4.4.c) were in an intermediate zone regarding the inhibition of the PN-AMX processes by FA, contributing to a certain inhibition of the anammox activity and the subsequent buildup of nitrite of the system.

4.5. Conclusions

The batch activity experiments performed indicated that the pig slurry can at least produce an inhibition up to 44.4% in the maximum specific anammox activity. The synergistic combination of factors that characterized the fed pig slurry, namely the conductivity (6 - 8 mS/cm) and the sCOD/N ratio (0.18 - 1.14 g/g), could have contributed to the long-term anammox inhibition. The presence of NOB activity in the available inoculum hindered the fast start-up of the PN-AMX processes for the treatment of the pig slurry, despite the presence of sufficient anammox activity. A strategy based on the limitation of the DO concentration and the promotion of inhibitory levels of free ammonia (FA)

showed to be suitable to suppress the nitrite oxidizers. Nevertheless, a strict aeration control at low levels (0.07 - 0.12 mg O_2/L) is mandatory to balance both the partial nitritation and anammox processes. Despite the presence of significant NOB activity in the start-up of the process, a NRR of 0.1 g N/(L·d) was achieved, with a contribution of the possible heterotrophic denitrification to the removal of nitrogen of less than 10%.

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Chapter 5

Treatment of Organic Fraction of Municipal Solid Waste (OFMSW) digestate with the PN-AMX processes

Summary

Treating the organic fraction of municipal solid waste (OFMSW) can be performed by coupling the anaerobic digestion (AD), to diminish the organic matter content and produce biogas, and the partial nitritation-anammox (PN-AMX) processes for nitrogen removal. On the other hand, a former ammonia stripping (AS) pre-treatment already proved to be beneficial for the AD, although the related higher sCOD/N ratios in the digestate can be harmful for the subsequent anammox process. In the present study, the operation of two PN-AMX sequencing batch reactors (SBRs) fed with and without AS pre-treated OFMSW digestate, respectively, was assessed. The increasing proportion of OFMSW fed showed similar decreases in the specific anammox activity (90%) for both SBRs, indicating no differences over the anammox activity whether the AS pre-treatment is implemented or not. The SBR treating 100% AS digestate achieved better effluent quality (127 ± 88 vs. 1050 ± 23 mg N/L) but for a lower nitrogen removal rate (58 ± 8 vs. 687 ± 32 g N/(L·d)) compared to the SBR without pre-treatment. Still, the latter required successive re-inoculations in order to obtain high nitrogen removal rates. Changes in the nitrogen removal and in the microbial communities were mainly correlated to sCOD/N ratios in the OFMSW, being Candidatus *Brocadia* and Candidatus *Scalindua* identified as the main anammox species for both reactors, with the former as the dominant one. The results proved the AS to be a suitable pre-treatment, despite the higher sCOD/N ratios in the OFMSW digestate, achieving good synergy between the PN-AMX and heterotrophic denitrification processes.

OUTLINE

5.1. Introduction

More than 2.01 billion metric tonnes of municipal solid wastes were generated globally in 2017, being the major part of it preferably destined to landfilling instead of processed for its conversion into useful resources (Panigrahi and Dubey, 2019). The anaerobic digestion (AD) is a commonly applied technology for the valorization of the organic fraction of municipal solid wastes (OFMSW), diverting a significant part of the organic waste away from sanitary landfills, which are still recognized as the most common ultimate disposal method for municipal solid waste (Renou et al., 2008). The AD can contribute to the gradual reduction in the amount of landfilled organics, besides producing methane in the process (Malamis et al., 2014). At European level, approximately 70 Mt/y of OFMSW are produced (Scaglione et al., 2017). The AD is already tackling 25% of the biological treatment, i.e. around 20% of all municipal solid waste disposal in Europe, and Spain occupies the second place regarding the largest capacity of AD installed (1,600,000 ton) (Baere and Mattheeuws, 2012).

The wide application of AD results in the production of high quantities of anaerobic digestates, which are normally separated into a solid and a liquid fraction (Malamis et al., 2014). The liquid fraction is often characterized by high concentrations of nitrogen content, recalcitrant organic substances and even metals (Renou et al., 2008; Wang et al., 2016; Wu et al., 2009). Consequently, an effective, cost-saving post-treatment is required to comply with the discharge legislation (Malamis et al., 2014; Walker et al., 2011).

The biological treatment is still the main approach for the removal of nutrients and organics from these digestates due to the lower cost, especially when including the AD (Wu et al., 2016a). In the biological treatment, the nitrogen is removed by reducing it to gaseous form, which means no secondary pollution compared with the physico-chemical systems. Among the available biological processes (nitrification, denitrification and anammox), different configurations can be adopted using as a guide the ratio between the chemical oxygen demand and the nitrogen concentrations (g COD/g N) in the digestate. Daigger et al. (2014) estimated adequate values of 3.5 - 4.0 g COD/g N for nitrification-denitrification, 2.0 - 2.5 g COD/g N for nitritation-denitritation and 0.5 g COD/g N for partial nitritation-anammox processes (PN-AMX). On other hand, Pedizzi et al. (2018) observed that a pre-AD treatment, such as the ammonia stripping (AS) at 75 °C, can provide a suitable COD/N ratio for applying the nitritation-denitritation process, while the digestate with no pre-treatment was more adequate to be treated by the PN-AMX processes. Zhang et al. (2016a) studied the effect of the thermal pre-treatment prior to the AD over the subsequent PN-AMX process, concluding that the dissolved organics produced provoked a decrease in the nitrogen removal from 97% (without pre-treatment) to 72% (with pre-treatment). Later, Zhang et al. (2018) demonstrated that this negative effect inhibited the activity of aerobic ammonia oxidizing bacteria (AOB) rather than that of the anammox bacteria.

All these studies implicitly suggest that the use of such pre-treatments to improve the AD performance is not beneficial for post-treatments such as the PN-AMX processes. However, the PN-AMX processes have several advantages compared with nitrification-denitrification, such as lower energy requirements (57% lower oxygen consumption) and much lower organic matter requirements (86% reduction) (Malamis et al., 2014). Therefore, shedding light over the applicability limits of the PN-AMX processes when treating OFMSW digestates (with and without pre-treatment) is of great interest and lacks dedicated research studies.

5.2. Objectives

In the research study presented in this Chapter, the biological nitrogen removal with the PN-AMX processes in a single unit was assessed to treat the digestate from the anaerobic digestion of the liquid fraction of the OFMSW with and without an ammonia stripping pre-treatment. The increasing sCOD/N ratio for both digestates was evaluated as a key factor to elucidate the nitrogen removal by the different processes involved: nitrification, anammox and denitrification.

5.3. Materials and methods

5.3.1. Operation of the reactors

5.3.1.1. Experimental set-up

Two laboratory sequencing batch reactors (SBRs) with working volumes between 1.5 - 1.7 L were used. The aeration system for each of the reactors consisted of a diaphragm pump (Laboport N86, KNF) which supplied air through a diffuser located at the bottom of the reactor, promoting the aeration with the formation of small bubbles. The aeration inside the reactor provided both a good mixture and the dissolved oxygen (DO) necessary to carry out the biological aerobic processes. The DO concentration was measured periodically with a DO probe (Hach Lange LD01) and was manually regulated by changing the opening degree of an air valve located in the gas inlet conduction. The SBRs were operated at a temperature range between 30 - 33 °C by means of a thermostatic bath. The operational cycles were of 6 h and distributed in different phases (Table 5.1).

Granular sludge from a PN-AMX full-scale plant (ELAN® technology) treating the reject digestate from the sludge anaerobic digester in the WWTP of Guillarei (Galicia) (Vázquez-Padín et al., 2014) was used as inoculum for both SBR. The initial sludge concentration was 4.4 g VSS/L and its specific anammox activity (SAA) was 0.342 ± 0.021 g N/(g VSS·d) at 30 °C.

Table 5.1. Distribution of the operational cycle (min) in the different experimental periods for both SBRs fed with ammonia-stripping pre-treated and non pre-treated OFMSW (AS-SBR and nAS-SBR, respectively).

Days AS-SBR	Days	Feeding	Aeration	Idle phase*	Settling	Withdrawal
0 - 19	0 - 19	15	315		15	15
23 - 168	23 - 213	15	255	60	25	5
169 - 228	214 - 300	15	315		15	15

* Neither aeration nor stirring were provided during the idle phase

The wastewater fed to both SBRs consisted in the effluent of two anaerobic digesters (AD) operated in the same laboratory facilities, treating the liquid fraction of the OFMSW from a treatment plant in Barcelona (Spain). Full description of the ADs can be found in Pedizzi et al. (2018). A scheme of the complete treatment processes before both SBRs is depicted in Figure 5.1.

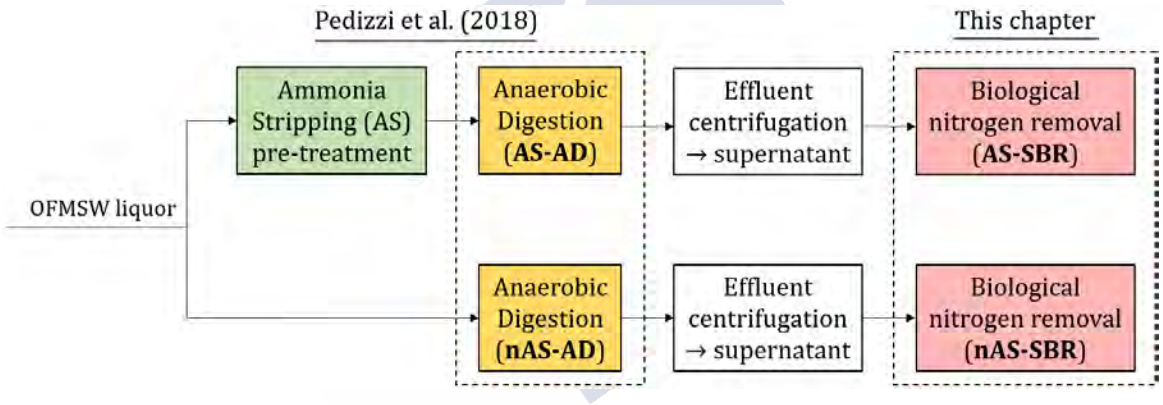

Figure 5.1. Treatment processes scheme for the OFMSW liquor before the biological nitrogen removal step studied in the present Chapter. AS-SBR: SBR fed with ammonia-stripping pre-treated OFMSW; nAS-SBR: SBR fed with OFMSW without ammonia stripping pre-treatment.

In one of the AD the OFMSW fed was previously treated in an ammonia stripping (AS) column at 75 °C to decrease its ammonium concentration, and the subsequent digestate was fed to the reactor called AS-SBR. The other AD was fed with the OFMSW without AS pre-treatment, and the correspondent digestate was fed to the reactor named nAS-SBR. The effluent of each AD was centrifuged upon reception (45 min at 14,000 rpm and 4 °C) to separate the anaerobic sludge, and stored at 4 °C before its use to preserve the characteristics of the wastewater.

5.3.1.2. Operational periods

Each AD was step-wise fed starting with reject wastewater from a municipal WWTP and progressively increasing the proportion of the liquid fraction of OFMSW in the feeding. Consequently, the relative proportion of treated OFMSW digestate fed to the subsequent SBRs for the nitrogen removal progressively increased as well. Despite the long hydraulic retention time (HRT) of the ADs (20 - 40 days) and the mismatch between the proportion of OFMSW fed to each AD and subsequent SBR, all OFMSW proportions hereinafter will refer to the known value fed to the AD. The operation of both SBR was divided in different stages regarding this percentage (Table 5.2).

Table 5.2. Operational conditions of both SBRs and characterization of the pre-digested OFMSW supernatant used as feeding in the different stages.

	***AS-SBR (with** ammonia stripping pre-treatment)*				
	Stage I_{AS}	Stage II_{AS}	Stage III_{AS}	Stage IV_{AS}	Stage V_{AS}
Days	0 - 33 [a]	34 - 96	97 - 142	143 - 240	241 - 300
Temperature (°C)	32.3 ± 0.8	32.6 ± 1.4	32.8 ± 0.8	32.0 ± 0.7	32.2 ± 1.0
HRT (d)	2.61 ± 0.09	4.36 ± 0.40	4.62 ± 0.33	4.80 ± 0.26	35.5 ± 0.0
DO (mg O_2/L)	0.059 ± 0.034	0.061 ± 0.026	0.068 ± 0.019	0.13 ± 0.12	4.15 ± 0.82
pH	8.5 ± 0.2	8.4 ± 0.3	8.2 ± 0.1	8.4 ± 0.3	8.1 ± 0.1
% OFMSW fed to previous AD step	0 - 10	10 - 35	35 - 60	60 - 100	100
NH_4^+ (mg N/L)	731 ± 55	905 ± 90	1304 ± 177	1187 ± 128	1290 ± 500
IC (mg IC/L)	611 ± 32	666 ± 78	1041 ± 132	1391 ± 200	1973 ± 341
tCOD (mg tCOD/L)	891 ± 201	2131 ± 622	3783 ± 1153	8200 ± 1936	7159 ± 462
sCOD (mg sCOD/L)	299 ± 54	1283 ± 424	2400 ± 600	6633 ± 1457	6433 ± 401
Ratio sCOD/N influent (g/g)	0.40 ± 0.08	1.37 ± 0.41	1.80 ± 0.35	5.54 ± 1.51	5.70 ± 1.72
Ratio N/IC influent (g/g)	1.18 ± 0.10	1.14 ± 0.51	1.26 ± 0.13	0.90 ± 0.14	0.76 ± 0.33
Conductivity (mS/cm)	-	12.3 ± 1.3	15.5 ± 2.0	24.3 ± 3.9	28.7 ± 1.6
	***nAS-SBR (without** ammonia stripping pre-treatment)*				
	Stage I_{nAS}	Stage II_{nAS}	Stage III_{nAS}	Stage IV_{nAS}	Stage V_{nAS}
Days	0 - 36 [a]	37 – 58 [b]	59 – 93 [c]	94 - 164	165 – 228 [c]
Temperature (°C)	32.6 ± 0.6	33.1 ± 0.5	30.7 ± 1.1	32.6 ± 0.9	33.0 ± 1.6
HRT (d)	2.72 ± 0.40	4.52 ± 0.15	23.6 ± 0.0	23.8 ± 0.6	4.5 ± 0.0
DO (mg O_2/L)	0.054 ± 0.029	0.054 ± 0.016	4.0 ± 0.5	2.5 ± 1.4	0.086 - 0.714
pH	8.6 ± 0.3	8.6 ± 0.4	8.3 ± 0.1	8.5 ± 0.2	8.2 ± 0.1
% OFMSW fed to previous AD step	0 - 10	10 - 30	30 - 40	40 - 60	100
NH_4^+ (mg N/L)	740 ± 62	1065 ± 142	1527 ± 149	1382 ± 275	3759 ± 307

Table 5.2 (continued)

	***nAS-SBR (without** ammonia stripping pre-treatment)*				
	Stage I_{nAS}	Stage II_{nAS}	Stage III_{nAS}	Stage IV_{nAS}	Stage V_{nAS}
Days	0 - 36 [a]	37 – 58 [b]	59 – 93 [c]	94 - 164	165 – 228 [c]
IC (mg IC/L)	616 ± 30	800 ± 43	979 ± 53	1249 ± 124	4918 ± 522
tCOD (mg tCOD/L)	938 ± 235	1507 ± 50	2262 ± 70	2515 ± 508	7896 ± 1092
sCOD (mg sCOD/L)	336 ± 82	919 ± 232	1644 ± 357	1822 ± 325	6570 ± 1011
Ratio sCOD/N influent (g/g)	0.44 ± 0.11	0.80 ± 0.14	1.07 ± 0.21	1.40 ± 0.20	1.79 ± 0.10
Ratio N/IC influent (g/g)	1.18 ± 0.15	1.31 ± 0.11	1.61 ± 0.10	1.01 ± 0.09	0.78 ± 0.08
Conductivity (mS/cm)	-	-	14.9 ± 1.6	15.2 ± 2.5	41.8 ± 2.6

[a] The OFMSW started to be supplemented in the feeding from day 28 onwards.

[b] From day 58 onwards, the operation of the nAS-SBR was stopped for 45 days. Therefore, day 59 corresponds with the subsequent first operational day.

[c] Partial reinoculation in Stage III_{nAS} and complete reinoculation in Stage V_{nAS}.

DO: dissolved oxygen; HRT: hydraulic retention time; IC: inorganic carbon; sCOD: soluble COD. tCOD: total COD.

The experimental period with increasing proportion of OFMSW for the AS-SBR lasted 240 days (Stages I_{AS} to IV_{AS}) and the operation with 100% of OFMSW was of 60 days (Stage V_{AS}). For the nAS-SBR, the same experimental equivalent periods lasted 164 days (Stages I_{nAS} to IV_{nAS}) and 64 days (Stage V_{nAS}), respectively. Between Stage II_{nAS} and Stage III_{nAS}, the nAS-SBR was not fed for 45 days and only stirring was provided in order to promote the nitrite consumption by the anammox bacteria (this period was not taken into account as operational days). The reactor was re-started in Stage III_{nAS} and partially re-inoculated with a mixture of granular sludge from the original inoculum and the previous biomass from Stage II_{nAS}, providing 2.9 g VSS/L inside the reactor and a SAA of 0.291 ± 0.015 g N/(g VSS·d) at 30 °C. In Stage V_{nAS} the reactor was again re-inoculated with the inoculum from the same source as in Stage I_{nAS}, providing 5.45 g VSS/L inside the reactor and a SAA of 0.103 ± 0.003 g N/(g VSS·d) at 30 °C.

5.3.2. Analytical methods

Analytical determination of ammonium (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N), soluble chemical oxygen demand (sCOD), conductivity, pH, total suspended solids (TSS) and volatile suspended solids (VSS) was carried out according to the Standard Methods (APHA/AWWA/WEF, 2012). Total Organic Carbon (TOC) concentration was determined by a Shimadzu analyzer (TOC-L_{CSN}, automatic sample injector Shimadzu ASI-L) as the difference between the total carbon (TC) and the inorganic carbon (IC) concentrations. Cation and anion concentrations were determined by ion

chromatography with an Advanced Compact IC system (861, Metrohm), CO_2 suppressor (MCS 853, Metrohm) and a sample processor/injector (838, Metrohm). For all these previous measurements the samples were filtered by 0.45 μm, except for TSS and VSS. Full description of the analytical methods is provided in Chapter 2 (Section 2.1).

The concentrations of metals were measured in raw and filtered (0.45 μm) samples of the influent and effluent for both reactors at days 167 (Stage IV_{AS}) and 122 (Stage IV_{nAS}). Biomass samples from both reactors were also collected for the corresponding sampling days, as well as biomass stored from the initial inoculum. The total and dissolved concentrations of metals were determined in triplicate through ICP-MS spectroscopy (Agilent 7700x). Full description of the analytical methods is provided in Chapter 2 (Section 2.1.4).

5.3.3. Biomass activity measurements

The SAA, expressed as g N/(g VSS·d), was assessed in batch tests at 30 °C by periodically measuring the headspace overpressure due to the production of nitrogen gas by anammox bacteria, with a differential pressure transducer (Centerpoint Electronics). The methodology described by Dapena-Mora et al. (2007) was followed, which is fully described in Chapter 2, Section 2.3.2.1. The SAA tests were performed during the operation of both reactors to monitor the anammox activity, as well as before the long-term operation to elucidate the effect of the OFMSW with and without pre-treatment over the SAA of the biomass. In all these tests, both ammonium (as NH_4Cl) and nitrite (as $NaNO_2$) were added as substrates, with the latter as the sole substrate when OFMSW (instead of phosphate buffer solution - PBS) was used as liquid medium due to the ammonium excess in the OFMSW liquor. Three consecutive feedings were performed for each test by adding a new dose of substrate once the previous one had been consumed to evaluate the possible negative or positive effect of the repeated exposition of the biomass to the tested liquid medium.

Furthermore, the specific denitrifying activity (SDA) of the biomass was measured at some points during the operation of both reactors, following the procedure in Buys et al. (2000). The headspace overpressure, in this case due to the production of nitrogen and carbon dioxide gases by denitrifying bacteria, was also measured with a pressure transducer. Organic matter (as $NaHCO_3$), together with either nitrite (as $NaNO_2$) or nitrate (as $NaNO_3$), were added as substrates, with the latter ones as the sole substrate when OFMSW (instead of PBS) was used as liquid medium due to the excess of organic matter in the OFMSW liquor.

5.3.4. Molecular analysis for identification of bacterial populations

Biomass samples from different operational days were taken from AS-SBR (days 1, 34, 79, 114, 253, 265 and 330) and nAS-SBR (days 1, 34, 69, 104, 181, 193 and 228). Samples were stored in 2 mL-aliquots and immediately frozen at -20 °C. Total genomic DNA was extracted using the Stool DNA Isolation KIT (Norgen. Thorold, Canada). Total DNA concentrations were quantified in a Qubit fluorometer (Thermo Fisher Scientific Waltham, MA, USA) and checked for size integrity by standard electrophoresis. The V3V4 hypervariable region of the 16S rRNA gene was amplified for *Bacteria* with the primer pair S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A (Klindworth et al., 2013). Then, samples were analyzed by Illumina following the procedure described in Braz et al. (2018). The methodologies used for DNA extraction and sequencing are fully described in Chapter 2 (Section 2.4.2).

5.3.5. Calculations

The nitrogen loading rate (NLR), nitrogen removal rate (NRR) and total nitrogen (TN) removal efficiency were estimated based on nitrogen balances and the anammox process stoichiometry (with the rates being expressed as g N/(L·d)), following eq. 2.5, eq. 2.7 and eq. 2.12, respectively (Chapter 2, Section 2.5.1.1 and 2.5.1.2). The maximum nitrogen removal percentage by a possible heterotrophic denitrification (HD) processes was determined based on the mass balance of the soluble chemical oxygen demand (sCOD), following eq. 2.14 (Section 2.5.1.2). Since part of the organic matter can be consumed for growth and/or aerobic oxidation due to the presence of oxygen, this maximum TN removal percentage considers only the maximum "potential" or contribution for the HD, not the actual.

The concentration of free ammonia (FA, NH_3) was calculated at the operational temperature based on the NH_4^+ concentrations and the pH in the bulk liquid (i.e., in the effluent of the reactor) according to Anthonisen et al. (1976) (eq. 2.15, Chapter 2, Section 2.5.1.3).

5.4. Results

5.4.1. Characteristics of the OFMSW digestate with and without AS pre-treatment

As shown in Table 5.2, and regarding the characteristics of both digestates with 100% of OFMSW proportion, similar sCOD concentrations were observed (6433 ± 401 mg sCOD/L for Stage V_{AS}, 6570 ± 1011 for Stage V_{nAS}), although a lower fraction of degradable organic matter is to be expected in the AS digestate, according to Pedizzi et al. (2018). The nAS digestate had higher ammonium concentrations (3759 ± 307 mg NH_4^+-N/L) compared to the AS digestate (1290 ± 500 mg NH_4^+-N/L), which were closer to the average concentrations reported on the main research studies treating leachates (1470 ± 1660

mg NH_4^+-N/L) and OFMSW effluents (1120 ± 1020 mg NH_4^+-N/L), as summarized in Table 1.15 (Chapter 1). The ammonium concentration of the nAS digestate is above the reported range (200 - 3000 mg NH_4^+-N/L), with only a few exceptions surpassing this value: 3400 - 5700 mg NH_4^+-N/L (Vilar et al., 2010) and 5.97 mg NH_4^+-N/L (Gabarró et al., 2012).

Similarly to the ammonium concentrations, inorganic carbon (IC) concentration was lower for the AS digestate (1973 ± 341 mg IC/L) compare to the nAS digestate (4918 ± 522 mg IC/L), and thus the nitrogen to inorganic carbon (N/IC) ratio was similar for both digestates (0.76 ± 0.33 g/g and 0.78 ± 0.08 g/g, respectively). However, this N/IC ratio for both digestates was on average above the reported threshold for limitation of ammonium removal to 50% due to alkalinity (1 g N/g IC, Pedrouso et al. (2017)) as the proportion of OFMSW in the feeding increased from Stages I_{AS} to IV_{AS} and I_{nAS} to IV_{nAS}, respectively (Table 5.2).

On the other hand, the concurrent increase of the sCOD concentration in both digestates was higher compared to the increase of the ammonium concentration. This favoured a progressive increase of the sCOD/N ratio above the reported threshold for heterotrophic denitrification via nitrite (1.71 g COD/g N, Jenni et al. (2014)): 5.70 ± 1.72 g/g and 1.79 ± 0.10 g/g for the AS and nAS digestates, respectively (Table 5.2). Except for the immature or young leachates, the reported sCOD/N ratios for the mature landfill leachate varies in the range 0.4 - 3.3 g sCOD/g N, with an average ratio of 1.70 ± 0.77 g sCOD/g N (Table 1.15.a). On the other hand, the high average ratio for the AS digestate (5.70 ± 1.72 g/g) is above the average values for OFMSW effluents (4.55 ± 0.20 g sCOD/g N), which is owed to the extremely high sCOD concentrations before the AD (Pedizzi et al., 2018). The observed range for the AS digestate (2.9 - 8.4 g/g) was comparable to a handful of reported previous studies: 2.8 - 7.2 g/g (Shalini and Joseph, 2018), 7 - 8 g/g (Zhu et al., 2013) and 5.8 - 14.7 g/g (Akgul et al., 2013).

Only 11% and 36% of the research studies in Table 1.15 reported measured conductivity values when treating landfill leachate and OFMSW digestates, respectively. However, the conductivity rather than the salinity or alkalinity may be the main factor responsible for the inhibition of anammox bacteria when treating these wastewater streams, with a IC_{50} value of 6.1 mS/cm (Scaglione et al., 2017). In the present research work, the average conductivity for the AS digestate (28.7 ± 1.6 mS/cm) is close to the average of reported values for the same wastewater stream (30.8 ± 3.7 mS/cm, Table 1.15), whereas the nAS digestate showed an average conductivity (41.8 ± 2.6 mS/cm) between the reported average values and some extremely high conductivities above 70 mS/cm (Gabarró et al., 2012; Ganigué et al., 2009).

In conclusion, the OFMSW digestates treated in the present Chapter are characterized for its high strength, mainly regarding the NH_4^+-N and sCOD concentrations, as well as the sCOD/N ratio, N/IC ratio

and the conductivity. These parameters will exert its influence over the biological removal of nitrogen with the PN-AMX processes, as it will be discussed hereinafter.

5.4.2. Effect of ammonia stripping and thermal pre-treatments over the SAA

Before the long-term operation of the PN-AMX processes treating the OFMSW digestate, batch assays were conducted to elucidate the effect of OFMSW with and without pre-treatments over the SAA of the biomass (granular sludge from the ELAN® PN-AMX full-scale plant).

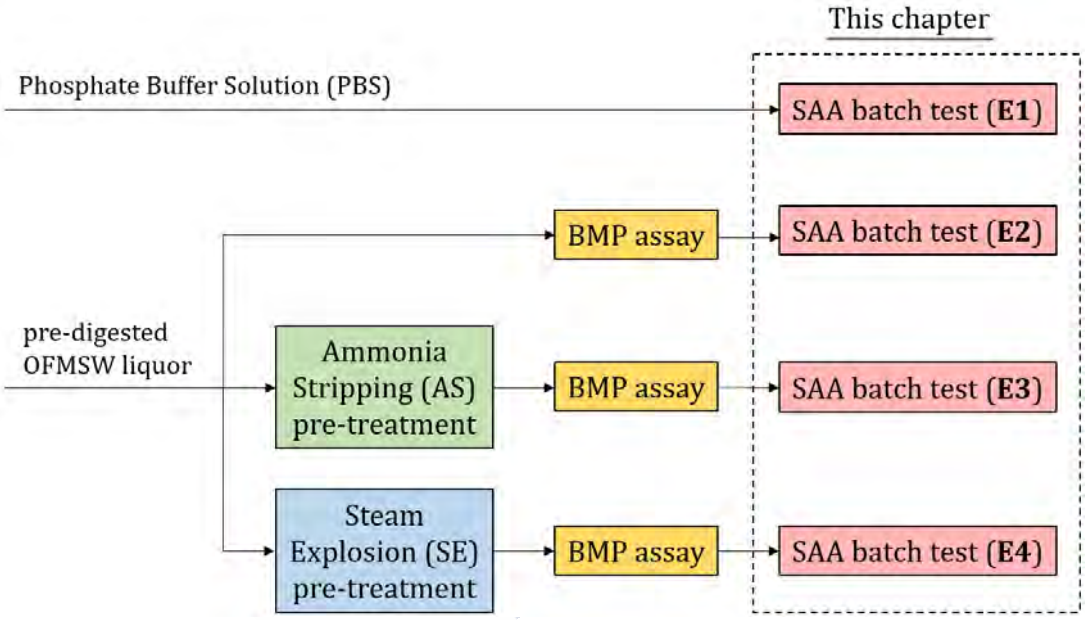

Figure 5.2. Pre-treatments applied to the pre-digested OFMSW liquor used as liquid medium before the performance of initial specific anammox activity (SAA) batch tests. The biomethane potential (BMP) test evaluates anaerobic biodegradability of any substrate fed into anaerobic digesters.

The batch activity tests were performed using as liquid medium a phosphate buffer solution (PBS), which served as control (E1), and with OFMSW coming from biomethane potential (BMP) tests, to guarantee the absence of organic matter in the pre-digested liquor, in three different conditions: without pre-treatment (E2), and with ammonia stripping (E3) and steam explosion (E4) as pre-treatments (Figure 5.2).

Table 5.3. Results of the SAA tests with synthetic medium (E1) and the OFMSW after biomethane potential batch tests (BMP) without previous treatment (E2), and with two types of pre-treatment: ammonia stripping (E3) and steam explosion (E4).

	E1	E2	E3	E4	
Type of liquid medium	PBS	OFMSW	OFMSW	OFMSW	
Pre-treatment of the OFMSW	-	BMP	AS pre-treatment + BMP	SE pre-treatment + BMP	
Nitrogen concentration in the liquid medium					
NH_4^+ (mg N/L)	70	4400	800	1000	
NO_2^- (mg N/L)	70	70	70	70	
SAA (g N/(g VSS·d))	SAA_{PBS}	SAA_{OFMSW}	SAA_{OFMSW}	SAA_{OFMSW}	
First feeding	0.551 ± 0.009	0.197 ±	0.273 ± 0.007	0.258 ± 0.003	*p = 0.000*
Second feeding	0.569 ± 0.070	0.209 ±	0.235 ± 0.004	0.229 ± 0.017	*p = 0.142*
Third feeding	0.612 ± 0.036	0.219 ±	0.252 ± 0.011	0.211 ± 0.005	*p = 0.018*
SAA reduction (%)[a]					
First feeding	-	64.2	50.4	53.2	
Second feeding	-	63.3	58.7	59.8	
Third feeding	-	64.2	58.8	65.5	
	p = 0.199	*p = 0.142*	*p = 0.003*	*p = 0.037*	

[a] Reduction percentage in the anammox activity regarding the maximum SAA value with PBS (E1).

BMP: biomethane potential, PBS: phosphate buffer solution, OFMSW: Organic Fraction of Municipal Solid Waste, AS: ammonia stripping, SE: steam explosion.

The parameter "p", determined through an ANOVA test, indicates the significance level of the results obtained between different experiments (E1 - E4, right column) and between consecutive feedings (1^{st}, 2^{nd}, 3^{rd} feedings, bottom column). Statistically significant deviations between mean values occur for $p < 0.05$.

From the obtained results (Table 5.3), the OFMSW digestate with no pre-treatment (E2) already reduced by 64% the SAA (0.219 ± 0.014 g N/(g VSS·d)) compared to the control (E1, 0.612 ± 0.036 g N/(g VSS·d)) after the third feeding, and up to 58.8% and 65.5% when ammonia stripping (E3) and steam explosion (E4) were used as pre-treatments, respectively. Furthermore, the variation in the SAA reduction percentage between successive feedings was statistically significant (i.e., $p < 0.05$) only when these pre-treatments were applied ($p = 0.003$ and 0.037 for E3 and E4, respectively), suggesting a more relevant long-term effect in the reduction of the anammox activity when applying these pre-treatments.

Both at the end of the first feeding, and at the end of the batch tests (after the third feeding), the SAA divergence between the tests was statistically significant ($p = 0.000$ and 0.018, respectively), as opposed for the second feeding, where there were no statistically significant differences ($p = 0.142$).

The highest SAA was obtained for the ammonia stripping pre-treatment case, E3 (0.252 ± 0.011 g N/(g VSS·d)), i.e. improving by 13% and 16% the SAA at the end of tests E2 and E4, respectively. However, in the three tests the SAA was under 50% of the SAA measured in the reference test (E1), which indicates that a negative effect occurs in all cases. On the other hand, the AS can help avoiding the inhibition by free ammonia in the thermophilic AD by reducing the ammonia concentration by 55%, as pointed out by Pedizzi et al. (2018). Therefore, the AS was validated as the most appropriate pre-treatment in order to successfully evaluate the applicability of the PN-AMX processes to the nitrogen removal from pre-treated OFMSW liquors.

5.4.3. Nitrogen removal with AS pre-treatment: AS-SBR

Due to the increase in the proportion of OFMSW digestate fed to the reactor, the concentration of ammonium progressively increased during the operation of the AS-SBR (Figure 5.3.a). After an initial decrease in the removal efficiency of total nitrogen (TN) during Stage I_{AS} (0 - 10% of OFMSW), the nitrogen loading rate (NLR) was decreased from 0.3 g N/(L·d) to 0.2 g N/(L·d) at the beginning of Stage II_{AS} (10 - 35% of OFMSW) (Figure 5.3.b), and the nitrogen removal increased to 78 ± 6%. The NOB activity was present during Stage I_{AS}, as inferred from the build-up of nitrate up to 220 mg NO_3^--N/L (Figure 5.3.a) and the ratio between nitrate produced and ammonium oxidized was 3-fold higher than the stoichiometric value for the PN-AMX processes (0.11 g/g) (Figure 5.3.c). The NOB activity was still observed during Stage II_{AS} (0.11 - 0.40 g/g, up to 212 mg NO_3^--N/L).

At the beginning of Stage I_{AS} the low IC concentration in the effluent of the AS-SBR between days 8 and 18 (3.4 - 5.6 mg IC/L) provoked a sharp decrease of the pH values in the reactor from 8.3 down to 5.3 (Figure 5.4), which probably hindered the activity of both AOB and anammox bacteria and provoked the nitrogen removal to decrease from 80% to 30% (Figure 5.3.b).

Similarly to the concentration of ammonium, the concentration of organic matter progressively increased during the operation (Figure 5.5.a). The OFMSW digestate was fed during Stage I_{AS} from day 28 onwards, in a proportion of 10% (Table 5.2), providing a sCOD/N ratio suitable for the PN-AMX processes (0.40 ± 0.08 g/g) (Figure 5.5.b). During Stages I_{AS} and II_{AS}, the fed concentration of organic matter already increased from 270 to 1864 mg sCOD/L, while the removal of organic matter decreased from 46.0 ± 7.7% (Stage I_{AS}) to 34.9 ± 5.6% (Stage II_{AS}, Figure 5.5.a). The sCOD/N ratio increased from 0.3 to 2.0 g sCOD/g N during Stages I_{AS} and II_{AS}, and the potential contribution of HD to the TN removal concurrently increased from 3.7% to 11.1% (Figure 5.5.b).

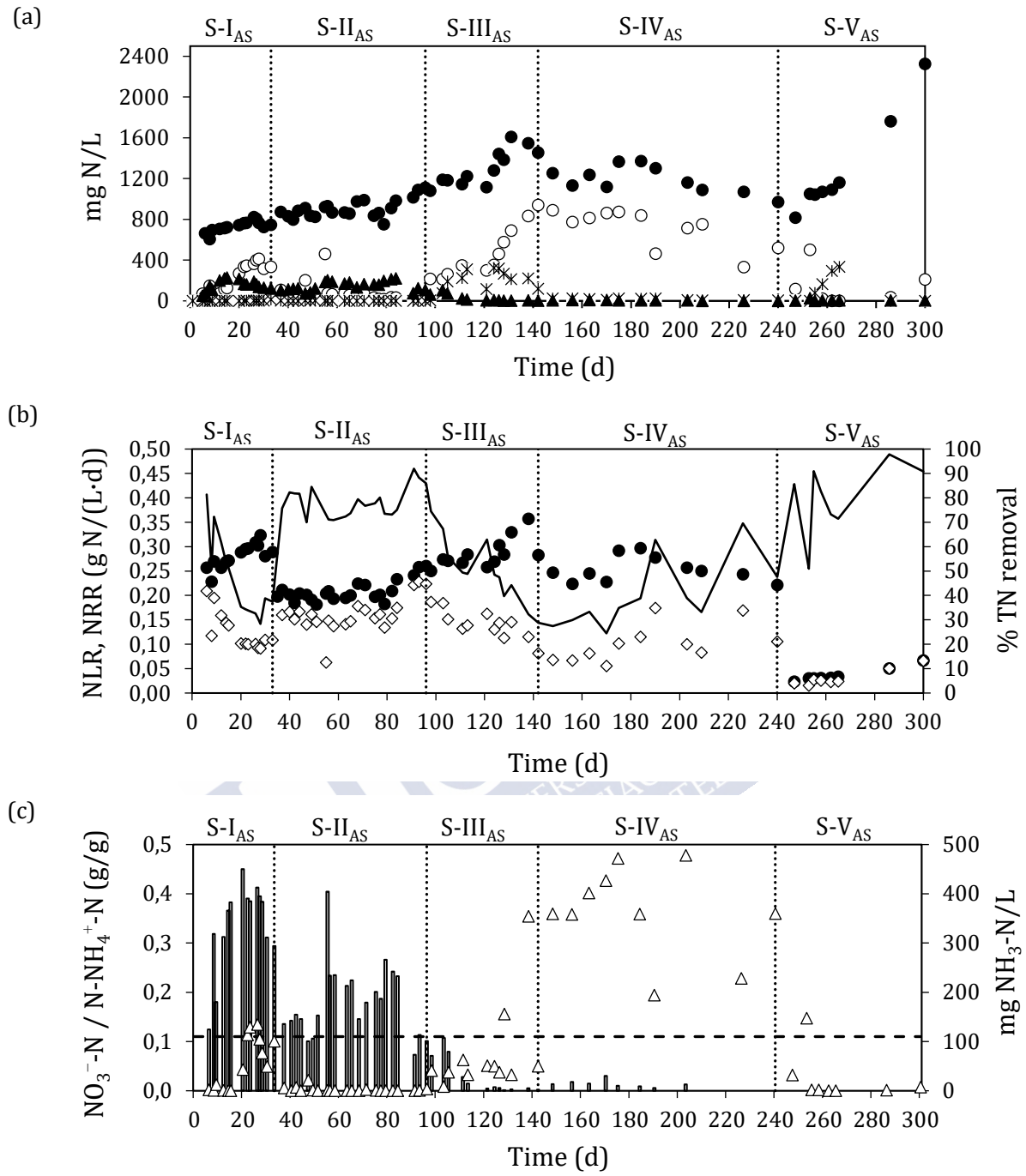

Figure 5.3. Data from the operation of AS-SBR (Stages I_{AS} to V_{AS}): (a) concentration of NH_4^+-N in the influent (●), and NH_4^+-N (○), NO_2^--N (∗) and NO_3^--N (▲) in the effluent (mg N/L); (b) Nitrogen Loading Rate, NLR (g N/(L·d), ●), Nitrogen Removal Rate, NRR (g N/(L·d), ◇) and % of total nitrogen (TN) removal (—); (c) nitrate produced to ammonium oxidized ratio (g NO_3^--N/g NH_4^+-N, ▯), concentration of free ammonia, FA (mg NH_3-N/L, △) and stoichiometric nitrate produced to ammonium consumed ratio for the PN-AMX processes (0.11 g N/g N) (- - -).

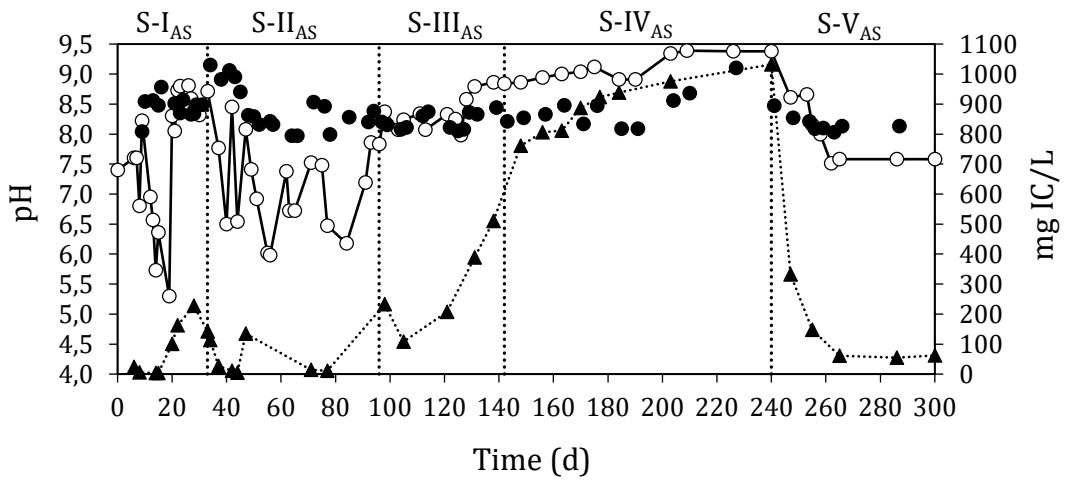

Figure 5.4. Concentration of inorganic carbon (IC) in the effluent (mg IC/L) from the AS-SBR (▲) and pH of the influent (●) and effluent (○) for the AS-SBR.

During Stage III$_{AS}$ (35 - 60% of OFMSW), the nitrogen removal rate (NRR) and the nitrogen removal efficiency decreased to 0.08 g N/(L·d) and 29%, respectively (Figure 5.3.b). The negligible nitrate concentration and the increasing nitrite concentration (121 - 323 mg NO_2^--N/L, Figure 5.3.a) indicate a decrease in the activity of nitrite consumers (NOB and anammox), probably inhibited by the high free ammonia (FA) concentrations (33 - 354 mg NH_3-N/L, Figure 5.3.c). The low organic matter removal (12.5 ± 7.4%) implied a low HD potential contribution to the TN removal (< 6%, Figure 5.5.b) together with the low availability of dissolved oxygen for its aerobic oxidation to take place.

In Stage IV$_{AS}$ (60 - 100% of OFMSW), both the sCOD concentration and the sCOD/N ratio drastically increased from 3000 to 8000 mg sCOD/L (Figure 5.5.a) and from 2.7 to 8.4 g/g (Figure 5.5.b), respectively, probably promoting the competition of aerobic heterotrophs with AOB for the oxygen. Consequently, the FA concentration in the effluent remained high (358 - 524 mg NH_3-N/L, Figure 5.3.c), hindering the activity of both NOB and anammox bacteria. Nevertheless, the nitrogen removal efficiency was able to recover from 30% to 69% as the NLR slightly decreased (Figure 5.3.b). Both the sCOD removal and nitrogen removal by HD increased (30% and 38%, respectively). At the end of Stage IV$_{AS}$, the liquid medium inside the reactor was washed to reduce the concentration of FA and sCOD, and the applied NLR was lowered below 0.030 g N/(L·d).

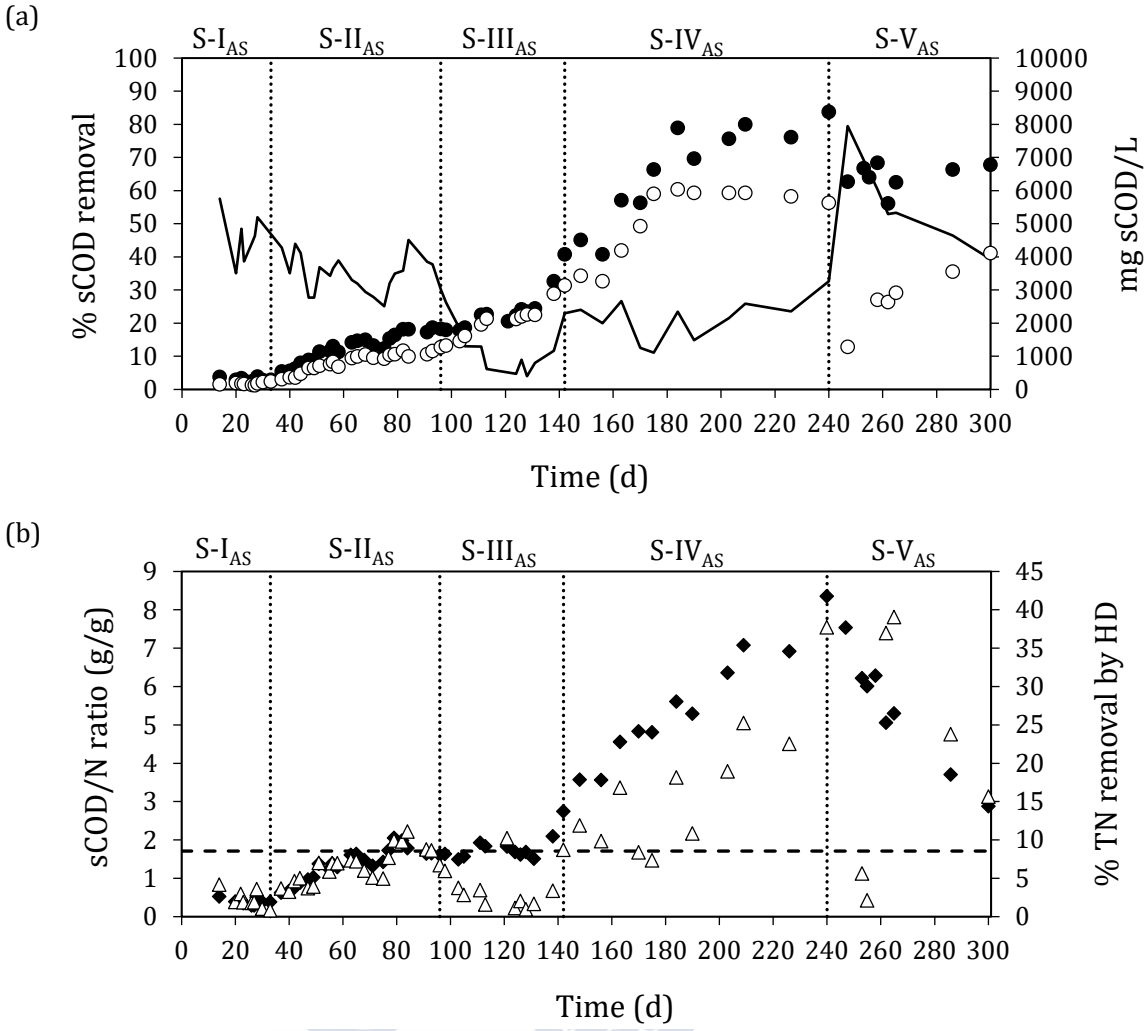

Figure 5.5. (a) Concentration of soluble COD (sCOD) in the influent (●) and effluent (○) (mg sCOD/L), and % of sCOD removal (—); (b) ratio sCOD/N in the influent (g sCOD/g N, ◆), maximum % of TN removal by possible heterotrophic denitrification (HD) (△) for the AS-SBR, and sCOD/N ratio threshold for complete denitritation (1.71 g sCOD/g N, Jenni et al. (2014)) (- - -) .

In Stage V_{AS} (100% of OFMSW), the sCOD/N ratio decreased from 8.4 to 2.9 g sCOD/g N (Figure 5.5.b) since the increase of the contact time during the AS pre-treatment (from 4 to 8 h) improved the methanization in the AD (Pedizzi et al., 2018) and thus lowered the C/N ratio in the digestate used to fed the AS-SBR. The potential HD contribution decreased from 39% to 16%, while the depletion of the effluent IC concentration from 1032 mg IC/L to 61 mg IC/L (Figure 5.4) dropped the pH and FA concentrations from 9.0 to 7.6 and below 30 mg NH_3-N/L, respectively. These conditions, together with the lower NLR (0.07 g N/(L·d)), probably promoted the efficiency of nitrogen removal up to 94% within 59 days (Figure 5.3.b), by coupling the anammox and HD processes, despite the higher concentrations of nitrogen and organic matter.

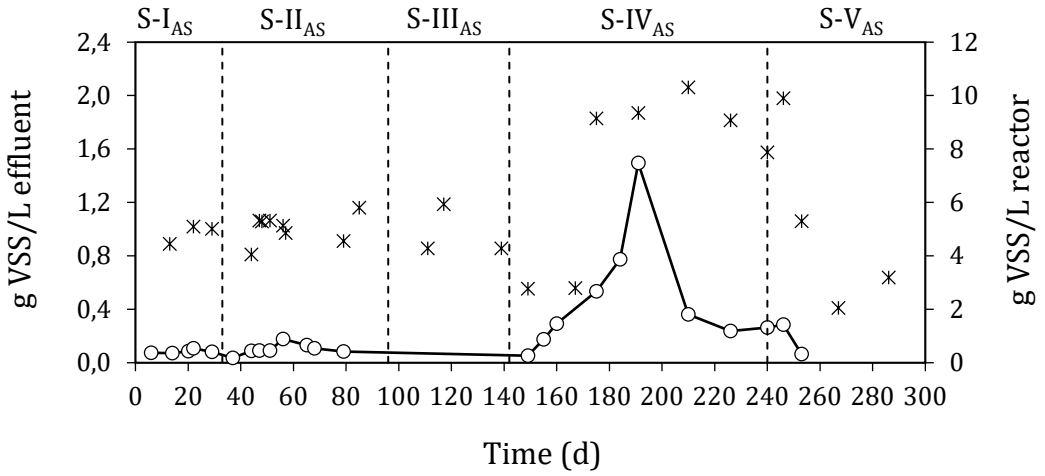

Figure 5.6. Concentration of effluent solids (g VSS/L, ○) and reactor solids (mg VSS/L, ✳) for the AS-SBR.

Regarding the solids, the digestate fed to the AS-SBR had solids concentrations between 0.18 - 0.74 g VSS/L (Figure 5.6). The effluent had less than 0.20 g VSS/L during Stages I_{AS}-III_{AS} and V_{AS}, with a sharp increase during Stage IV_{AS} from 0.05 to over 1.4 g VSS/L. Similarly, the biomass concentration inside the reactor was between 3.2 and 5.9 g VSS/L for Stages I_{AS}-III_{AS} and V_{AS}, and increased to 10 g VSS/L during Stage IV_{AS}. The high solids concentrations achieved inside the reactor and in the effluent in Stage IV_{AS} coincided with the high increase in the organic matter concentration fed (Figure 5.5.a).

5.4.4. Nitrogen removal without AS pretreatment: nAS-SBR

Equally to the operation of the AS-SBR, the progressive increasing proportions of OFMSW digestate fed to the nAS-SBR provoked increasing concentrations of ammonium in the feeding (Figure 5.7.a), although the higher ammonium concentrations in the nAS digestate (without ammonia stripping pre-treatment) favoured a faster concentration increase compared to the AS digestate. The concentration of ammonium remained at an average of 731 ± 55 mg NH_4^+-N/L during Stage I_{nAS} (0 - 10% of OFMSW), and later increased to 1167 mg NH_4^+-N/L during Stage II_{nAS} (10 - 30% of OFMSW). A removal of TN of 66.5 ± 8.4% was achieved during Stage I_{nAS} (Figure 5.7.b).

The ratio between nitrate produced to ammonium oxidized was above the stoichiometric values during Stage I_{AS} (0.15 - 0.25 g/g, Figure 5.7.c), suggesting an active presence of NOB from the inoculum, similarly to the AS-SBR. During Stage II_{nAS}, the air supplied was diminished (< 0.05 mg O_2/L) to reduce the NOB activity, but both ammonium and nitrite accumulated inside the reactor (Figure 5.7.a) and the nitrogen removal efficiency decreased to 21%.

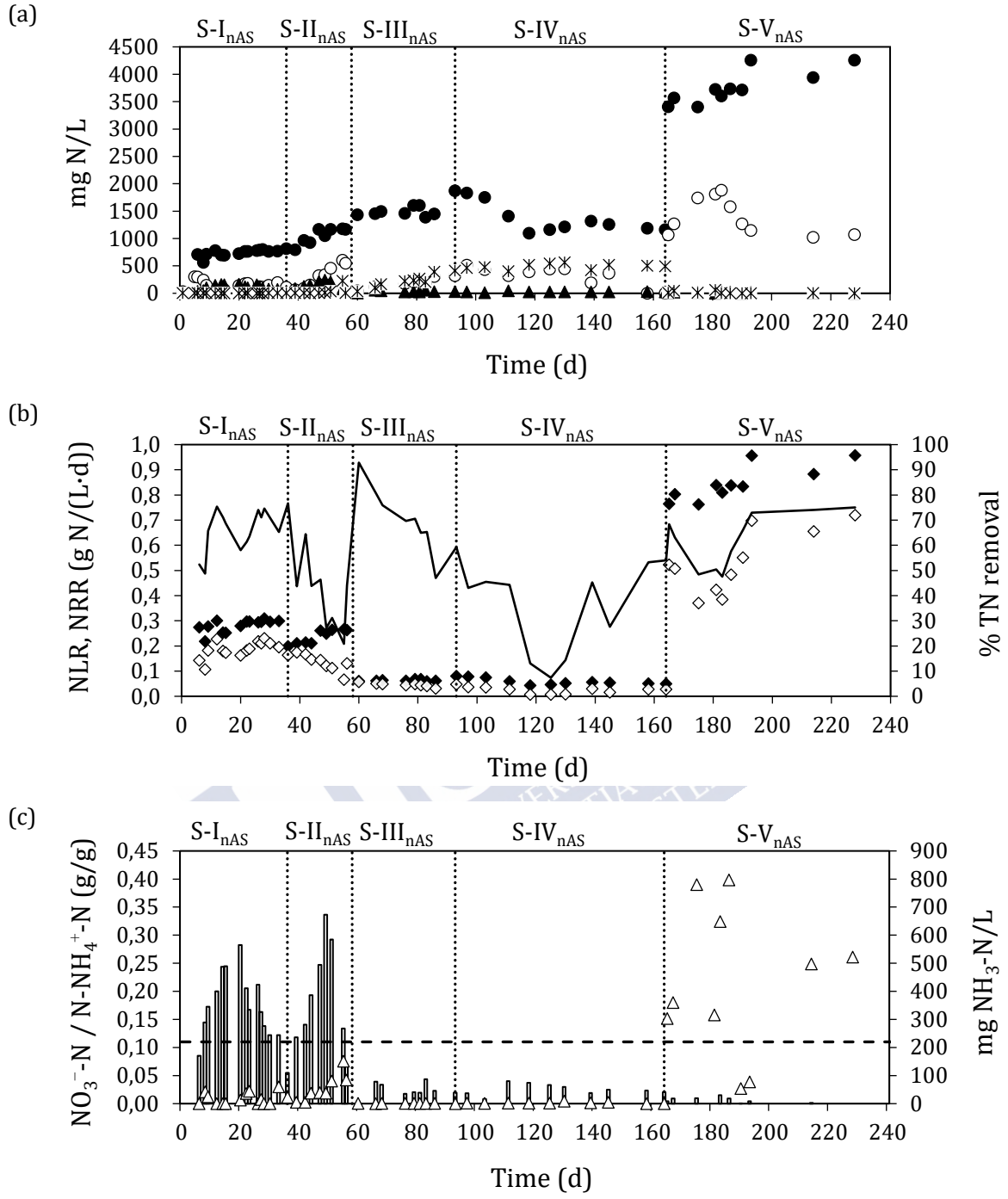

Figure 5.7. Data from the operation of nAS-SBR (Stages I_{nAS} to V_{nAS}): (a) concentration of NH_4^+-N in the influent (●), and NH_4^+-N (○), NO_2^--N (∗) and NO_3^--N (▲) in the effluent (mg N/L); (b) Nitrogen Loading Rate, NLR (g N/(L·d), ◆), Nitrogen Removal Rate, NRR (g N/(L·d), ◇) and % of total nitrogen (TN) removal (—); (c) nitrate produced to ammonium oxidized ratio (g NO_3^--N/g NH_4^+-N, ▯), concentration of free ammonia, FA (mg NH_3-N/L, △) and stoichiometric nitrate produced to ammonium oxidized for the PN-AMX processes (0.11 g N/g N) (- - -).

During Stage I_{nAS}, the low IC concentrations in the effluent were below 10 mg IC/L and the pH concurrently decreased to 5.3 by day 19 (Figure 5.8). Still, the removal of TN was able to remain in the range 58 - 77% at the end of Stage I_{nAS}. During Stage III_{nAS} (30 - 40% of OFMSW), the TN removal efficiency increased to 80 - 90% due to the decrease of the NLR down to 0.06 g N/(L·d) (Figure 5.7.b) and the previous re-inoculation. Nevertheless, the IC concentrations in the effluent below 30 mg IC/L favored again the decrease of both the pH (from 8.2 to 6.1, Figure 5.8), while the nitrogen removal efficiency decreased to 47% (Figure 5.7.b).

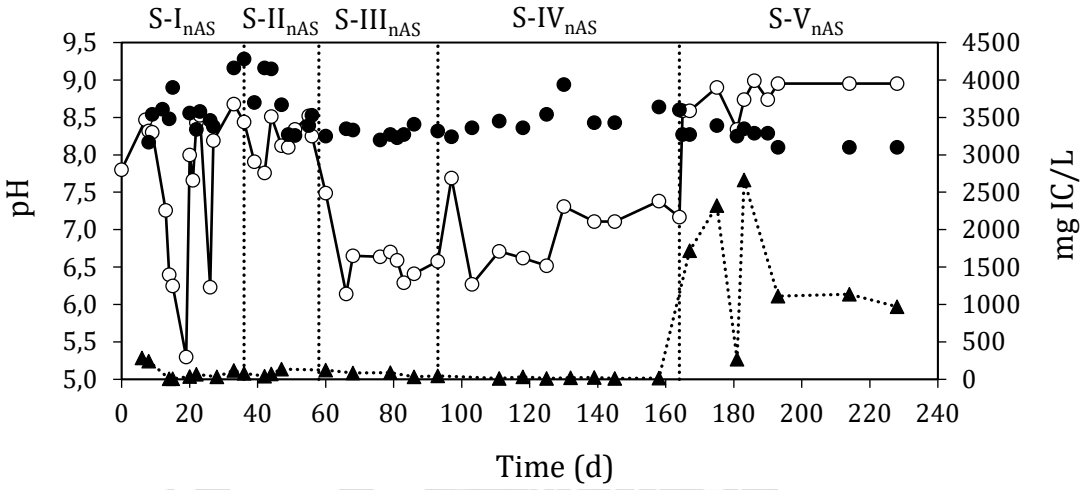

Figure 5.8. Concentration of inorganic carbon (IC) in the effluent (mg IC/L) from the nAS-SBR (▲) and pH of the influent (●) and effluent (○) for the nAS-SBR.

Similarly to the concentration of ammonium, the concentration of organic matter progressively increased during the operation (Figure 5.9.a). From Stage I_{nAS} to Stage III_{nAS}, the fed concentration of organic matter increased from 235 to 1904 mg sCOD/L. Besides, the concentrations in the OFMSW liquor were similar for both digestates for the same OFMSW proportion fed (35 - 40%), as opposed to the concentration of ammonium. The average removal of organic matter increased from the range 18 - 64% for Stages I_{nAS} and II_{nAS} to a higher range of 34 - 88% during Stage III_{nAS} (Figure 5.9.a). The increase of the sCOD/N ratio was smoother compared to the AS digestate, going from 0.3 to 1.4 g sCOD/g N during Stages I_{nAS} to III_{nAS}, and the potential contribution of HD to the TN removal concurrently increased from 0.9% to 12.0% (Figure 5.9.b).

During Stage IV_{nAS} (40 - 60% of OFMSW), the pH remained low inside the reactor (6.5 - 7.0) and favored FA concentrations below 5 mg NH_3-N/L (Figure 5.7.c). The average organic matter removal was 14.4 ± 8.9% (Figure 5.9.a) and the HD potential contribution to nitrogen removal was below 5%

(Figure 5.9.b). The nitrogen removal efficiency was able to recover from 7 to 53% at the end of Stage IV_{nAS} (Figure 5.7.b).

In Stage V_{nAS} (100% of OFMSW), the sCOD/N ratio was on average 1.79 ± 0.10 g/g (Figure 5.9.b). The nitrogen removal efficiency initially decreased to 47% but later recovered in 10 days to 75%, i.e. similar values were obtained compared to Stage I_{nAS} for 3-fold higher NLR (0.80 - 0.96 g N/L·d, Figure 5.7.b). The negligible NO_3^--N/NH_4^+-N ratio (Figure 5.7.c) suggested FA inhibition of the possible NOB present in the re-inoculation at this stage. The removal of organic matter was variable (24 - 72%, Figure 5.9.a) as well as the potential contribution of HD to the nitrogen removal (5.5 - 18.3%, Figure 5.9.b).

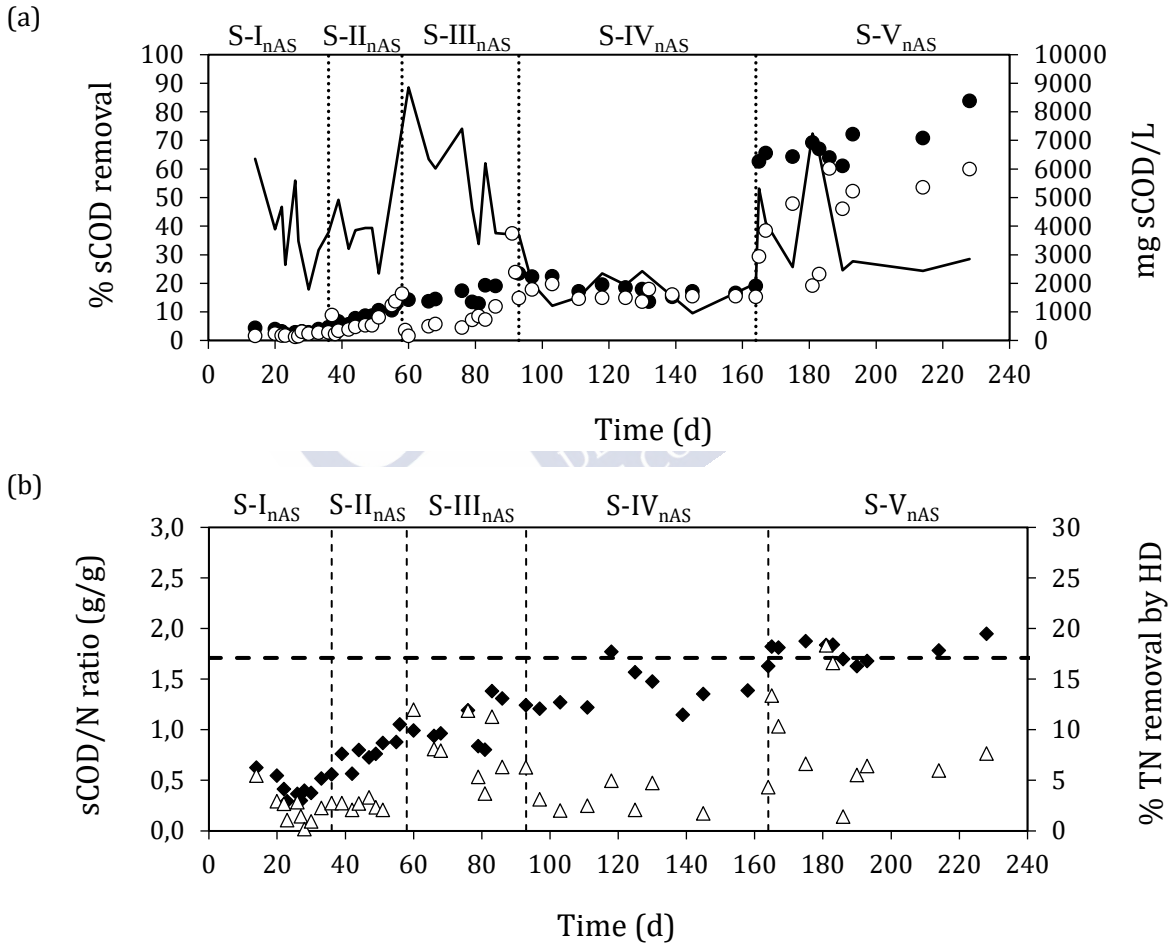

Figure 5.9. (a) Concentration of soluble COD (sCOD) in the influent (●) and effluent (○) (mg sCOD/L), and % of sCOD removal (—); (b) ratio sCOD/N in the influent (g sCOD/g N, ◆), maximum % of TN removal by possible heterotrophic denitrification (HD) (△) for the nAS-SBR and sCOD/N ratio threshold for complete denitritation (1.71 g sCOD/g N, Jenni et al. (2014)) (- - -).

Regarding the solids, the digestate fed to the nAS-SBR had concentrations between 0.19 - 0.71 g VSS/L (Figure 5.10). The effluent had less than 0.24 g VSS/L during Stages I_{nAS}-III_{nAS}, and later increased to 0.6 g VSS/L during Stages IV_{AS}-V_{AS}. The biomass concentration inside the reactor was variable during the entire operation, between 0.8 and 10.0 g VSS/L, which can be explained by the different operational conditions and the re-inoculation events.

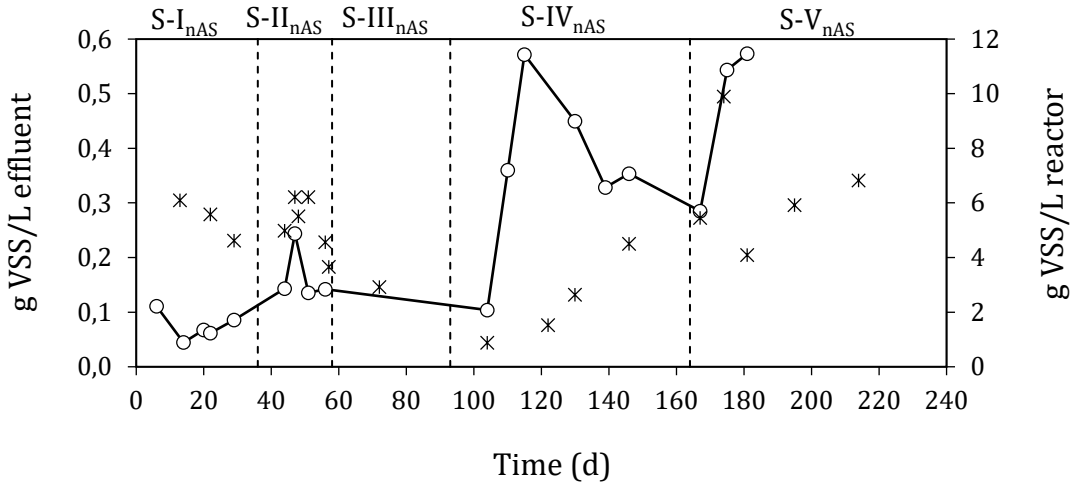

Figure 5.10. Concentration of solids (g VSS/L) in the effluent (○) and in the reactor (∗) (mg VSS/L) for the nAS-SBR.

5.4.5. Performance comparison between both SBR: anammox activity and effluent quality

5.4.5.1. Evolution of anammox activity treating OFMSW

Although the characteristics of both digestates were not ideal for the anammox process (namely, the sCOD/N ratio), the nitrogen removal via the PN-AMX route was promoted as much as possible by regulating the operational conditions in both SBR. However, the SAA measured throughout the operational time in batch tests (with PBS as liquid medium), showed a loss tendency up to 90%, as the % of fed OFMSW increased up to 100%, which was similar despite the presence or absence of the AS pre-treatment (Figure 5.11). Furthermore, sporadic SAA batch tests were performed comparing the SAA with PBS and OFMSW as liquid medium, showing an activity loss of 81.1% (day 48, Stage II_{AS}), 93.8% (day 57, Stage II_{AS}) and 90.4% (day 57, Stage II_{nAS}) when using OFMSW as liquid medium for the batch test.

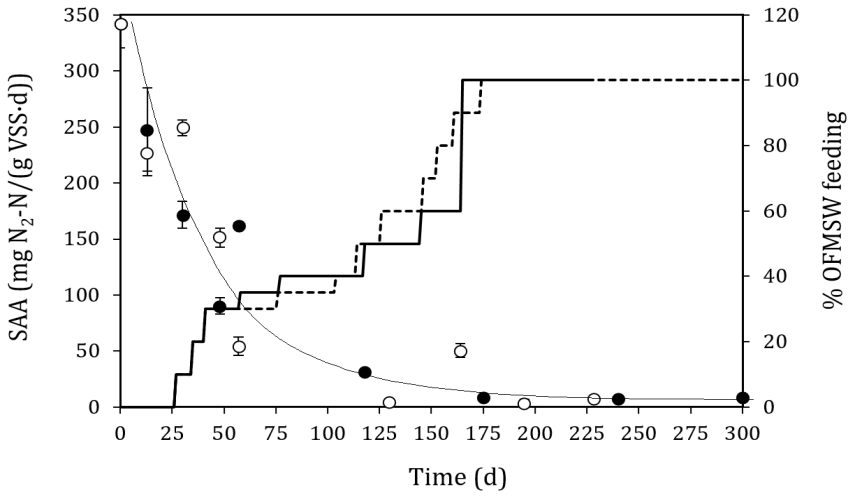

Figure 5.11. Evolution of the SAA values with the operational time for the AS-SBR (●) and nAS-SBR (○), and evolution of the % of OFMSW fed to each anaerobic digester previous to the operation of the AS-SBR (– –) and nAS (––).

On the other hand, the ratio VSS/TSS was similar for both reactors (67.4 ± 5.8% for AS-SBR and 66.2 ± 5.8% for nAS-SBR, Figure 5.12), with are moderate values compared to previously reported ratios (92 - 94%, Johansson et al., 2017). Since the VSS/TSS ratio was similar to these values already in the inoculum for both reactors, and it was maintained in the same range during both operations (Figure 5.12), no relation between the VSS/TSS ratio of the feeding and the possible precipitation issues on the biomass could be inferred.

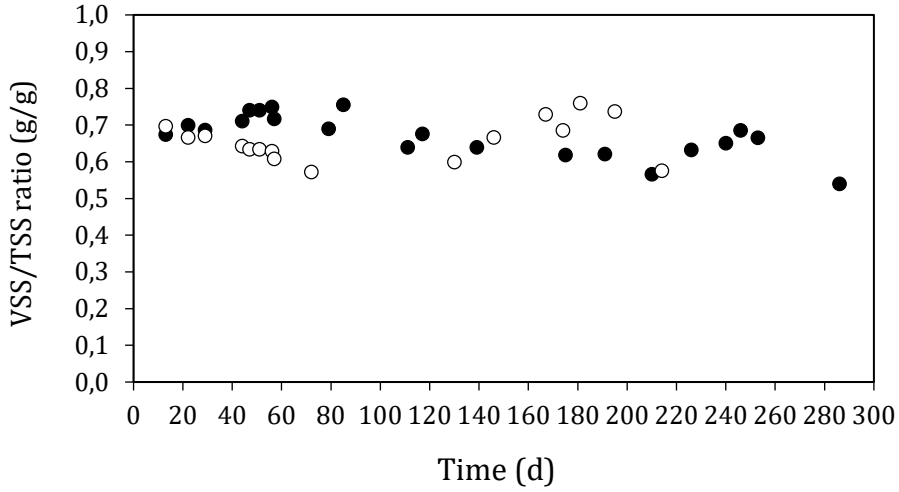

Figure 5.12. Evolution of the VSS/TSS ratio (g VSS/g TSS) with operational time for AS-SBR (●) and nAS-SBR (○).

In conclusion, these results indicate that in the continuous long-term operation, the application of such pre-treatment did neither improve nor worsen the harmful effect of the OFMWS digestate over the anammox activity.

5.4.5.2. Effluent quality

The comparison between the performances of both SBR is not straightforward, as the behavior of the nitrogen removal processes differed and motivated different operational conditions. However, the effluent quality constitutes a good indicator of the appropriateness of the AS pre-treatment. Table 5.4 shows different scenarios for effluent quality comparing AS-SBR and nAS-SBR: treating the 100% of OFMSW digestate (A), and treating the highest comparable sCOD concentration (B), NLR (C), sCOD/N ratio (D) and ammonium concentration (E) in the feeding.

Table 5.4. Comparison of the performance and effluent quality between AS-SBR and nAS-SBR treating 100% of OFMSW (A) and the highest comparable sCOD concentration (B), NLR (C), sCOD/N ratio (D), and ammonium concentration in the feeding (E).

Scenario	Stage (Days)	Effluent quality					Performance of PN-AMX processes		
		NH_4^+-N (mg/L)	NO_2^--N (mg/L)	NO_3^--N (mg/L)	TN (mg/L)	sCOD (mg/L)	TN_{rem} (%)	$sCOD_{rem}$ (%)	NRR (mg N/L·d)
A & B OFMSW[a] 100%	V_{AS} 276 - 300	122 ± 88	0.5 ± 0.1	4.6 ± 0.1	**127 ± 88**	**2833 ± 278**	94 ± 3	43 ± 4	58 ± 8
	V_{nAS} 200 - 228	1046 ± 25	1.3 ± 0.8	1.7 ± 0.7	1050 ± 23	5680 ± 319	75 ± 1	26 ± 2	687 ± 32
C NLR 40-50 mg N/(L·d)	V_{AS} 253 - 300	20 - 210	0.5 - 332	0 - 4.6	**39 - 337**	2870 ± 959	85 ± 10	55 ± 14	32 ± 17
	IV_{nAS} 118 - 164	367 ± 104	507 ± 46	23.6 ± 2.2	793 ± 227	**1546 ± 124**	31 ± 20	22 ± 2	7 - 30
D sCOD/N 1.8 g/g	III_{AS} 113 - 124	321 ± 28	316 ± 9	3.9 - 13.3	**579 ± 141**	**1827 ± 520**	53 ± 8	5 ± 1	140 ± 20
	V_{nAS} 165 - 228	1385 ± 334	0.3 - 10.1	3.5 - 26.1	1409 ± 346	4848 ± 1056	62 ± 11	23 ± 8	370 - 720
E NH_4^+-N 1200 mg/L	IV_{AS} 143 - 198	841 ± 42	19.7 ± 5.2	5.9 ± 1.1	990 ± 45	5744 ± 1120	59 ± 5	20 ± 6	20 - 80
	IV_{nAS} 118 - 164	367 ± 104	507 ± 46	23.6 ± 2.2	**793 ± 227**	**1546 ± 124**	13 - 54	22 ± 2	30 - 70

Values highlighted in **bold** correspond to the lower TN and sCOD concentrations in the effluent for each scenario.

[a] This scenario coincides with the scenario with the highest comparable sCOD concentration treated by both reactors (B), which was 6433 ± 401 mg sCOD/L for AS-SBR and 6570 ± 1011 mg sCOD/L for nAS-SBR.

In scenario A (which coincided with scenario B), the average inlet ammonium concentration (2300 mg N/L) was lowered to 127 ± 88 mg N/L in the effluent of AS-SBR, removing more than 90% of the total nitrogen. On the other hand, the average inlet ammonium concentration in the nAS-SBR (4098 mg N/L) decreased to approximately 1000 mg N/L, removing 75% of total nitrogen. Therefore, a better effluent quality in terms of TN was achieved in the AS-SBR, as well as in terms of organic matter (2822 ± 278 mg sCOD/L compared to 5680 ± 319 mg sCOD/L) for a NRR 10-fold lower than in the nAS-SBR. However, the higher NRR in the nAS-SBR was probably owed to the re-inoculation in Stage V_{nAS}.

In scenario C (similar NLR of 0.04 - 0.05 g N/(L·d)), the effluent quality was better in terms of TN concentration for the AS-SBR (39 - 337 mg N/L), while lower sCOD concentrations were achieved for the nAS-SBR (1546 ± 124 mg sCOD/L). In scenario D (similar sCOD/N ratio of 1.8 g/g), the lower effluent values of TN and sCOD concentrations were achieved in the AS-SBR. However, in the case of scenario E (similar ammonium concentrations around 1200 mg NH_4^+-N/L), the effluent of the nAS-SBR presented lower TN and sCOD concentrations.

Following the comparison in Table 5.4, the implementation of the AS pre-treatment before the anaerobic digestion would not be always advisable. Nevertheless, it is important to remark that the improvement in the effluent quality is comparatively higher when the AS pre-treatment is applied, and the AS pre-treatment is clearly recommended when treating 100% OFMSW. Still, none of the scenarios fulfilled the discharge limits imposed in the industry regarding the concentration of ammonium (47 mg NH_4^+-N/L), TN (90 mg TN/L) and organic matter (1500 mg COD/L). This compliance is crucial to conform to the existing EU legislation concerning the application of digestate to land (Council Directive 91/676/EEC, 1991) and the discharge of treated effluent to water recipients (Council Directive 91/271/EEC, 1991) (Malamis et al., 2014), although it has been rarely achieved previously by the sole application of biological treatments to the treatment of leachate-like effluents (Wu et al., 2015; L. Wu et al., 2016a).

5.4.6. Molecular analysis

5.4.6.1. Bacterial diversity and phylogenetic variations

Bacterial populations were analyzed for samples taken in the different stages during the operation of AS-SBR and nAS-SBR. Regarding the bacterial community similarity at OTU level across different time points, the community structure was dynamic with time, shifting during the different stages (Figure 5.13). Samples collected during the operation of AS-SBR were placed in two main clusters, corresponding to samples collected from Stage I_{AS} to IV_{AS} (< 100% proportion of OFMSW in the feeding) and from Stage V_{AS} (100% OMFSW). On the other hand, samples from the nAS-SBR were

clustered in two main groups, following the re-inoculation of the reactor from the same source (Stage I_{nAS} and day 181) and the remaining samples (Stages III_{nAS}, IV_{nAS} and V_{nAS}).

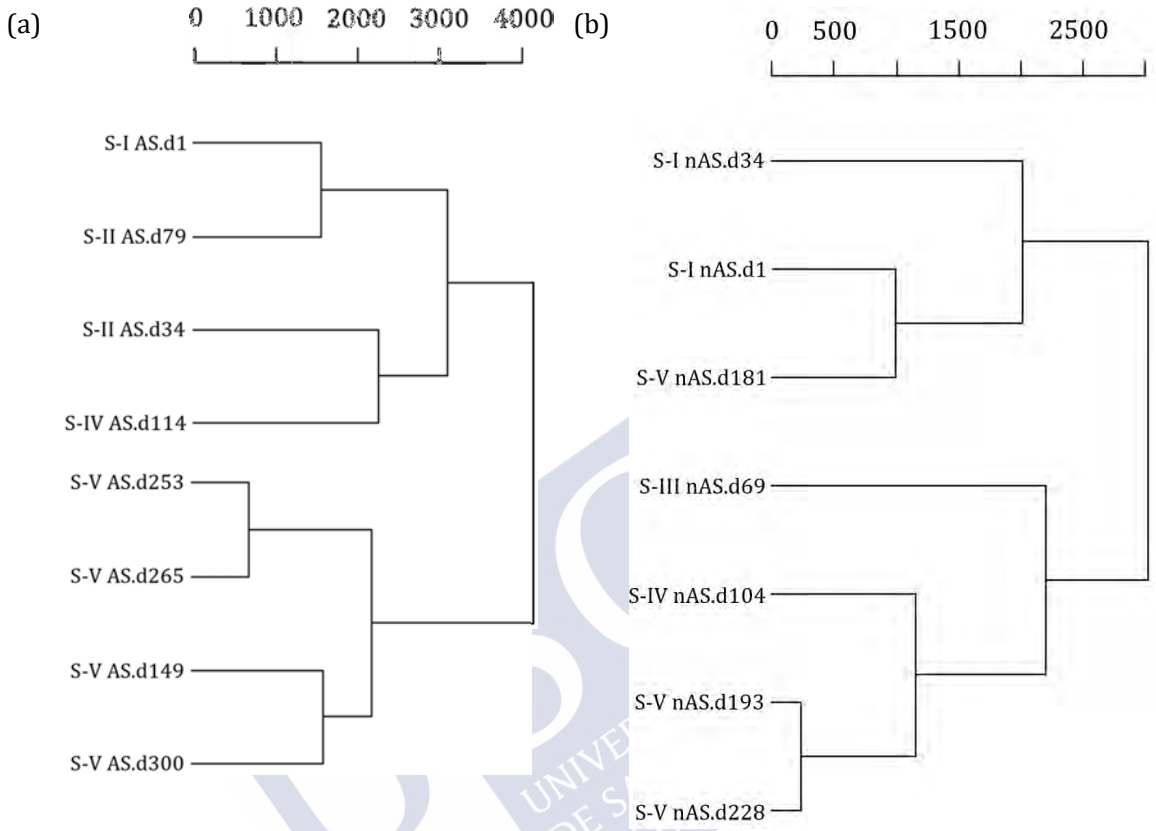

Figure 5.13. Dendrogram of samples collected during the operation of AS-SBR (a) and nAS-SBR (b) illustrating the similarity level of the different samples..

Divergences among samples during the operation of both reactors were analyzed based on phylogenetic variations by PCoA and then by applying db-RDA (Figure 5.14). The first two axes of the constrained redundancy analysis, i.e. RDA1 (32%) and RDA2 (17%), explained almost 50% of the phylogenetic variations of the microbial communities. The sCOD/N ratio, the concentration of PO_4^{3-} and the conductivity were the operational parameters that best explained the variations in the bacterial communities.

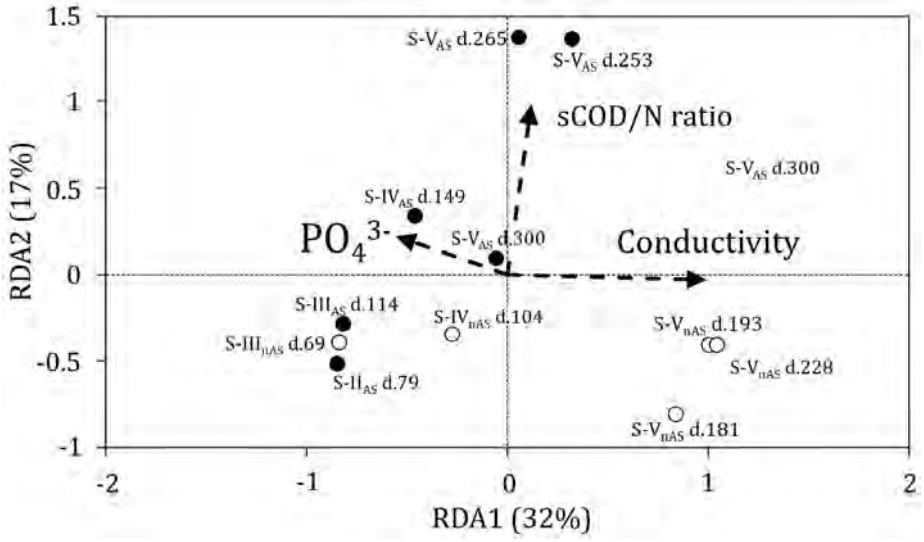

Figure 5.14. Distance-Based Redundancy Analysis (db-RDA) showing the parameters that best explain the variation in microbial communities in the AS-SBR (●) and nAS-SBR (○).

5.4.6.2. Bacterial taxonomy and evolution

The bacterial community was composed by 29 phyla in AS-SBR and 28 phyla in nAS-SBR, and the 10 most abundant represented 96.1 ± 2.2 and 96.4 ± 2.1 in relative abundance (RA, %) of the total *Bacteria*, respectively (Figure 5.15). Even though the same inoculum was used at the beginning in both reactors, the evolution of the bacterial communities was clearly different. *Proteobacteria* (mainly *Betaproteobacteria* and *Gammaproteobacteria*), *Bacteroidetes* and *Firmicutes* were the dominant phyla and contributed to more than 50% of the RA in all samples.

Classes *Betaproteobacteria* (RA of 14.2 ± 4.1%) and *Gammaproteobacteria* (RA of 10.7 ± 7.8%) showed great resistance to operational shifts in the AS-SBR, while they decreased in the case of nAS-SBR from Stage IV_{nAS} to Stage V_{nAS}. Microorganisms from phylum *Bacteroidetes* mainly belonged to classes *Sphingobacteriia* and *Flavobacteria*. The RA of class *Sphingobacteriia* gradually increased in AS-SBR up to 27.1 ± 5.9% during Stage V_{AS}, while it was maintained in nAS-SBR at a lower value of 4.9 ± 1.2%, except for a peak on day 104 (23.1%). The opposite behavior was observed for the class *Flavobacteria*, which decreased its RA in the AS-SBR from 23.9 ± 3.8% in the inoculum to 3.7% by day 34. In the nAS-SBR, the RA of *Flavobacteria* increased during Stages IV_{nAS} and V_{nAS} and maintained at 10.3 ± 5.4%, despite the same washout effect at the beginning of Stage I_{nAS}.

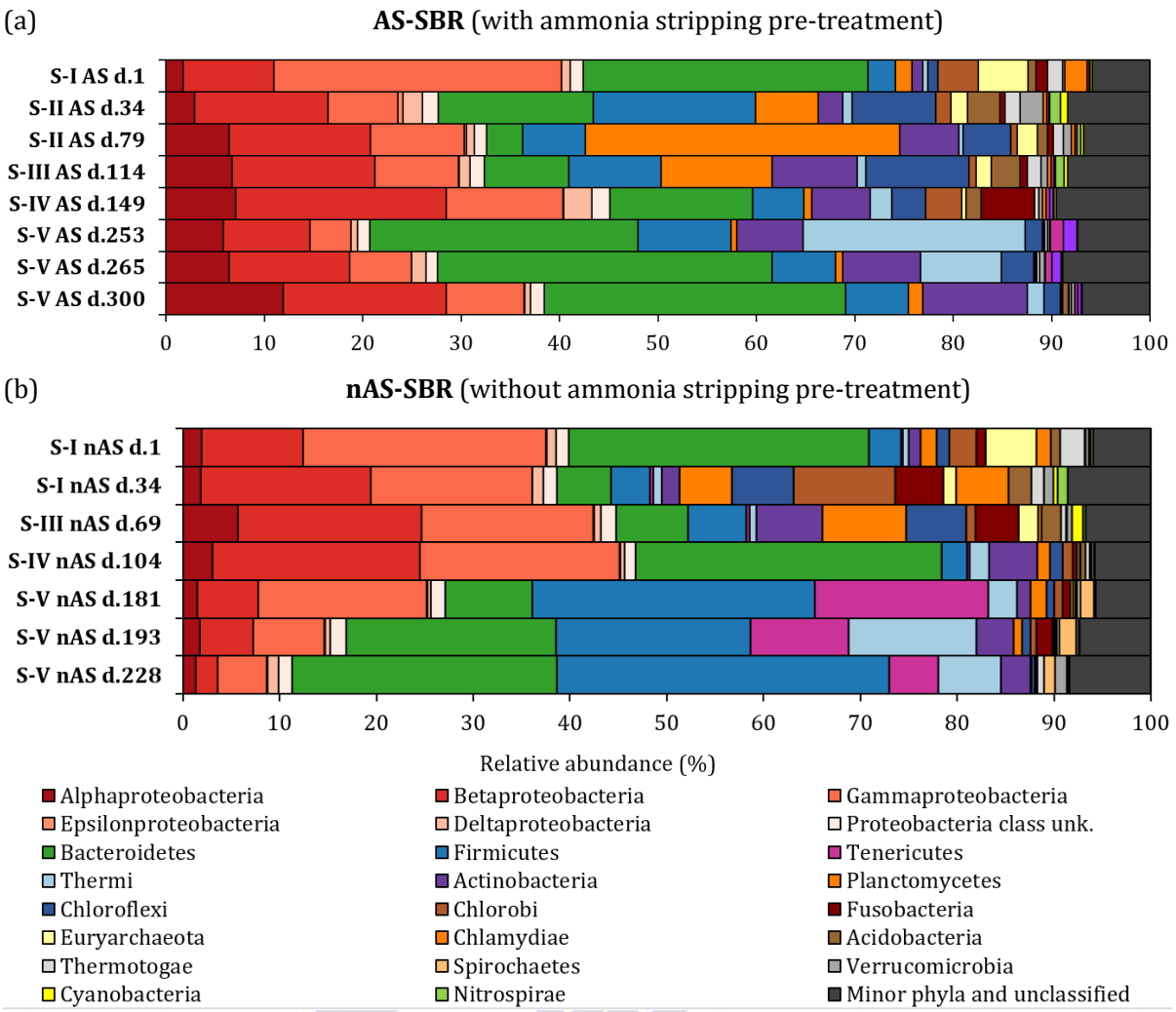

Figure 5.15. Taxonomic profile and corresponding relative abundances (RA) of the 10 most abundant phyla identified in the bacterial community during the operation of (a) AS-SBR and (b) nAS-SBR. For *Proteobacteria* phylum, the different classes are shown. Sample labels indicate the Stage of each reactor followed by the operational day.

On the other hand, Candidatus *Brocadia* and Candidatus *Scalindua* were identified as the main anammox species for both reactors, with the former as the dominant one (RA of 87.4 ± 1.5% for AS-SBR, 88.5 ± 1.9% for nAS-SBR). The temporal changes of the major anammox, AOB, NOB and denitrifiers for both reactors is depicted in Figure 5.16. The RA of the class *Brocadiae* in AS-SBR and nAS-SBR reached its maximum on day 79 (31.1% and 8.1%, respectively) and further decreased from this point on (0.05% by day 300 and 0.01% by day 228, respectively). These results are in agreement with the SAA decreasing values throughout the operational time observed in both reactors (Figure 5.11). Main identified genera for denitrifying bacteria were *Thauera*, *Paracoccus*, *Burkholderia*, *Comamonas*, *Thiobacillus* and *Pedobacter*. The RA tendency of the denitrifiers in AS-SBR showed an opposite tendency in comparison with the main anammox genera. The availability of organic carbon

can lead to the shift between the PN-AMX and denitrification processes (Li et al., 2018), and in this case the increasing sCOD/N ratio in the feeding (see Figure 5.5.b) contributed to this shift.

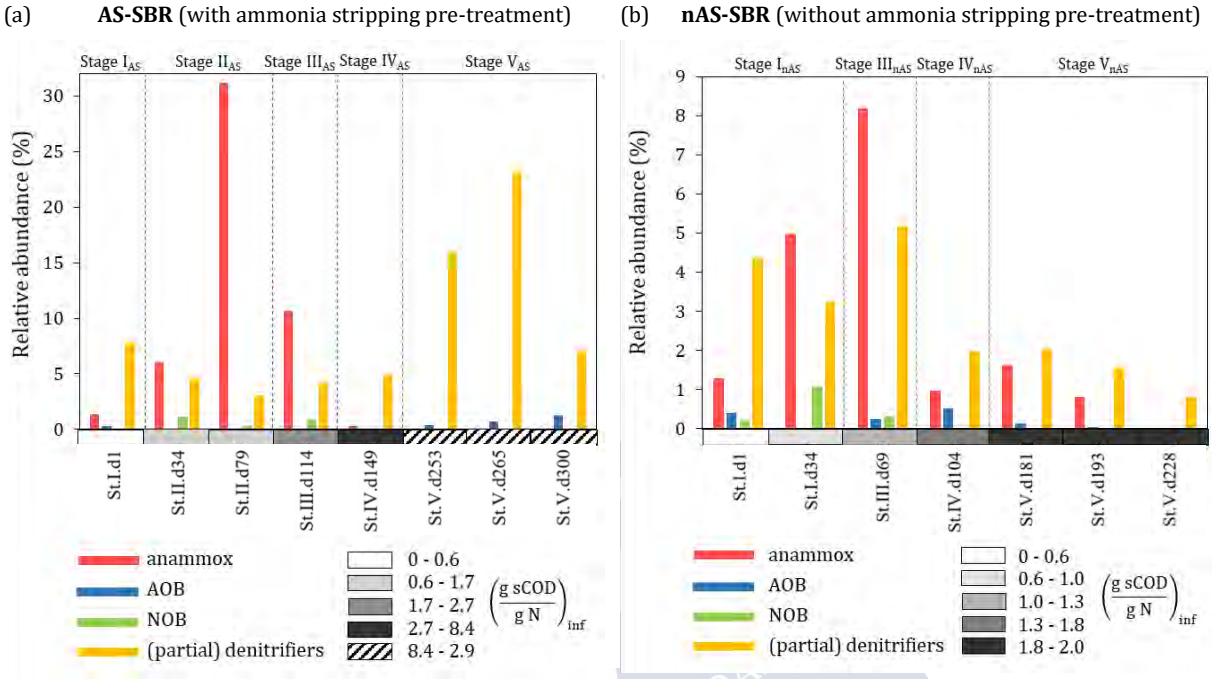

Figure 5.16. Temporal changes of the major anammox bacteria (genera Candidatus *Brocadia* and Candidatus *Scalindua*), AOB (genera *Nitrosospira*, *Nitrosovibrio* and *Nitrosococcus*), NOB (genera *Nitrospira*) and denitrifiers (main genera: *Thauera*, *Paracoccus*, *Burkholderia*, *Comamonas*, *Thiobacillus* and *Pedobacter*; *Denitrificans* sp.). Sample labels indicate the Stage of each reactor followed by the operational day.

5.5. Discussion

5.5.1. Partial nitritation and suppression of NOB activity

Regarding the inhibition of NOB by free ammonia (FA), no agreement has been reached about the inhibitory threshold (Wang et al., 2016), with previously reported ranges of 0.082 - 0.82 mg NH_3-N/L (Anthonisen et al., 1976), 0.8 - 22 mg NH_3-N/L (Kim et al., 2010) and 10 - 22 mg NH_3-N/L (Jianlong and Ning, 2004). On the other hand, Fernández et al. (2012) showed an IC_{50} value of 38 mg NH_3-N/L for anammox bacteria. Malamis et al. (2014) stated that the characteristics of the OFMSW digestates favor the inhibition of NOB in downstream biological nitrogen removal processes due to the high concentration of ammonium and the temperature. Ganigué et al. (2007), for example, reported little accumulation of nitrate up to 0.4 mg N/L for FA concentrations of 5.58 mg N/L.

In the present study, the NOB were already present in the inoculum of both reactors, as shown by the observed NOB activity (see Figure 5.3.c and Figure 5.7.c) and the microbiological analyses (samples

S-I AS d.1/nAS d.1, Figure 5.15). The relative abundance of *Nitrospira* sp. reached by day 34 a maximum of 1.1% for both reactors (Figure 5.16), while the NOB activity was barely significant during Stages III_{AS} and III_{nAS} due to FA inhibition, with relative abundances for *Nitrospira* sp. of 0.88% (AS-SBR) and 0.31% (nAS-SBR). From Stages IV_{AS} and IV_{nAS} on the relative abundance dropped below 0.02% until the end of the operation for both reactors. These results are in line with previous research studies addressing the NOB suppression when treating landfill leachate by controlling the free ammonia (FA), pH and DO concentration (Li et al., 2014a; Spagni et al., 2014; Wu et al., 2016b).

As to the cause of the FA inhibition, Kulikowska and Bernat (2013) found that low DO values (0.7 mg O_2/L) were the main NOB inhibitor when treating landfill leachate. Li et al. (2013) and Sobotka et al. (2015) underlined the importance of the intermittent aeration for effectively NOB inhibition. In the present study, the DO concentration values were in general below 0.1 mg O_2/L for both reactors (Table 5.2), which directly influenced the oxidation of ammonium and hence the remaining FA concentration inside the reactor. The link between high FA concentrations and low DO concentrations can be seen for example during Stage III_{AS} (from day 124 on), where a decrease in the DO concentration from 0.10 mg O_2/L to 0.05 mg O_2/L provoked both the concentration of ammonium and the FA in the reactor to rise from 300 to 938 mg NH_4^+-N/L (Figure 5.3.a) and from 50 to 350 mg NH_3-N/L (Figure 5.3.c), respectively. However, care must be taken with this strategy since these high FA concentrations can also inhibit both the anammox and heterotrophic denitrifying bacteria (Malamis et al., 2014). For example, the high FA concentrations during Stages III_{AS}-IV_{AS} probably contributed to the decreasing nitrogen removal efficiency (from 75% to 24%, Figure 5.3.b), considering a threshold of 150 mg NH_3-N/L as a starting point for anammox inhibition (Aktan et al., 2012). This shows the importance of a tight control in the DO concentration for achieving NOB suppression without excessive limitation of AOB activity and consequently accumulation of remaining ammonium.

Other factors that could be responsible for the NOB inhibition from Stage III on for both SBR is the increase in the organic matter concentration. This can help developing aerobic and denitrifying heterotrophic organisms, which can compete by the oxygen and the nitrite with the NOB, respectively.

On the other hand, the inorganic carbon (IC) as carbon source for the PN-AMX processes constitutes an important factor affecting their microbial interactions (Ma et al., 2015), whereas the ratio between the nitrogen and the IC (N/IC) indicates the extent of the partial nitritation process: for N/IC ratios higher than of 1 g N/g IC, the alkalinity restricts the ammonium oxidation below 50% values (Pedrouso et al., 2017). Durán et al. (2014) found a 50% oxidation of ammonium for the partial nitration process treating pig slurry when adjusting the NH_4/IC ratio in the feeding to 0.82 g N/g C.

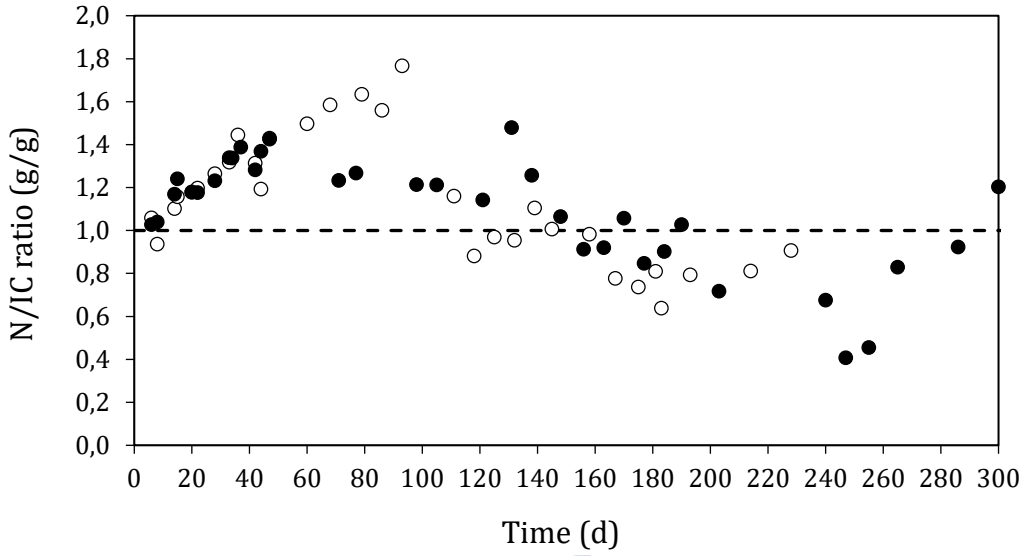

Figure 5.17. Ratio N/IC (g/g) in the influent throughout the operation of AS-SBR (●) and nAS-SBR (○), and N/IC ratio threshold (1 g N/g IC) below which 50% or more of the ammonium can be oxidized (- - -).

As can be seen in Figure 5.17, the N/IC ratio was above 1 g N/g IC for the major part of the AS-SBR and nAS-SBR respective operations, as the proportion of OFMSW in the feeding increased. In the case of the nAS-SBR, the N/IC ratio matched this threshold from day 120 (Stage IV_{nAS}) onwards, which coincides with the operational period (including Stage V_{nAS}) where the removal efficiency of total nitrogen (TN) was able to recover from 10% to 75% (Figure 5.7.b). On the other hand, the N/IC ratio for the AS-SBR was in a lower range, with an average value around the threshold, for Stages IV_{AS} (0.90 ± 0.14 g/g) and V_{AS} (0.76 ± 0.33 g/g) (Table 5.2). Similarly to the nAS-SBR, the recovery of the removal efficiency of TN from Stage IV_{AS} onwards (from 27% to 91 - 97% on Stage V_{AS}, Figure 5.3.b) agrees with the concurrent low N/IC ratios.

However, high removal efficiencies of TN (regarding each entire operation) were achieved also for higher N/IC ratios, as it is the case for Stage II_{AS} (78.4 ± 6.0%, Figure 5.3.b) for 1.14 ± 0.51 g N/g IC, and Stage III_{nAS} (72.3 ± 10.6%, Figure 5.7.b) for 1.61 ± 0.10 g N/g IC. As it will be discussed in the next Chapter, differences in the removal of nitrogen for N/IC ratios in the same range arise when comparing the treatment of pre-digested OFMSW liquors with and without the presence of membranes in the previous anaerobic digestion step (permeates in Chapter 6 and digestates in Chapter 5, respectively). Still, the removal of nitrogen during Stage III_{nAS} decreased during Stage III_{nAS} from 93% to 59% (Figure 5.7.b) as the N/IC ratio increased from 1.5 g/g to the highest observed value for the nAS-SBR (1.8 g/g, Figure 5.17). Overall, the relation between the N/IC ratio (Figure 5.17) and the removal efficiency of total nitrogen (Figure 5.3.b and Figure 5.7.b) confirms the positive effect of the former over the removal of nitrogen for values closer to the threshold for alkalinity limitation (1 g/g).

5.5.2. Organic matter effect and heterotrophic denitrification

Both the presence of organic matter and the aeration provided in the SBR can promote the growth of aerobic heterotrophs. However, in order to simplify the calculations and study the possible competition between anammox and denitrifying bacteria, the organic matter was assumed to be removed by heterotrophic denitrifiers, and their maximum potential contribution to nitrogen removal was estimated for AS-SBR and nAS-SBR (Figure 5.5.b and Figure 5.9.b, respectively).

Although heterotrophic denitrifiers can help anammox bacteria by consuming the organic matter and supplying nitrite for the latter (Li et al., 2018), they can also outcompete the anammox bacteria if enough organic matter is present due to the faster growth rates and more favorable thermodynamics of their metabolic pathway (Li et al., 2016b). Organic matter concentrations above 200 mg COD/L (Li et al., 2016a) and above 300 mg COD/L (Chamchoi et al., 2008; Wang et al., 2019) were considered inhibitory for the anammox bacteria when treating pig slurry and synthetic wastewater, respectively, while the activity of anammox bacteria predominates over the heterotrophic denitrification in PN-AMX systems for COD/N ratios below 2 g COD/g N (Lackner et al., 2014, 2008). Jenni et al. (2014) pointed out a ratio of 1.71 g COD/N as a minimum threshold for complete denitrification via nitrite.

In the present study, the evolution of nitrogen removal and the abundance of anammox bacteria in the biomass from the reactors correlated with the increasing sCOD/N ratio in the feeding. During Stages I_{AS} and II_{AS}, the relative abundance of anammox increased up to 31.1% (Figure 5.16.a) for sCOD/N ratios below 1.7 g sCOD/g N (Figure 5.5.b), corresponding to 0.058 ± 0.028 g sCOD/g TN consumed and 36.3 ± 9.0% of organic matter removal. The specific denitrifying activity ($SDA_{PBS\text{-}NO3}$) for day 85, for example, was 24.5 ± 1.4 mg N/(g VSS·d), approximately half the SAA_{PBS} for this day regarding the SAA evolution in Figure 5.11, and in agreement with the lower relative abundance for denitrifiers in Figure 5.16.

Later, the removal efficiency of total nitrogen dropped from 74% to 29% during Stage III_{AS} (Figure 5.3.b) as the sCOD/N ratio increased to 2.7 g sCOD/g N (0.012 - 0.153 g sCOD/g TN consumed, 12.5 ± 7.4% of organic matter removal). Both the accumulation of nitrite over 300 mg NO_2^--N/L (Figure 5.3.a) and the drop in the relative abundance of the class *Brocadiae* to 10.6% during Stage III_{AS} suggest that the anammox bacteria were not able to compete with the denitrifiers, which were favored by the increasing sCOD/N ratio and removed up to 10% of the total nitrogen (Figure 5.5.b). Furthermore, the increase of the biomass inside the reactor during Stage IV_{AS} (from 2.8 to 10.3 g VSS/L), as well as the sharp solids increase in the effluent (from 0.05 to 1.5 g VSS/L, Figure 5.6) seems to match with a fast development of denitrifying activity in this stage.

Wang et al. (2019) pointed out the importance of COD concentration rather than COD/N ratio for determining the nitrogen removal pathways, with 300 mg COD/L as a threshold for 50% loss of

anammox activity (Wang et al., 2019). Nevertheless, the removal of total nitrogen remained over 80% during Stage II_{AS} (Figure 5.3.b) for a sCOD concentration already 4-fold the reported threshold (1283 ± 424 mg sCOD/L), and only started to decrease during Stage III_{AS} as the sCOD/N ratio reached and surpassed the reported inhibition threshold for denitrification via nitrite (1.71 g COD/g N, Jenni et al. (2014) (Figure 5.5.b). Furthermore, the sCOD/N ratio defines the microbial communities mainly for the AS-SBR (not the nAS-SBR), as seen in Figure 5.14, probably due to the smoother increase only up to 2 g sCOD/g N in the nAS-SBR (Figure 5.9.b). Therefore, the sCOD/N ratio rather than the sCOD concentration seems to better describe the performance and evolution of both anammox and denitrifying bacteria.

The relative abundance of anammox bacteria decreased to 0.05% (Figure 5.16.a) as the sCOD/N ratio increased from 2.7 to 8.4 g sCOD/g N during Stage IV_{AS} (Figure 5.5.b) (0.100 - 0.471 g sCOD/g TN consumed, 21.5 ± 6.5% of organic matter removal), which determined the bacterial community change for Stage V_{AS} (days 253 and 265, Figure 5.14). The $SDA_{PBS\text{-}NO3}$ at the end of Stage IV_{AS} (day 240) was of 2.9 ± 0.3 mg N/(g VSS·d), while the $SDA_{PBS\text{-}NO3}$ (9.1 ± 0.5 mg N/(g VSS·d)) was 19.7% higher than the SAA_{PBS} for the same day (7.6 ± 0.3 mg N/(g VSS·d)). Furthermore, higher relative abundances for denitrifying bacteria were observed for Stage IV_{AS} and Stage V_{AS}: 20.1 ± 10.6% (*Sphingobacteriaceae*, phylum *Bacteroidetes*), 8.4 ± 2.5% (families *Comamonadaceae*, phylum *Alphaproteobacteria*) and 16.3 ± 5.2% (*Sphingomonadaceae*, phylum *Betaproteobacteria*). All of them have been previously found as dominant bacterial populations in the denitrifying microbial community in aerobic granules (Adav et al., 2010).

However, a good synergy between anammox and denitrification processes would occur for more moderate sCOD/N ratios, where the denitrifiers could help consuming both the nitrite produced by AOB and the nitrate produced by anammox and NOB (Li et al., 2016b; Miao et al., 2016). Chen et al. (2016), for example, reported that anammox activity gradually recovered by reducing the influent C/N ratio from 2.3 to 1.25 g COD/g N. During Stage V_{AS}, the total nitrogen removal efficiency increased above 90% (Figure 5.3.b) as the sCOD/N ratio decreased from 8.5 to 2.9 g sCOD/g N (Figure 5.5.b) (with 0.002 - 0.070 g sCOD/g TN consumed) and the NLR was reduced to 0.07 g N/(L·d). From this 90% of TN removal, a maximum of 35% could be owed to the denitrifying bacteria (Figure 5.5.b). In line with the coupling anammox and denitrifying bacteria, the biomass concentration inside the reactor was lower (3 - 5 g VSS/L, Figure 5.6) after the washout of Stage IV_{AS}, suggesting a better selection of the biomass responsible for nitrogen removal in the reactor. Likewise, the relative abundance of the main genera of denitrifying bacteria raised from 4.9% in Stage IV_{AS} to 23.1% in Stage V_{AS} (Figure 5.16.a). Therefore, the sCOD/N ratio both favored the denitrification pathway and improved the removal of total nitrogen through the synergy between anammox bacteria and heterotrophic denitrifiers.

As to the nAS-SBR operation, the higher abundance of anammox bacteria (8.1%) occurred for sCOD/N ratios below 1.71 g COD/g N (day 69, Figure 5.16.b), corresponding to 0.013 ± 0.004 g sCOD/g TN consumed and 55.8 ± 18.7% of organic matter removal. Accordingly, the $SDA_{PBS\text{-}NO3}$ (0.97 ± 0.01 mg N/(g VSS·d)) was 97% lower than the SAA_{PBS} (32.5 ± 9.7 mg N/(g VSS·d)) at the end of Stage II_{nAS} (day 58), while the $SDA_{OFMSW\text{-}NO3}$ (with OFMSW as liquid medium for the batch test) was 30% higher than the SAA_{OFMSW} (3.9 ± 1.5 mg N/(g VSS·d) compared to 3.0 ± 0.1 mg N/(g VSS·d)).

However, while in the AS-SBR the relative abundance of the anammox bacteria dropped after 36 days matching the sCOD/N ratio threshold for denitrification (days 77 to 113, Figure 5.5.b), in nAS-SBR it dropped already from Stages III_{nAS}-IV_{nAS} on, i.e. after less than 20 days at 1.3 g sCOD/g N (days 83 to 103, Figure 5.9.b) (0.012 - 0.005 g sCOD/g TN consumed, 33.7 ± 19.2% of organic matter removal). The reason for the low relative abundance of anammox bacteria from Stage IV_{nAS} on may be attributed instead to the low concentrations of IC substrate at this point (below 30 mg IC/L, Figure 5.8).

Likewise, the relative abundances for the denitrifying bacteria were lower during Stages II_{nAS}-IV_{nAS} compared to Stage IV_{AS} (maximal of 14.6%, 2.4% and 1.4% for families *Sphingobacteriaceae*, *Comamonadaceae* and *Sphingomonadaceae*, respectively), probably due to the smoother sCOD/N increase only up to 2 g sCOD/g N in the first case. The relative abundance of denitrifiers was higher during Stage IV_{nAS} (1.98%) compared to anammox bacteria (0.98%) (Figure 5.16), in agreement with the higher $SDA_{PBS\text{-}NO3}$ and $SDA_{PBS\text{-}NO2}$ at the end of Stage IV_{nAS} (5.5 ± 0.1 mg N/(g VSS·d) and 14.7 ± 0.5 mg N/(g VSS·d), respectively) compared to the SAA_{PBS} (5.0 ± 0.6 mg N/(g VSS·d)).

On the other hand, the utilization of the organic matter available in the feeding by the different biological processes was induced by the operational conditions. The low aeration avoided the development of NOB by FA inhibition and the inhibition of anammox bacteria, but also limited the organic matter removal by the aerobic pathway. The avoided accumulation of nitrite and/or nitrate inside the SBR probably hindered the heterotrophic denitrification pathway as well, although average organic matter removal efficiencies of 28% (Figure 5.5.a) and 33% (Figure 5.9.a) were observed for AS-SBR and nAS-SBR, respectively. Despite the little difference, these results are in line with the hypothesis of a more biodegradable organic matter fraction in the digestate when no AS pre-treatment is applied (Pedizzi et al., 2017).

5.5.3. Presence of metals in the OFMSW

The industrial wastewater types like the landfill leachate or the OFMSW are characterized for including complex components, like metals, that could directly or indirectly affect the anammox

activity (Li et al., 2018). Table 5.5 summarizes the complete list of metals determined for the samples from day 167 (Stages IV_{AS} and V_{nAS}).

Table 5.5. Total concentration of metals (µg/L) measured in the OFMSW fed for days 167 (AS-SBR, Stage IV_{AS}) and 122 (nAS-SBR, Stage IV_{nAS}).

Metal	AS-SBR	nAS-SBR	Metal	AS-SBR	nAS-SBR
Fe	6137 ± 2	4946 ± 4	**Ni**	286 ± 2	98.0 ± 4.2
Al	6427 ± 3	4124 ± 4	**Cr**	115 ± 1	40.7 ± 4.6
Zn	371 ± 2	451 ± 4	**As**	95.8 ± 1.0	56.3 ± 6.0
Cu	225 ± 1	372 ± 4	**Mn**	137 ± 1	161 ± 5
Ti	413 ± 3	235 ± 5	**Pb**	49.0 ± 1,0	41.3 ± 1.9
Metal	AS-SBR	nAS-SBR	Metal	AS-SBR	nAS-SBR
Co	42.4 ± 1.1	20.7 ± 4.2	**Hg**	1.2 ± 4.1	2.5 ± 2.5
V	50.8 ± 1.6	20.0 ± 3.9	**Cd**	1.7 ± 9.8	1.9 ± 9.8
Mo	14.2 ± 2.8	11.2 ± 11.8	**Be**	0.3 ± 24.3	0.5 ± 26.4
Se	27.3 ± 7.8	6.4 ± 16.8			
Ag	5.1 ± 6.8	7.3 ± 3.8			

All the measured values are within the reported ranges for metals in leachates (see Table 1.10 in Chapter 1), except for Al (4.1 - 6.4 mg/L) which was above the reported range of 0.020 - 2.000 mg/L. On the other hand, several metals frequently studied along with the anammox process like Cu, Zn, Cr and Ni were below the reported IC_{50} values (Table 1.11, Chapter 1).

The dissolved fraction of metals is known to provoke inhibition over the anammox bacteria (Li et al., 2015). In this case, the dissolved fraction of metals present in the influent and effluent was very similar for both reactors, with the exception of Cu and Ni for the nAS-SBR, which increased in the effluent by 89% and 47% compared to the influent, respectively (day 122, Figure 5.18.a). The average fraction of dissolved metals was higher for the AS-SBR than for the nAS-SBR, for example 72% opposite to 36% for the case of Al in Figure 5.18.b. However, no inhibitory effects over the PN-AMX processes from dissolved metals can be related to their performance on day 167, according to the values of Table 5.5.

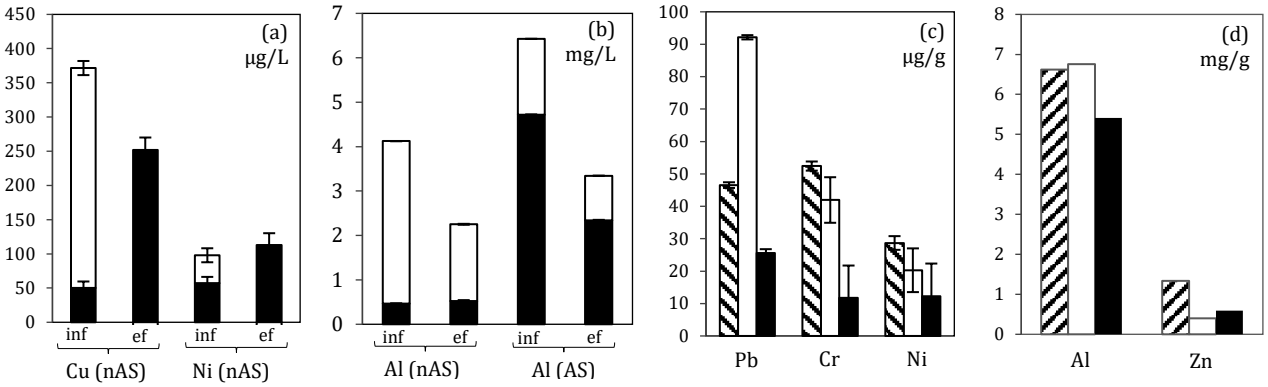

Figure 5.18. Concentration of the most relevant metals measured at days 167 (AS-SBR) and 122 (nAS-SBR): (a) particulated (▢) or dissolved (■) copper and nickel concentrations (µg/L) in the influent and effluent of nAS-SBR, (b) particulated (▢) or dissolved (■) aluminum concentration (mg/L) in the influent and effluent of nAS-SBR and AS-SBR, (c) lead, chromium and nickel concentrations in the biomass (µg/g) for day 167 (AS-SBR, ▢), day 122 (nAS-SBR, ▨) and the inoculum (■), (d) aluminum and zinc concentrations in the biomass (mg/g) for day 167 (AS-SBR, ▢), day 122 (nAS-SBR, ▨) and the inoculum (■).

Regarding the accumulation of metals in the biomass, the metals at lower concentration levels (range of µg/g) showed higher differences between the higher concentration for the day 167 and the concentration in the inoculum (in terms of percentage), e.g. 3.6-fold for Pb (AS-SBR), 4.4-fold for Cr (nAS-SBR) and 2.3-fold for Ni (nAS-SBR) (Figure 5.18.c). On the other hand, the metals present at higher concentrations (range of mg/g) showed lower differences between the biomass on day 167 and the inoculum, e.g. maximum differences of 1.2-fold for Al (AS-SBR) and 2.3-fold for Zn (nAS-SBR) (Figure 5.18.d). In the case of the nAS-SBR, the concentrations of adsorbed metal onto the biomass on day 122 (Figure 5.18.d) could influence the biological processes taking place in the granules, but these concentrations were similar between the biomass from the inoculum and on days 167 (AS-SBR) and 122 (nAS-SBR). This is in agreement with the similar VSS/TSS ratio during the operation for both reactors (67 - 68%). Thus, no detrimental effect over the PN-AMX processes could be inferred from the presence of metals on the biomass.

5.5.4. Influence of salinity and conductivity

In the present study, the maximum concentration of salt was 8.5 g NaCl/L for AS-SBR and 8.2 g NaCl/L for nAS-SBR (Figure 5.19). Previous research works reported IC_{50} inhibitory levels for anammox bacteria ranging from 5.4 g NaCl/L (Carvajal-Arroyo et al., 2013) to 13.5 g NaCl/L (Dapena-Mora et al., 2007), as well as IC_{50} values for AOB of 11.7 g NaCl/L (Hunik et al. (1992)). Val del Rio et al. (2018), on the other hand, observed certain instability of the PN-AMX processes when exposed to long-term salt concentrations up to 9 g NaCl/L. From these reported values, the salt concentrations

observed here were not expected to exert a significant influence over the PN-AMX processes during the operation of AS-SBR and nAS-SBR.

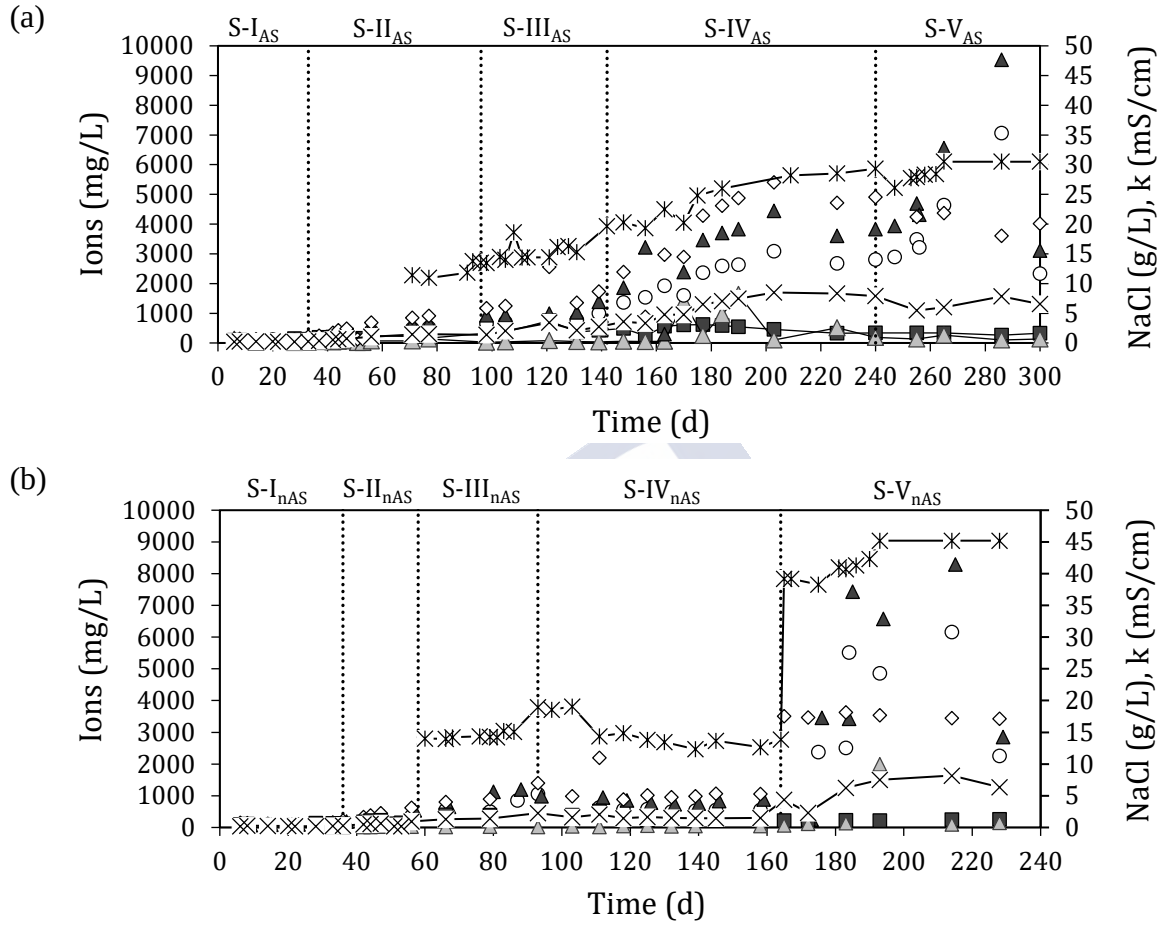

Figure 5.19. Concentration of salt (g NaCl/L, ×), conductivity (mS/cm, ✳) and the main ion concentrations (mg/L): sodium (Na^+, ○), potassium (K^+, ▲), chloride (Cl^-, ◇), phosphate (PO_4^{3-}, ■) and sulphate (SO_4^{2-}, ▲) for the feeding of AS-SBR (a) and nAS-SBR (b).

The concentration of ions in the feeding progressively increased along with the proportion of OFMSW liquor fed to the previous AD step (Figure 5.19). In particular, the concentration of phosphate ion (PO_4^{3-}) were higher during Stages III$_{AS}$ and IV$_{AS}$ (491 ± 158 mg PO_4^{3-}/L) and during Stages III$_{nAS}$ and IV$_{nAS}$ (428 ± 56 mg PO_4^{3-}/L) compared to the rest of the operation (256 ± 136 mg PO_4^{3-}/L for AS-SBR, 195 ± 61 mg PO_4^{3-}/L for nAS-SBR). Some authors found reversible inhibition above 190 mg PO_4^{3-}/L (Jetten et al., 1998) and up to a 80% decrease in the anammox activity at 306 mg PO_4^{3-}/L (Pynaert et al., 2003), while others observed long-term acclimation below 1532 mg PO_4^{3-}/L (Zhang et al., 2016) and no significant inhibition in the range 509 - 1462 mg PO_4^{3-}/L (Galvagno et al., 2016). Several IC_{50}

values were reported: 1900 mg PO_4^{3-}/L (Dapena-Mora et al., 2007) and 2403 (Carvajal-Arroyo et al., 2013). On the other hand, Egli et al. (2001) found no inhibition for 1900 mg PO_4^{3-}/L when treating landfill leachate with a rotating biological contactor supporting the anammox process, while Caffaz et al. (2008) observed reversible inhibition of anammox biomass (50%) when the phosphate concentration increased from 30 to 100 mg PO_4^{3-}-P/L.

In this case, and although being below the inhibitory reported values, the concentration of phosphate was sufficiently high to influence the microbial community in the AS-SBR (see Figure 5.14, day 149), agreeing with the drop in the anammox relative abundance bacteria observed for the same day (Figure 5.16.b). No big differences were found in the phosphate concentration between the influent and the effluent, indicating a low phosphate precipitation on the biomass.

Nevertheless, the conductivity rather than the single salts may be the main factor responsible for the inhibition of the anammox biomass, as stated by Scaglione et al. (2017), who found an IC_{50} value for the conductivity of 6.1 mS/cm when treating the liquid fraction of digestate from OFMSW liquors. Azari et al. (2017), on the other hand, successfully treated landfill leachate with the anammox process for a conductivity range of 1.1 - 25.2 mS/cm. In the present study, the maximum conductivity was 30.5 mS/cm for the AS-SBR digestate (Figure 5.19.a) and 45.2 mS/cm for the nAS digestate (Figure 5.19.b). No clear relation was found between the conductivity and both the specific nitrogen removal rate and the total nitrogen removal efficiency (Figure 5.20). However, the higher conductivity during Stage V_{nAS} did have an influence over the microbial community variation (days 181, 193 and 228, Figure 5.14). Similarly to the high ion concentrations, the good nitrogen removal efficiencies achieved in the end (see Figure 5.3.b and Figure 5.7.b) suggest a minor effect of the conductivity on the long-term performance of the PN-AMX processes coupled to the heterotrophic denitrification, as suggested by previous research studies (Ruscalleda et al., 2010).

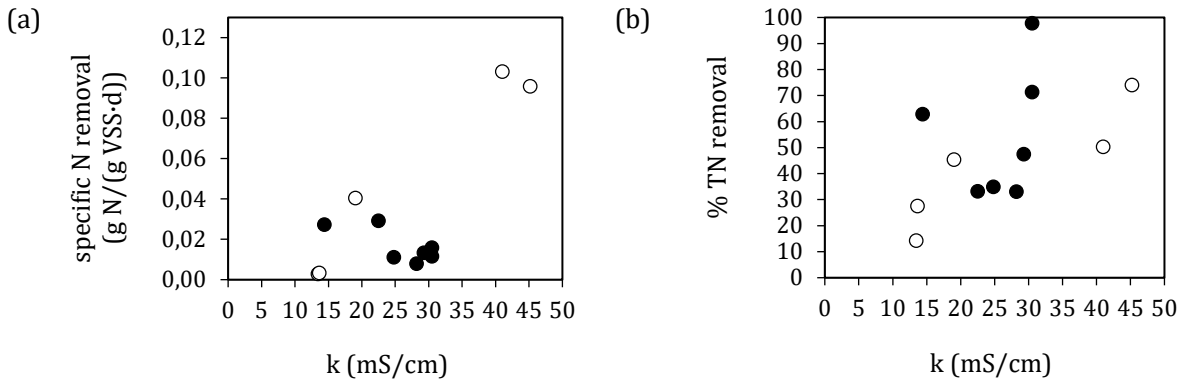

Figure 5.20. Relation between the conductivity (k, mS/cm) in the feeding and (a) the specific nitrogen removal rate (g N/(g VSS·d)), (b) the efficiency of nitrogen removal (%), for the operation of AS-SBR (●) and nAS-SBR (○).

5.5.5. Biological nitrogen removal in OFMSW

The research work presented in this chapter constitutes the first one addressing the operation of the one-stage PN-AMX processes after an ammonia stripping step for the treatment of the liquid fraction of digested OFMSW. As can be seen in Table 5.6 and Table 1.15.b (Chapter 1), only a handful of research studies addressed the application of the anammox process alone to OFMSW liquors, either as a review (Malamis et al., 2014), through batch assays with anammox biomass (Scaglione et al., 2017) or with the operation of an anammox reactor (Scaglione et al., 2015). The works of Caffaz et al. (2008) and Lotti et al. (2019) represent the only two research works addressing the application of the partial nitritation-anammox (in two separate stages) to this type of wastewater streams.

Table 5.6. Inhibition thresholds of organic matter (expressed as COD) reported for anammox-based processes, partial nitritation and denitrification treating leachate-like wastewater streams.

Ref.	Treatment process scheme	LL age	Reactor volume (L)	NH_4^+-N (mg N/L) before N removal step	COD/N (g C/g N) before N	Maximum % of TN removal achieved[a]
Partial nitritation-anammox (PN-AMX), 1-stage						
This	PN + AMX (AS-SBR)	OFMSW	1.7	815 - 2324	2.8 - 8.4	91 - 98%
This	PN + AMX (nAS-SBR)	OFMSW	1.7	3759	1.8	73 - 75%
[1]	PN-AMX	Old	6.5	1000 - 3000	0.8	85
[2]	PN-AMX + Ozonation + GAC	Mature	6 + 10 + 0.27	244 - 819	2.24 - 2.91	85.9
[3]	OMR + PN-AMX	Inmature	9 + 10	1154 ± 66	7.9	90
Partial nitritation-anammox (PN-AMX), 2-stage						
[4]	DN + PN + AMX	Mature	4.25	1900 - 2000	2.8 - 3.9	24.6 (DN)+49.6
[5]	PN + OMR + AMX	Mature	10.5 + 5.5	1240	1.6 - 1.9	94
[6]	PN + AMX	Mature	10 + 10	3000	1	93
[7]	pre-treat. + PN + AMX	Mature	10 + 10 + 13	250	2.4 - 3.2	93 (AMX)
[8]	OMR + PN + AMX	Mature	8.25 + 15 +	400 - 410 (1:5	1.45	54.4
[9]	pre-treat. + PN + AMX	Mature	12 + 12 + 15	2000	1.1	90 (AMX)
[10]	PN + AMX	Old	66.5 + 5	500 - 1000 (1:5	1.05	81 - 93
[11]	PN + AMX	Mature	52.0 + 4.4	1040 ± 322	1.75	85 (AMX)
[12]	PN-AMX (Panammox®) + AOPs	Mature	400	994 ± 96	5.74	87 - 89
[13]	OMR + PN + AMX	OFMSW	30	ns	ns	95
Anammox (AMX)						
[14]	AcS + N-DN + UF + AMX (GAC)	Mature	ns	600 - 900	2.4 - 3.6[b]	94
[15]	AMX-DN	Mature + raw	20	1000 - 3000	1 - 1.2	90

Table 5.6 (continued)

Ref.	Treatment process scheme	LL age	Reactor volume (L)	NH_4^+-N (mg N/L) before N removal	COD/N (g C/g N) before N	Maximum % of TN removal achieved[a]
Partial nitritation (PN)						
[16]	PN	Old	2.4	2050 (TAN)	2.83	50 - 70
[17]	PN	Mature	52	1170 ± 220	1.86	97
[18]	PN	Several	3.0 - 4.5	688 - 1748	2.23 - 3.88	85
Nitrification-Denitrification (N-DN)						
[19]	AcS + N-DN	ns	10 + 10	1104	5.62	95
[20]	N-DN	ns	5	340 ± 14	2.15	78
Aged Refuse Bioreactor (ARB)						
[21]	ARB	ns	-	1237 - 1506	1.8	81
[22]	ARB	Old	-	1903 ± 299	1.22	90

[a] maximum % of total nitrogen (TN) removal achieved for the combined treatment, unless otherwise is specified.
[b] Influent of AS step

AcS: activated sludge. A/O: anoxic/oxic. AOPs: advanced oxidation processes. ARB: aged refuse bioreactor. COD: chemical oxygen demand. DN: denitrification. GAC: granular activated carbon. LL: landfill leachate. N-DN: nitrification-denitrification. ns: not specified. OMR: organic matter removal. SBR: sequencing batch reactor. UASB: upflow anaerobic sludge blanket. UF: ultrafiltration.

References: [1] Vo and Nguyen (2016), [2] Gao et al. (2015), [3] Zhu et al. (2013), [4] Wu et al. (2016b), [5] Wang et al. (2016), [6] Miao et al., 2016, [7] Miao et al. (2015), [8] Wu et al. (2015), [9] Miao et al. (2014), [10] Phan et al. (2014), [11] Li et al. (2014b), [12] Anfruns et al. (2013), [13] Caffaz et al. (2008), [14] Azari et al. (2017), [15] Wang et al. (2017), [16] Spagni et al. (2014), [17] Li et al. (2014a), [18] Li et al. (2013), [19] Wang et al. (2013), [20] Kulikowska and Bernat (2013), [21] Wang et al. (2014), [22] Xie et al. (2013).

From Table 1.15.b (Chapter 1), the total nitrogen removal efficiencies for the nitrification-denitrification (N-DN) and partial nitritation-denitritation (PN-DN) processes treating OFMSW effluents are in the range 80 - 98%, whereas Caffaz et al. (2008) obtained 95% removal efficiencies with the application of a 2-stage PN-AMX process system. In the present research study, the PN-AMX processes were able to adapt to the increasing proportion of OFMSW in the feeding, reaching high nitrogen removal efficiencies with AS pre-treatment (94% for a NLR of 0.05 g N/(L·d)) and without AS pre-treatment (75% for a NLR of 0.96 g N/(L·d)) harsh liquor conditions, regarding nitrogen concentration, sCOD/N ratio and conductivity.

5.5.6. Strategies to improve the BNR of OFMSW liquors

Regarding the PN-AMX processes as one of the main biological nitrogen removal (BNR) processes for the treatment of high strength wastewater like the OFMSW, several studies addressed whether a

one- or two-stage PN-AMX processes should be applied. On one hand, a two-stage PN-AMX system should be preferred when a significant amount of biodegradable organic matter is present since a previous PN reactor could remove the biodegradable organic matter from the liquor. This would avoid the growth of heterotrophic denitrifiers and enhance the ammonium removal in the subsequent anammox step (Li et al., 2014b, 2013; Phan et al., 2014). Miao et al. (2014) implemented a treatment step before the PN-AMX process in order to decrease the concentration of organic matter in the leachate and reduce the negative effect on the anammox process. Miao et al. (2016) used a two-stage system (PN-SBR and subsequent anammox SBBR) to treat mature landfill leachate, in order to achieve a fast start-up and make use of the more robust biofilms systems. Wang et al. (2013) pointed out that previous research studies treating landfill leachate with a single-stage biological process had efficiencies all below 80%, which suggests the difficulty in realizing the advanced nitrogen removal in a single-stage process. However, the main research works summarized in Table 1.15 (Chapter 1) show an average nitrogen removal efficiency for 2-stage PN-AMX processes of 90.8 ± 7.8% (16 research studies), compared to 82.3 ± 4.9% for 1-stage PN-AMX processes (4 research studies). Furthermore, the average value for SNAD processes (i.e., 1-stage PN-AMX processes including the heterotrophic denitrification as a third-party nitrogen removal pathway) is 89.0 ± 9.8%, close to the average for 2-stage PN-AMX processes.

Hence, the single-stage system is still to be considered. Its simplicity of operation require less control and results in a better volumetric rate of nitrogen removal (Malamis et al., 2014). Furthermore, it has been also successfully assessed to treat mature landfill leachate (Gao et al., 2015a; Shalini and Joseph, 2018; Vo and Nguyen, 2016; Xu et al., 2010). Besides, the variation in the composition of wastewater, which is a challenge for process stability during treatment of leachates, can provoke an unsuitable effluent of the partial nitritation for the anammox process regarding the nitrite to ammonium ratio (Li et al., 2014b). On the other hand, one possible alternative in order to reduce the impact of the COD/N ratio over the anammox process would be to recirculate part of the treated wastewater after the anammox process to the anaerobic digestion step (Malamis et al., 2014), which would dilute the OFMSW fed and adjust the pH of the feeding.

However, the removal efficiency of COD from digestates reported in the literature is highly variable (52-94%, Malamis et al., 2014), and relies on the maximum ability of anaerobic bacteria to degrade the organic compounds (Pedizzi et al., 2018). Hence, another alternative would be to have a high-rate aerobic process prior to the PN-AMX processes, in order to further decrease the concentration of organic matter after the anaerobic digestion, since certain organic compounds which are not anaerobically degraded can be oxidized under aerobic conditions.

In the case of the OFMSW liquor treated in the present study, the main contributor to the destabilization of the anammox process (besides NOB activity) was the sCOD/NH_4^+-N ratio, although

some authors attached more importance to the COD concentration rather than the COD/N ratio in order to maintain the anammox stability (Wang et al., 2019). Therefore, it is important in the first place to lower the organics content in the OFMSW liquor through and adequate pre-treatment combined with the anaerobic digestion, which would avoid more complex PN-AMX systems (two-stage, additional intermediate steps, etc.) (Li et al., 2018). If the one-stage PN-AMX processes is entirely dedicated to the removal of nitrogen, the total nitrogen (TN) discharge limits could be more easily met.

5.6. Conclusions

In the continuous operation of the PN-AMX system treating OFMSW digestate, with and without ammonia stripping pre-treatment, a progressive reduction in the specific anammox activity up to 90% was observed, indicating no differences regarding the presence or absence of the AS pre-treatment. The performance of the nitrogen removal for both reactors and the changes in the microbial communities could be correlated mainly to the increasing sCOD/N ratio in the OFMSW: over 1.7 g sCOD/g N, a shift between anammox and denitrifying bacteria was observed. Nevertheless, the removal of nitrogen was able to reach 94% in the AS-SBR for a sCOD/N ratio up to 4-fold higher (2.8 - 8.5 g/g) than in the nAS-SBR, with a relative abundance of 20% for denitrifiers. The AS-SBR achieved better effluent quality (127 ± 23 vs. 1050 ±23 mg N/L) for a lower nitrogen removal rate (58 ± 8 vs. 687 ± 32 mg N/(L·d)) compared to the nAS-SBR, although the latter required successive re-inoculations in order to obtain higher removal rates. These results proved the AS to be a suitable pre-treatment when treating OFMSW digestates with the PN-AMX processes, despite the related higher sCOD/N ratios in the wastewater, achieving good synergy between the PN-AMX and heterotrophic denitrification processes.

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Chapter 6

Treatment of Organic Fraction of Municipal Solid Waste (OFMSW) permeate with the PN-AMX processes

Summary

The partial nitritation-anammox (PN-AMX) processes applied to the anaerobically digested permeate from the liquid fraction of the organic fraction of municipal solid waste (OFMSW) was evaluated, regarding the characterization of an OFMSW digestate after an AnMBR. The research work presented in this Chapter complements the research discussed in Chapter 5 on the treatment of an OFMSW digestate after a conventional anaerobic digestion (AD) (without membrane) with the PN-AMX processes. The results from the laboratory scale experiments allowed to directly correlate the efficiency of nitrogen removal to the nitrogen to inorganic carbon (N/IC) ratio in the permeate. This parameter, which limit the partial nitritation up to 50% ammonium oxidation above 1 g N/g IC, was on average higher in the OFMSW permeate (1.2 - 2.2 g N/g IC) compared to the OFMSW digestate. The partial nitritation was thus hindered and achieved variable removal efficiencies between 40 and 80%. The application of an aerobic biological pre-treatment achieved successful reduction of the organic matter concentration and sCOD/N ratio, but the N/IC ratio increased over 4 g N/g IC. After that, the external supplementation of IC to achieve N/IC closer to the aforementioned threshold allowed for the recovery of the nitrogen removal from 22% to 60%.

OUTLINE

6.1. Introduction

The anaerobic digestion (AD) is a commonly applied technology for the valorization of the organic fraction of municipal solid wastes (OFMSW) (Paritosh et al., 2018; Renou et al., 2008; Tyagi et al., 2018). The AD implies lower costs and environmental impacts compared to the existing disposal methods (Razavi et al., 2019), and is currently diverting 20% of all municipal organic waste disposal in Europe away from sanitary landfills (Baere and Mattheeuws, 2012).

The liquid fraction of the digestate produced in the AD is characterized by high concentrations of nitrogen and organic matter (Renou et al., 2008), and therefore needs an adequate post-treatment to comply with the discharge legislation (Malamis et al., 2014). Compared to traditional systems like the nitrification-denitrification, the combination of the partial nitritation and the anaerobic ammonium oxidation (anammox) processes (PN-AMX) constitutes a novel and cost-effective alternative for the removal of nitrogen from ammonium-rich wastewaters (Scaglione et al., 2017). The PN-AMX processes has several advantages worth considering compared with nitrification-denitrification, such as lower energy requirements (57% lower oxygen consumption) and much lower organic matter requirements (86% reduction) (Malamis et al., 2014).

Regarding the conventional infrastructure for the anaerobic digestion reactors, one main disadvantage is the long HRTs and large reaction volumes required. However, the interest has rised during the last years on the feasibility of combining the AD with membrane technology, implementing anaerobic membrane bioreactors (AnMBRs) for the removal of organic matter (Ma et al., 2018; Pretel et al., 2016). Regarding the valorization of the OFMSW liquors, the application of AnMBRs may allow for the treatment of large organic loading rates, COD removal efficiencies and biogas production, besides lowering the operational costs (Ma et al., 2018; Trzcinski et al., 2010; Trzcinski and Stuckey, 2016, 2009). Furthermore, and despite the fouling and cleaning issues related to the membranes, they can greatly improve the retention of suspended solids inside the anaerobic reactor, thus lowering the solids concentrations for the subsequent PN-AMX post-treatment (Trzcinski et al., 2010). On the other hand, a large amount of dissolved COD can be retained on the polarization layer of the membrane surface, providing a concentration of soluble COD up to 3-fold lower in the permeate (Stephenson et al., 2011). Since the ammonium is not oxidized during the AD, lower sCOD concentrations and sCOD/N ratios would characterize the effluent of the AnMBR compared to those systems without membrane, which is more advantageous for the removal of nitrogen with the PN-AMX processes (Li et al., 2016).

As opposed to the sCOD/N ratio, the nitrogen to inorganic carbon (IC) ratio are supposed to increase after the AnMBR. As a carbon source for the ammonium oxidizing bacteria (AOB) and anammox bacteria, insufficient IC concentrations in the feeding can affect the performance of the PN-AMX processes (Chen et al., 2012). If the alkalinity is too high (N/IC ratio above 1 g N/g C), only up to

50% of the ammonium can be oxidized (if enough DO is provided), while higher N/IC ratios restrict the partial oxidation of ammonium below this % threshold (Pedrouso et al., 2017). However, scarce research literature is available regarding the effect of inorganic carbon and N/IC ratio over the PN (Guisasola et al., 2007) and PN-AMX (Chen et al., 2012) processes, and no previous studies addressed the effect a permeate from an AnMBR treating the OFMSW on the removal of nitrogen in post-treatments like the PN-AMX processes.

6.2. Objectives

The main objective is to evaluate the application of the PN-AMX processes in a single unit for the removal of nitrogen from OFMSW permeates obtained from a full-scale anaerobic digestion membrane bioreactor (AnMBR). The applicability limits of the PN-AMX processes when treating this kind of digestates, regarding the presence of organic matter and the potential alkalinity limitations related to the nitrogen to inorganic carbon (N/IC) ratio, are explored.

Furthermore, the feasibility of applying a pre-treatment before the PN-AMX processes was assessed, with the objective of lowering the organic matter concentration in the OFMSW permeate and achieve more favourable conditions in the liquor for the nitrogen removal post-treatment.

6.3. Materials and methods

6.3.1. Operation of the reactors

6.3.1.1. Experimental set-up

A laboratory sequencing batch reactor (SBR) with a working volume of 5 L was used. The aeration system consisted in a diaphragm pump (Laboport N86, KNF) which supplied air through a non-return valve located at the bottom of the reactor, promoting the aeration with formation of small bubbles. The aeration inside the reactor provided the dissolved oxygen (DO) necessary to carry out the biological aerobic processes. The dissolved oxygen (DO) concentration was periodically measured with a DO probe (Hach Lange LD01) and was manually regulated by changing the opening degree of an air valve located in the gas inlet conduction. In order to provide gentle stirring inside the SBR, a mechanical stirrer was used (80 rpm). The temperature in the SBR was maintained in a range between 29 - 33 °C by means of a thermostatic bath.

Granular sludge from a PN-AMX full-scale plant (ELAN® technology) treating the reject digestate in the WWTP of Guillarei (Galicia) (Vázquez-Padín et al., 2014) was used as inoculum for both laboratory experiments, with an initial sludge concentration for Stage I-A of 3.8 g VSS/L (specific

anammox activity (SAA) of 0.267 ± 0.021 g N/(g VSS·d) at 30 °C), and an initial sludge concentration for Stage I-B of 7.9 g VSS/L (SAA of 0.717 ± 0.014 g N/(g VSS·d) at 30 °C).

The wastewater fed to the reactor in both laboratory experiments consisted in the effluent of an anaerobic membrane bioreactor (AnMBR) treating the liquid fraction of the OFMSW in the 'Ecoparc 2' treatment plant in Montcada i Reixac (Barcelona, Spain). All the available permeate for the feeding came from an initial industrial batch (1 m^3) collected at the treatment plant, and was stored at room temperature. No centrifugation was required before feeding it to the SBR due to the negligible solid concentration in the permeate.

6.3.1.2. Operational periods

The operation of the SBR was divided in five stages (I-A and II-A for the first experiment, and I-B, II-B and III-B for the second experiment), according to the applied HRT (Table 6.1). In the first experiment, A (94 days), the dilution of the OFMSW permeate with tap water was maintained at a 1:5 ratio and no pre-treatment was applied to the permeate. Two different stages were considered (Stage I-A and II-A) depending on the HRT of the cycle, which changed from 6 h to 12 h (see Table 6.2). On the other hand, an aerobic pre-treatment (as it will be indicated in the next section) was applied to the OFMSW permeate at the beginning of the second experiment (69 days), prior to the PN-AMX processes (Stage I-B). This pre-treatment was later removed (Stage II-B) and the dilution of the permeate was decreased to 1:3. This dilution ratio was maintained in Stage III-B, and inorganic carbon was supplemented (as $NaHCO_3$) to the OFMSW permeate in order to lower the N/IC ratio in the feeding.

Table 6.1. Operational conditions for both laboratory experiments and characterization of the OFMSW permeate fed to the SBR throughout the stages of operation.

	1st experiment (A)		***2nd experiment (B)***		
	Stage I-A	Stage II-A	Stage I-B [a]	Stage II-B	Stage III-B [b]
Days	0 - 74	75 - 94	0 - 14	15 - 36	37 - 69
Operational conditions					
Temperature (°C)	30.2 ± 1.4	29.0 ± 2.8	31.4 ± 0.9	29.9 ± 1.9	31.2 ± 2.0
HRT (d)	8.3	16.5	2.5	1.6	2.5
DO (mg O_2/L)	0.102 ± 0.093	0.134 ± 0.044	0.065 ± 0.036	0.073 ± 0.024	0.096 ± 0.005
pH	8.5 ± 0.2	8.2 ± 0.4	7.0 ± 1.3	8.9 ± 0.1	8.6 ± 0.3

Table 6.1 (continued)

	1st experiment (A)		*2nd experiment (B)*		
	Stage I-A	Stage II-A	Stage I-B [a]	Stage II-B	Stage III-B [b]
Days	0 - 74	75 - 94	0 - 14	15 - 36	37 - 69
OFMSW permeate feeding					
Dilution ratio	1:5	1:5	1:5	1:3	1:3
NH_4^+ (mg N/L)	813 ± 115	752 ± 22	695 ± 28	1248 ± 68	1275 ± 53
sCOD (mg sCOD/L)	865 ± 144	820 ± 65	55 - 1123	1677 ± 129	1584 ± 43
IC (mg IC/L)	665 ± 91	651 ± 56	108 ± 12	676 ± 303	1376 ± 9
Ratio sCOD/N (g/g)	1.05 ± 0.14	1.08 ± 0.11	0.08 - 1.54	1.32 ± 0.11	1.20 ± 0.04
Ratio N/IC (g/g)	1.24 ± 0.07	1.20 ± 0.09	1.9 - 6.8	2.2 ± 0.9	0.96 ± 0.05
Salt (g NaCl/L)	1.05 ± 0.50	0.46 ± 0.14	1.67 ± 0.66	1.22 ± 0.36	5.83 ± 0.51
Conductivity (mS/cm)	8.4 ± 1.1	7.2 ± 1.0	8.3 ± 0.6	11.1 ± 2.7	15.8 ± 1.8

[a] An aerobic pre-treatment was applied to the feeding during Stage I-B

[b] Inorganic Carbon (as $NaHCO_3$) was externally added to the feeding during Stage III-B

DO: dissolved oxygen, HRT: hydraulic retention time, IC: inorganic carbon, N: nitrogen, OFMSW: organic fraction of municipal solid waste, sCOD: soluble chemical oxygen demand.

The operational cycle length was initially of 6 h during Stage I-A, later doubled to 12 h during Stage II-A, and it was finally set on 3 h for the second experiment (Stages I-B to III-B). The distribution of the cycles in the different stages can be seen in Table 6.2.

Table 6.2. Distribution of the operational cycle phases (min) in both experimental periods.

Days	Stage	Cycle	Length (h)	Distribution of the cycle (min)
1st experiment (A)				
0 - 18	I-A	a	6	15 | 315 | 15 | 15
19 - 74	I-A	b	6	290 | 40 | 15 | 15
75 - 94	II-A	c	12	30 | 90 | 60 | 30 | 90 | 60 | 30 | 90 | 60 | 30 | 90 | 35 | 15 | 10
2nd experiment (B)				
0 - 69	I-B II-B III-B	d	3	10 | 155 | 10 | 5

▌→ feeding. ▌→ (feeding + aeration + mechanical stirring). ▌→ (aeration + mechanical stirring). ▌→ mechanical stirring. ▯ → settling. ▌→ discharge.

6.3.1.3. Aerobic pre-treatment batch assays

Before the second laboratory experiment (B), batch assays were conducted in a reactor of 1 L operated at room temperature. The purpose was lowering the concentration of organic matter and the

sCOD/N ratio before the PN-AMX processes, since the latter was found to be crucial for the performance of these processes in Chapter 5. Two different sets of batch assays were performed at room temperature (Table 6.3). In the first one, the OFMSW permeate was subjected to either aeration at a flow of 10 L/min (saturation point) or mechanical stirring (300 rpm) during 48 h, fixing the pH value at 8.5 and 10.0 in both cases. Whereas in the second one activated sludge at an aeration flow of 10 L/min (saturation point) with no further mechanical stirring was used to treat the OFMSW permeate and experiments were performed at pH values fixed at 6.0, 7.0, 8.0 and 9.0 for a duration between 91 and 120 h. The activated sludge came from an aerobic reactor in the WWTP of Silvouta (Galicia). The pH was adjusted using either HCl or NaOH when necessary, except for pH = 8.5 which was already the pH of the permeate. Several samples of the reaction medium were taken during each batch assay in order to monitor the evolution of different parameters: ammonium nitrogen (NH_4^+-N), total nitrogen (TN), sCOD, total organic carbon (TOC), inorganic carbon (IC), pH and conductivity (k).

Table 6.3. Operational conditions for the batch assays performed with the OFMSW permeate before its treatment with the PN-AMX processes during Stage I-B.

	PHYSICOCHEMICAL				***BIOLOGICAL***			
Batch assay	AER1	AER2	STIR1	STIR2	AS1	AS2	AS3	AS4
Aeration (saturation)	✓	✓			✓	✓	✓	✓
Mechanical stirring (300 rpm)			✓	✓				
Activated sludge					✓	✓	✓	✓
pH	8.5	10.0	8.5	10.0	6.0	7.0	8.0	9.0
Dilution of the OFMSW permeate	1:1	1:1	1:1	1:1	1:5	1:5	1:5	1:5
Duration of the assay (h)	48	48	48	48	120	120	120	91

AER: aeration. STIR: mechanical stirring. AS: activated sludge

6.3.2. Analytical methods

Analytical determination of ammonium (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N), soluble chemical oxygen demand (sCOD), conductivity, pH and volatile suspended solids (VSS) was carried out according to the Standard Methods (APHA/AWWA/WEF, 2012). Total Organic Carbon (TOC) concentration was determined by a Shimadzu analyser (TOC-L_{CSN}, automatic sample injector Shimazdu ASI-L) as the difference between the total carbon (TC) and the inorganic carbon (IC) concentrations. Cation and anion concentrations were determined by ion chromatography with an Advanced Compact IC system (861, Metrohm), CO_2 suppressor (MCS 853, Metrohm) and a sample processor/injector (838, Metrohm). For all these previous measurements the samples were filtered by 0.45 μm, except for VSS. Full description of the analytical methods is provided in Chapter 2 (Section 2.1).

The SAA, expressed as g N/(g VSS·d), was assessed in batch tests at 30 °C by periodically measuring the headspace overpressure due to the production of nitrogen gas by anammox bacteria with a differential pressure transducer (Centerpoint Electronics). The methodology described by Dapena-Mora et al. (2007) was followed, which is fully described in Chapter 2, Section 2.3.2.1. The SAA tests were performed using two different liquid medium: phosphate buffer solution (PBS) to determine the maximum activity (SAA_{PBS}), which serves as control, and AD pre-treated OFMSW to determine the reduction in the SAA in comparison with the control (SAA_{OFMSW}). In all these tests, both ammonium (as NH_4Cl) and nitrite (as $NaNO_2$) were added as substrates, with the latter as the sole substrate when the OFMSW instead of PBS was used as liquid medium due to the ammonium excess in the wastewater.

The COD fractionation was assessed through respirometric batch assays by periodically measuring the consumption of the DO concentration (from the saturation point) when treating the OFMSW permeate with activated sludge in a respirometer (BM-T+, Surcis). The methodology is fully described in Chapter 2 (Section 2.1.5).

6.3.3. Calculations

The nitrogen loading rate (NLR), the nitrogen removal rate (NRR) and the total nitrogen (TN) removal efficiency were estimated based on nitrogen balances and the anammox process stoichiometry (with the rates being expressed as g N/(L·d)), following eq. 2.5, eq. 2.7 and eq. 2.12, respectively (Chapter 2, Section 2.5.1.1 and 2.5.1.2). The maximum nitrogen removal percentage by a possible heterotrophic denitrification (HD) processes was determined based on the mass balance of the soluble chemical oxygen demand (sCOD), following eq. 2.14 (Section 2.5.1.2). Since part of the organic matter can be consumed for growth and/or aerobic oxidation due to the presence of oxygen, this maximum TN removal percentage considers only the maximum "potential" or contribution for the HD, not the actual.

The concentration of free ammonia (FA, NH_3) was calculated at the operational temperature based on the NH_4^+ concentrations and the pH in the bulk liquid (i.e., in the effluent of the reactor) according to Anthonisen et al. (1976) (eq. 2.15, Chapter 2, Section 2.5.1.3).

6.4. Results

6.4.1. Nitrogen removal of the OFMSW permeate (Stages I-A and II-A)

During Stages I-A and II-A, the concentration of ammonium in the 1:5 diluted OFMSW permeate fed to the reactor remained in general between 710 and 930 mg NH_4^+-N/L (Figure 6.1.a). The

concentration of ammonium in the effluent during Stage I-A tended to increase at several operational moments, despite the efforts to provide enough DO concentration in the reactor (see Table 6.1), and thus the feeding was sporadically stopped in order to stimulate the consumption of the remaining ammonium in the reactor. The effect of these feeding stops can be observed in the steep decrease of the ammonium concentration in the effluent for days 13 - 18, 34 - 39 and 64 - 67 (Figure 6.1.a). Furthermore, the 6-hours cycle was modified by day 19 by lengthening the feeding step along with the aeration + mechanical stirring (from cycle a to b, Table 6.2), in order to provide a step-wise feeding strategy and help consuming the remaining ammonium in the reactor. As a consequence of this modification of the cycle, the ammonium concentration in the effluent was able to progressively decrease from 436 mg NH_4^+-N/L (day 25) to 298 mg NH_4^+-N/L (day 70, Figure 6.1.a).

Still, both the removal of ammonium (42 - 82 %) and TN (38 - 80 %) varied in the same broad range during the first half of Stage I-A, for an applied nitrogen loading rate (NLR) between 0.06 and 0.11 g N/(L·d) (Figure 6.1.b). On the other hand, the accumulation of nitrite up to 90 mg NO_2^--N/L from day 30 on (except for day 61, Figure 6.1.a), which coincided with the progressive increase in the removal of ammonium from 42% to 78% (Figure 6.1.b), suggests a capacity for nitrite production by the ammonium oxidizing bacteria (AOB) higher than the capacity of the anammox to reduce this nitrite. In fact, the maximum SAA during Stage I-A decreased from 0.267 g N/(g VSS·d) (inoculum) to 0.098 0.267 g N/(g VSS·d) (day 25) and to 0.037 by day 74. The concentration of nitrate was negligible during Stage I-A, which points out both the low activity of the anammox bacteria as well as the presumably consumption of the produced nitrate by the heterotrophic denitrification (HD). The activity of the other nitrite consumers (the nitrite oxidizing bacteria, NOB) was probably hindered by the varying concentrations of free ammonia (FA) in the reactor (between 23 - 150 mg NH_3-N/L, Figure 6.1.c), which exceeded the reported inhibitory values for NOB (0.8 - 22 mg NH_3-N/L) (Anthonisen et al., 1976; Jianlong and Ning, 2004).

In order to help the PN-AMX processes treating the 1:5 diluted OFMSW permeate, the NLR was decreased at the beginning of Stage II-A to 0.045 g N/(L·d) (Figure 6.1.b) by doubling the cycle length (cycle c) (Table 6.2) and thus the HRT (Table 6.1). As opposed to cycle b, in cycle c the OFMSW permeate is fed only initially (3 min), followed by the aeration and stirring during 90 min (main reaction) and further stirring during 60 min, in a sequence which is repeated 4 times during the 12-hours cycle. In this way, the oxidation of ammonium during the partial nitritation is promoted when aeration is provided, followed by the consumption promotion of both ammonium and accumulated nitrite by the anammox bacteria when only stirring is provided.

During Stage II-A, the concentration of ammonium in the effluent was maintained below 150 mg NH_4^+-N/L (Figure 6.1.a), maintaining less inhibitory free ammonia (FA) concentrations (below 25 mg NH_3-N/L, Figure 6.1.c), although the concentration of nitrate in the effluent remained close to zero and

only increased to 32 mg NO_3^--N/L by day 80. The higher oxidation of ammonium was probably stimulated by the distribution of cycle c, which helped consuming the remaining ammonium in the reactor. The removal of TN slightly increased on average compared to Stage I-A (68 ± 11% compared to 56 ± 13%, Figure 6.1.b), with a nitrogen removal rate (NRR) of 0.031 ± 0.005 g N/(L·d) compared to Stage I-A (0.033 - 0.087 g N/(L·d)).

Nevertheless, the higher oxidation of ammonium during Stage II-A (87 ± 6%) probably surpassed the capacity of anammox bacteria to reduce the produced nitrite, which increased over 200 mg NO_2^--N/L. Similarly to Stage I-A, the negligible concentration of nitrate during Stage II, as well as the ratio between nitrate produced and ammonium oxidized below the stoichiometric value (0.11 g N/g N) for the PN-AMX processes (Figure 6.1.c), suggest again the occurrence of denitrifying bacteria activity.

Regarding the removal of carbonaceous compounds, the concentration of soluble COD (sCOD) in the 1:5 diluted OFMSW permeate was in general in the range 731-1097 mg sCOD/L during Stages I-A and II-A (Figure 6.2.a). Considering the feeding stops on days 13 - 18, 34 - 39 and 64 - 67 for oxidizing the remaining ammonium, the consumption of the organic matter was not so pronounced (Figure 6.2.a) as in the case of the ammonium (Figure 6.1.a). The removal of organic matter, except for the initial values, varied in an average range of 43.8 ± 7.4% during Stage I-A, which is close to the biodegradable fraction of the COD obtained through COD fractionation (47%).

The 2-fold higher HRT imposed to the reactor in Stage II-A (Table 6.1) may have favoured a slightly higher consumption of the organic matter, with sCOD concentrations in the effluent below 500 mg sCOD/L (Figure 6.2.a). The average sCOD to nitrogen (sCOD/N) ratio in the feeding was 1.06 ± 0.13 g/g during Stages I-A and II-A (Figure 6.2.b), which is below the threshold for complete denitrification via nitrite, or denitritation (1.71 g COD/g N, Jenni et al. (2014)), and thus no strong competition between the HD with the anammox process for the substrate nitrite should be expected. The possible maximum contribution of the HD to the removal of nitrogen was in the range 2 - 10% for the first experiment (Figure 6.2.b), according to the varying removal efficiencies of organic matter.

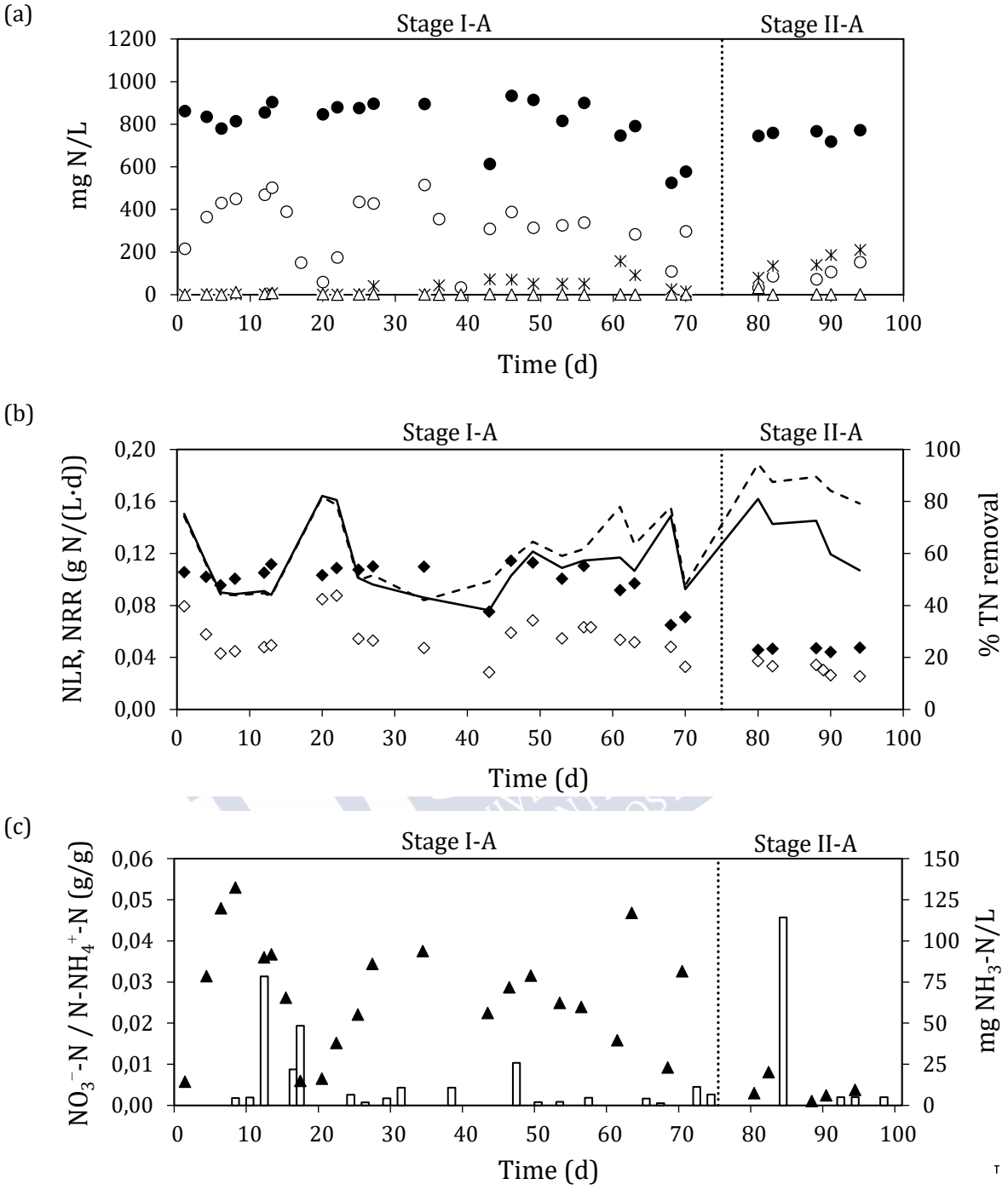

Figure 6.1. Data from the first laboratory experiment (Stages I-A and II-A): (a) concentration of NH_4^+-N in the influent (●), and NH_4^+-N (○), NO_2^--N (∗) and NO_3^--N (△) in the effluent (mg N/L); (b) Nitrogen Loading Rate, NLR (g N/(L·d), ●), Nitrogen Removal Rate, NRR (g N/(L·d), ◇) and % of total nitrogen (TN) removal (—) and % of NH_4^+-N removal (– –); (c) nitrate produced to ammonium oxidized ratio (g NO_3^--N/g NH_4^+-N, ▯), and concentration of free ammonia (mg NH_3-N/L) (▲).

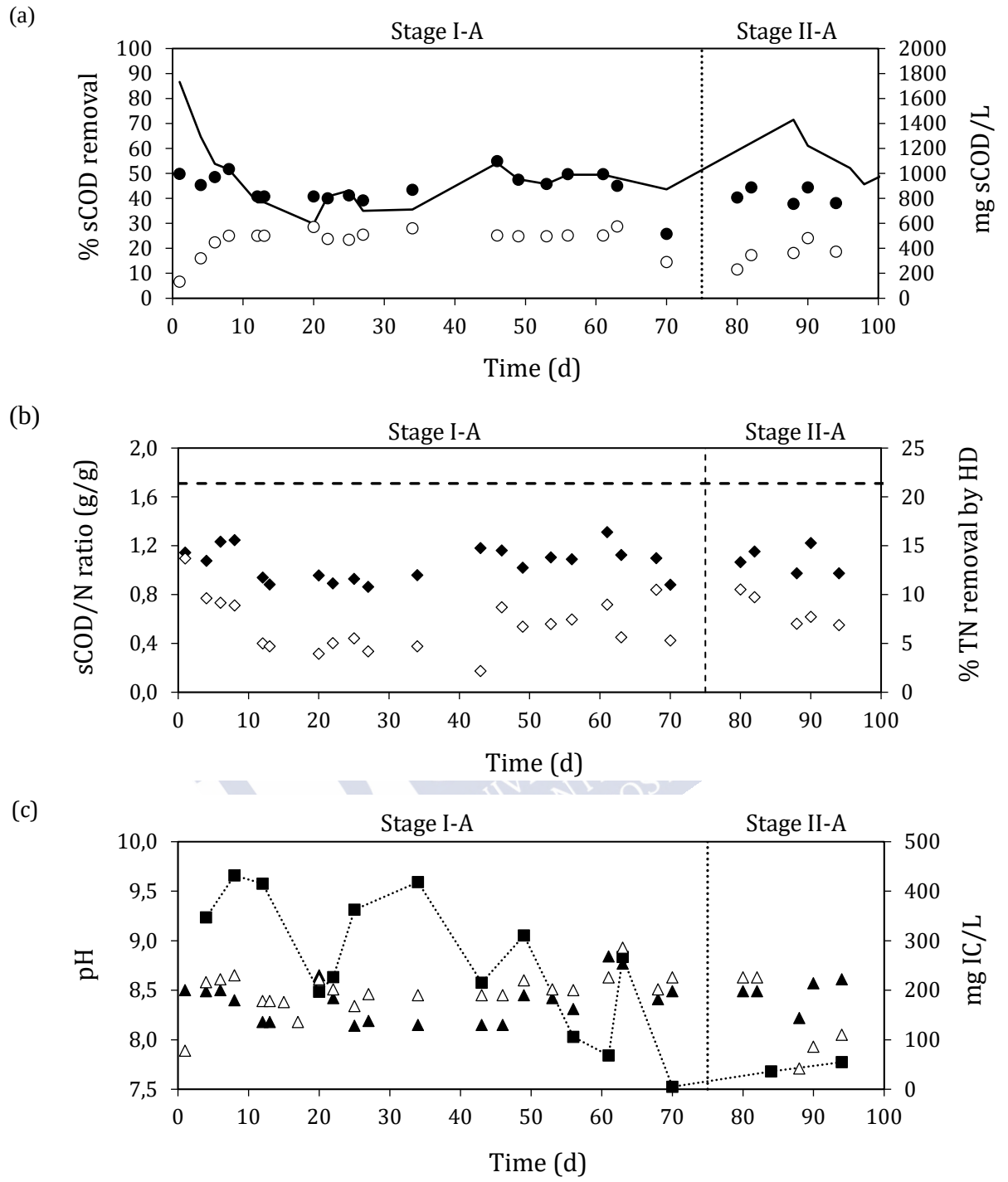

Figure 6.2. (a) Concentration of soluble COD (sCOD) in the influent (●) and effluent (○) (mg sCOD/L), and % of sCOD removal (—); (b) ratio sCOD/N in the influent (g sCOD/g N, ◆), maximum % of TN removal by possible heterotrophic denitrification (HD) (◇) and sCOD/N ratio threshold for complete denitritation (1.71 g sCOD/g N, Jenni et al. (2014)) (- - -); (c) Concentration of inorganic carbon (IC) in the effluent (mg IC/L, ■) and pH of the influent (▲) and effluent (△) for the first experiment (Stages I-A and II-A).

On the other hand, the trend of the concentration of inorganic carbon (IC) in the effluent (Figure 6.2.c) is similar to the concentration of ammonium in the effluent (see days 13 - 18, 34 - 39 and 64 - 67, Figure 6.1.a), indicating its consumption in the ammonium oxidation process during the operation. The concentration of IC was lower at the end of Stage I-A and during Stage II-A (below 68 mg IC/L, sporadically reaching 5 mg IC/L), but it was not low enough to provoke a drop in the operational pH, which was 8.4 ± 0.3 during the first experiment. However, as it will be discussed later, the nitrogen to inorganic carbon (N/IC) ratio may become crucial for the removal of nitrogen with the PN-AMX processes.

Regarding the solids, the OFMSW permeate fed to the SBR had a low solids concentration (0.03 - 0.12 g VSS/L), in agreement with the solids retention in the membrane of the AnMBR. The effluent had less than 0.10 g VSS/L during Stages I-A and II-A (Figure 6.3). The biomass concentration inside the reactor was between 3.2 and 5.1 g VSS/L for Stages I-A and II-A, and it only decreased to 2.1 g VSS/L by day 34 (Stage I-A).

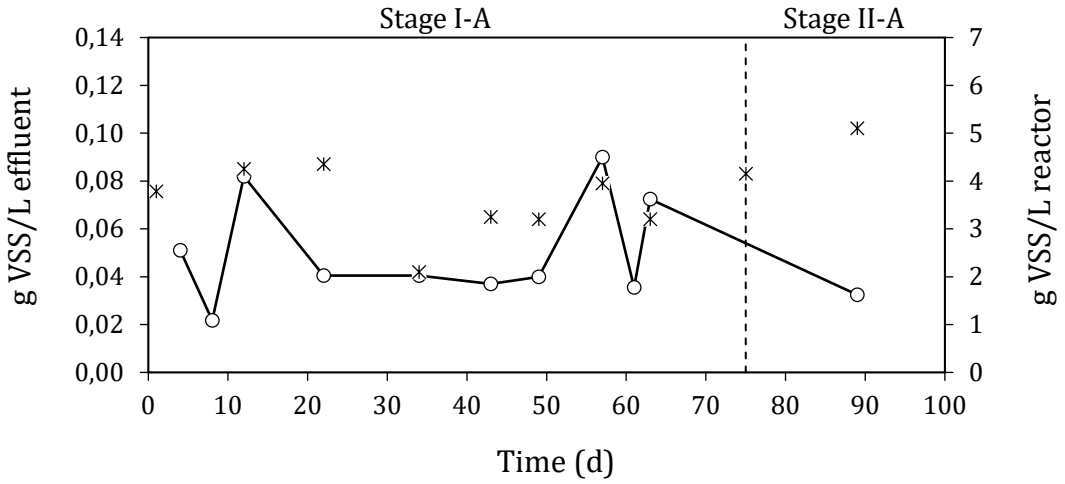

Figure 6.3. Concentration of solids (g VSS/L) in the effluent (○) and in the reactor (∗) (mg VSS/L) for the first experiment (Stages I-A and II-A).

6.4.2. Aerobic pre-treatment to lower the sCOD/N ratio of the OFMSW permeate

Regarding the oxidation of organic matter, the concentration of soluble COD (sCOD) did not decrease for the physicochemical pre-treatments, increasing instead from the initial concentration by 14.6 ± 1.1% (from 5342 to 6123 mg sCOD/L) for STIR1 and STIR2, by 24.1% for AER1 (pH = 10.0), and by 62.1% for AER2 (pH = 8.5) after 30 h (Figure 6.4.a), probably due to the hydrolysis of particulated COD, as observed by Pedizzi et al. (2018) working when stripping the OFMSW liquor. On the other

hand, the biological pre-treatments achieved an average sCOD oxidation of 34 ± 5% for pH between 7.0 and 9.0, with a final sCOD concentration after 30 h of 945 ± 41 mg sCOD/L (Figure 6.4.b). Considering that the biodegradable sCOD accounts for a 43.2% of the sCOD concentration, the biological pre-treatments were able to oxidize up to 79% of the biodegradable sCOD. In the case of AS1 (pH = 6.0), the concentration of sCOD stabilized around 790 mg sCOD/L after 30 h, removing 43% of sCOD, i.e. with AS1 after 30h, all the biodegradable sCOD is already oxidized.

With respect to the removal of ammonium, the mere application of aeration (up to saturation point) to the OFMSW permeate removed over 98% of ammonium due to its stripping within 24 h (from 3489 to 69 mg NH_4^+-N/L) regardless of the pH (8.5 or 10.0, Figure 6.4.c). At that time, 91% of ammonium removal (from 3489 to 310 mg NH_4^+-N/L) was achieved for STIR2, while only 70% (from 4009 to 1184 mg NH_4^+-N/L) was removed for STIR1 during the entire batch test (48 h). The good ammonium removal efficiencies obtained in the first case were to be expected, since the direct stripping of the ammonium to the gas phase takes place in these conditions (Pedizzi et al., 2018). In this aforementioned research work, which preceded the operation of the PN-AMX AS-SBR in Chapter 5, the authors achieved 92% of ammonium removal in the OFMSW digestate after 8 h of pre-treatment in a stripping column.

On the other hand, the achieved biological removal of ammonium in the aerated activated sludge batch assay was similar after 30 h (54.1 ± 2.6% on average) for a pH range of 7.0 - 9.0. In this case, the removal of ammonium was probably due to both the nitrification with the aerated activated sludge, and a certain stripping of ammonium favoured by the aeration. The ammonium concentration for pH = 6.0 (AS1) stabilized in the range 470 - 500 mg NH_4^+-N/L after 30 h (Figure 6.4.b), which is an ammonium concentration 19%, 35% and 66% higher than for pH = 9.0, 8.0 and 7.0, respectively.

In relation to the consumption of inorganic carbon (IC), no clear trend was observed for the physicochemical pre-treatments during the 48 h batch test: while it ended up decreasing down to 704 mg IC/L (AER1) and 1509 mg IC/L (STIR1), it increased up to 5030 mg IC/L (AER2) and 4777 mg IC/L (STIR2) (Figure 6.4.e). Due to the bicarbonate-pH equilibrium, the increase in the IC concentration through time in AER2 (aeration, pH = 10.0) was higher compared to pH = 8.5 (AER1), while for the biological pre-treatments, the lowest IC concentrations after 24 h (95 mg IC/L) were obtained at the lowest pH (6.0, Figure 6.4.f), also due to the more favourable pH for IC biological uptake.

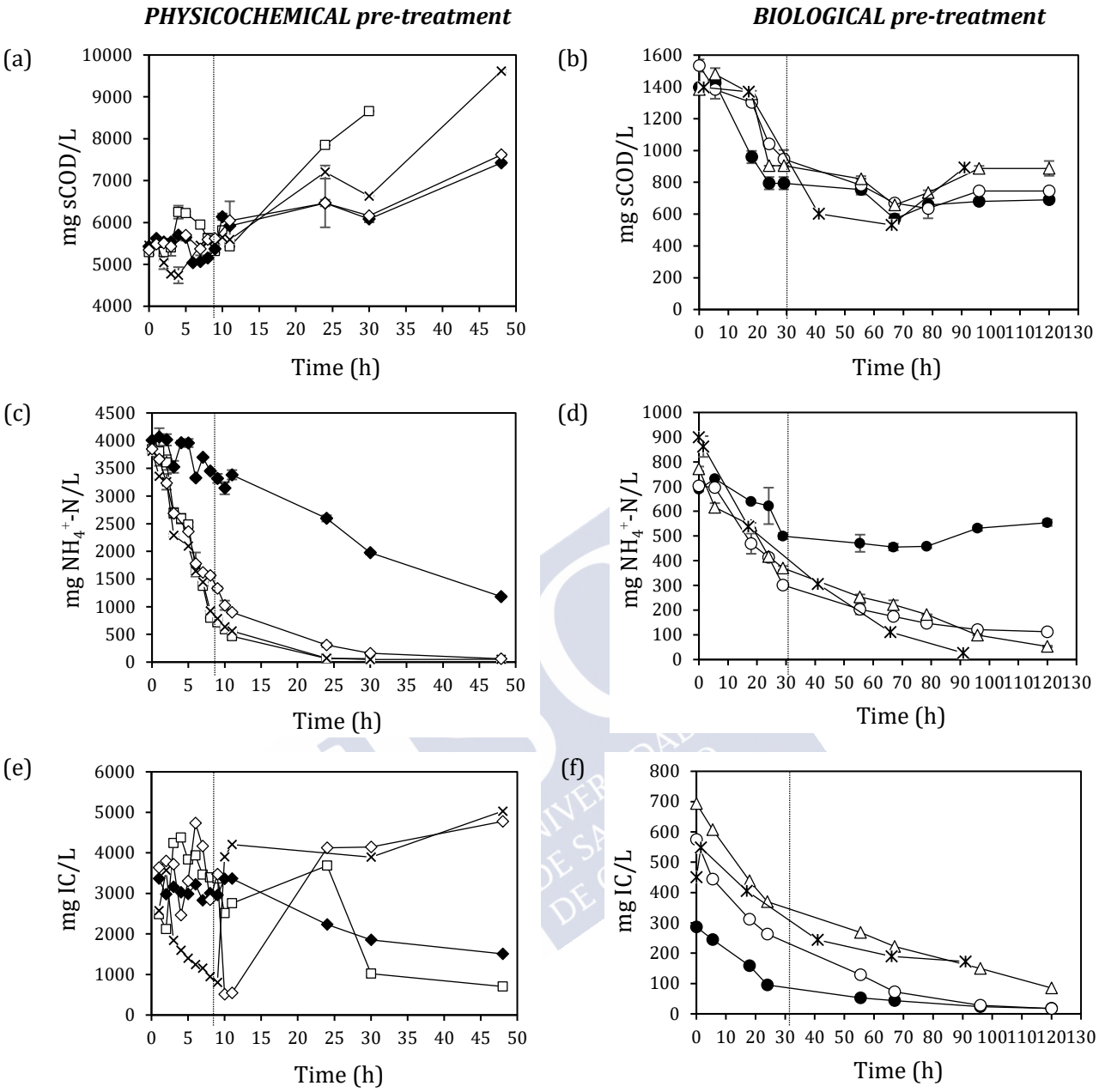

Figure 6.4. Experimental profiles of the parameters monitored during the physicochemical and biological pre-treatments applied to the OFMSW permeate before Stage I-B: concentration of organic matter, in mg sCOD/L (a,b), concentration of ammonium, in mg N/L (c, d) and concentration of inorganic carbon, in mg IC/L (e, f). The different symbols indicate the initial pH of the permeate for the biological batch tests (see Table 6.3): 6.0 (AS1, ●), 7.0 (AS2, ○), 8.0 (AS3, △) and 9.0 (AS4, ✳), and the initial pH and operational conditions for the physicochemical batch tests: aeration at pH = 8.5 (AER1, □), aeration at pH = 10.0 (AER2, ×), mechanical stirring with no pH control (pH = 8.5, STIR1, ◆) and mechanical stirring at pH = 10.0 (STIR2, ◇).

In conclusion, the application of only aeration or mechanical stirring provokes both a partial sCOD solubilization (15 - 62% after 48 h, Figure 6.4.a) and a rapid depletion of ammonium nitrogen (98% removal in 24 h, Figure 6.4.c). On the other hand, a moderate oxidation of organic matter (27.7 ± 10.5%, Figure 6.4.b) and ammonium (44.4 ± 3.3%, Figure 6.4.d) was achieved with the biological treatment for a pH range of 7.0 - 9.0 after 24 h. However, the sCOD/N ratio should be regarded as an important factor that can drive the performance of the PN-AMX processes, as previously discussed in Chapter 5.

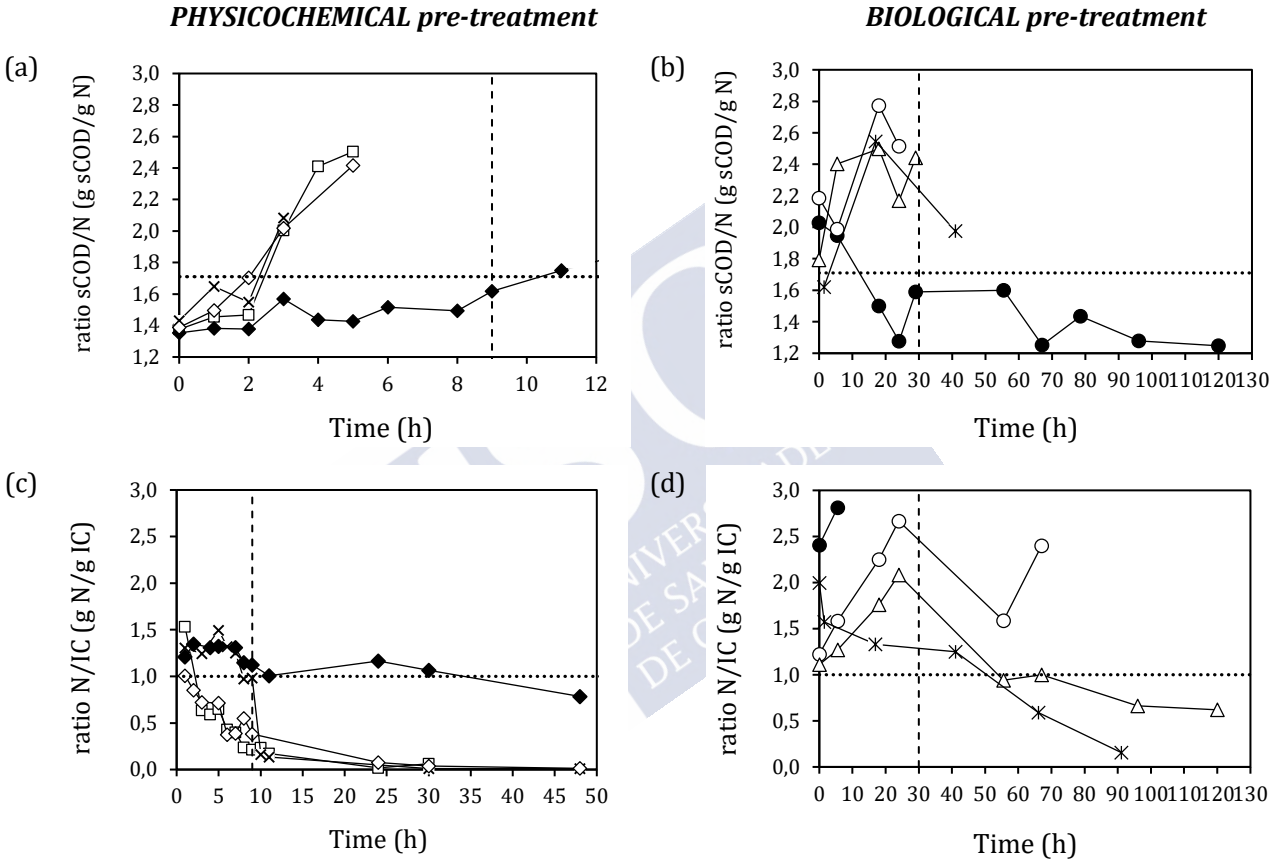

Figure 6.5. Experimental profiles of the ratios monitored during the physicochemical and biological pre-treatments applied to the OFMSW permeate before Stage I-B: ratio sCOD/N, in g sCOD/g N (a, b) and ratio N/IC, in g N/g IC (c, d). The different symbols indicate the initial pH of the permeate for the biological batch tests (see Table 6.3): 6.0 (AS1, ●), 7.0 (AS2, ○), 8.0 (AS3, △) and 9.0 (AS4, ✳), and the initial pH and operational conditions for the physicochemical batch tests: aeration at pH = 8.5 (AER1, □), aeration at pH = 10.0 (AER2, ×), mechanical stirring with no pH control (pH = 8.5, STIR1, ◆) and mechanical stirring at pH = 10.0 (STIR2, ◇). The horizontal dotted line in (a) and (b) represents the sCOD/N ratio threshold for complete denitritation (1.71 g COD/g N, Jenni et al. (2014)), while in (c) and (d) represents the N/IC ratio upper threshold to ensure at least 50% oxidation of the ammonium.

In Figure 6.5, only the sCOD/N and N/IC ratios below 3 g sCOD/g N and 3 g N/g IC, respectively, are represented to address the values close to the respective thresholds for complete HD via nitrite (1.71 g sCOD/g N) or 50% oxidation of ammonium by IC limitations (1 g N/g IC). For the physicochemical pre-treatments, the ammonium stripping rapidly increased the sCOD/N ratio over 1.71 g COD/g N in less than 3 h, except for STIR1 (Figure 6.5.a), while the N/IC ratio concurrently decreased below 1 g/g (AER1, AER2 and STIR2) or close to this threshold (STIR1, Figure 6.5.c). In the case of STIR1, and despite having a sCOD/N and N/IC ratio close to the threshold for the best case after 10 h, only 16% of ammonium was removed at that time (from 4009 to 3384 mg NH_4^+-N/L, Figure 6.4.c) and the concentration of organic matter increased by 15% (up to 7425 mg sCOD/L, Figure 6.4.a).

In the case of the biological pre-treatments, the sCOD/N ratio was already close or above the threshold for HD via nitrite at the beginning of the batch tests (Figure 6.5.b). For a pH range of 7.0 - 9.0, the sCOD/N ratio only increased due to the faster transformation of ammonium (around 50% oxidation after 20 - 30 h, from Figure 6.4.d) compared to the oxidation of organic matter (around 50% oxidation after 40 - 60 h, from Figure 6.4.b). However, as previously mentioned, the transformation of ammonium stabilized for pH = 6.0 (between 470 - 500 mg NH_4^+-N/L, Figure 6.4.b) and thus the sCOD/N ratio decreased to 1.3 g sCOD/g N after 20 h (Figure 6.5.b). On the other hand, the N/IC ratio was initially above 1 g N/g IC due to the characteristics of the OFMSW permeate, and while it ended up decreasing below 1 g/g after 60 and 90 h for AS4 and AS3, respectively (Figure 6.5.d), it rapidly increased over 4 g/g in the case of AS1 (pH = 6.0).

Table 6.4. Best case results for physicochemical and biological pre-treatments, regarding the optimal batch test length, ammonium and sCOD removal, and final characteristics of the OFMSW permeate after the pre-treatment.

	STIR1[b] **(stirring, pH = 8.5)**	**AS1**[b] **(biological, pH = 6.0)**
Optimal length (h)	9 - 10	25 - 30
% of NH_4^+-N removal	19.4 ± 3.1	19.4 - 35.2
% of sCOD removal	-5.1[a] - 3.4	43.1
NH_4^+-N (mg/L)	3231 ± 125	561 ± 86
sCOD (mg/L)	5261 ± 155	795
IC (mg/L)	511	95
pH	8.8	8.4 - 8.6
sCOD/N ratio (g/g)	1.6 - 2.0	1.3 - 1.6
N/IC ratio (g/g)	1.1	4.0 - 5.0

[a] The negative sign indicates increase (instead of decrease) of the % of sCOD removal with respect to the initial sCOD concentration due to particulate COD hydrolysis. [b] Dilution of the OFMSW permeate before physicochemical and biological pre-treatment: 1:1 and 1:5, respectively (see Table 6.3).

Table 6.4 summarizes the best cases according to the results obtained for the physicochemical and biological pre-treatments. In the former case, the mechanical stirring at pH = 8.5 (STIR1) was selected since the lowest sCOD/N ratios were obtained for an optimal duration of the pre-treatment of 10 h, which would avoid excessive increase of the sCOD/N ratio (Figure 6.5.a). On the other hand, a pH of 6.0 was selected as the optimal pH to pre-treat the OFMSW permeate with aerated activated sludge, since the lowest sCOD/N ratios were obtained after 25 - 30 h (Figure 6.5.b), although it is necessary to monitor the adequacy of the pH in the final feeding (diluted with tap water) for the subsequent PN-AMX processes. However, and despite the different dilution applied in each case, the hydrolysis of the organic matter with the physicochemical pre-treatments would lead to higher sCOD concentrations in the permeate (Figure 6.4.a), which would destabilize the removal of nitrogen by PN-AMX and favour the HD.

In conclusion, a biological pre-treatment with aerated activated sludge at pH = 6.0 was selected for the OFMSW permeate at the beginning of Stage I-B, with the objective of assessing the performance of the PN-AMX processes for lower organic matter and lower sCOD/N ratios. As it will be discussed later, the high N/IC ratio may become a collateral issue instead, due to the characteristics of the OFMSW permeate.

6.4.3. Nitrogen removal with aerobic pre-treatment and subsequent IC supplementation (Stages I-B to III-B)

Due to the higher anammox activity available at the beginning of Stage I-B (0.609 ± 0.014 g N/(g VSS·d)) compared to Stage I-A (0.253 ± 0.021 g N/(g VSS·d)), the cycle length was reduced from 6 h in cycle a (beginning of Stage I-A) to 3 h in cycle d (Stage I-B, Table 6.2). The ammonium concentration in the feeding was lower (695 ± 28 mg NH_4^+-N/L) compared to Stage II-A (752 ± 22 mg NH_4^+-N/L) (Table 6.1) due to the biological nitrification and ammonium stripping in the biological aerobic pre-treatment of the permeate.

The concentration of nitrite in the effluent was negligible during Stage I-B (Figure 6.6.a), partly owed to the decrease in the ammonium oxidation from 52% to 21%, very similar to the removal of total nitrogen for an applied NLR of 0.280 ± 0.012 g N/(L·d) (Figure 6.6.b). The concentrations of nitrate were again negligible as well, suggesting the presence of HD. On the other hand, the ratio between nitrate produced and ammonium oxidized was below the stoichiometric value for the PN-AMX processes (Figure 6.6.c), agreeing with a low activity of NOB and anammox bacteria and with the HD consuming the produced nitrate.

The time range for the aerobic pre-treatment applied to the feeding during Stage I-B (between 25 and 30 h) provoked that the concentration of organic matter decreased from 1123 mg sCOD/L to below

130 mg sCOD/L in the feeding (Figure 6.7.a) as well as the sCOD/N ratio from 1.54 g/g (close to the HD threshold) to 0.04 g/g (Figure 6.7.b). The concurrent low IC concentrations in the feeding during Stage I-B (108 ± 12 mg IC/L), compared to the previous operation, favoured the occurrence of low IC concentrations in the effluent (below 30 mg IC/L), which provoked a drop in the operational pH down to 5.4 by day 6 (Figure 6.7.c). This was probably the main cause for the decrease in the removal of TN and ammonium during Stage I-B.

As previously explained in the previous section, the N/IC ratio was already above the threshold for 50% limitation of the partial nitritation, and did not decrease during the aerobic pre-treatment at pH = 6.0 (Figure 6.5.d). For this reason, the removal of nitrogen (mainly from the partial nitritation) was hindered during Stage I-B, despite the lower sCOD concentrations and sCOD/N ratios fed at the end of Stage I-B (Figure 6.7.a/b, respectively), which were more favourable for the removal of nitrogen with the PN-AMX processes. The SAA for the anammox bacteria decreased from 0.717 g N/(g VSS·d) (day 0) to 0.482 g N/(g VSS·d) (day 15). Therefore, the implementation of the aerobic pre-treatment was abandoned after Stage I-B in order to provide higher IC concentrations in the feeding and avoid further limitations by alkalinity, and the dilution of the fed permeate waste increased to a 1:3 ratio.

Consequently, the concentration of ammonium increased to 1248 ± 68 mg NH_4^+-N/L during Stage II-B (Figure 6.6.a), with ammonium concentrations in the effluent up to 870 mg NH_4^+-N/L and negligible nitrite and nitrate concentrations in the effluent. The IC concentration in the effluent increased over 600 mg IC/L, which allowed the operational pH to recover from 5.4 to an average of 8.9 ± 0.1 (Figure 6.7.c). Both the removal of total nitrogen ad ammonium slightly increased up to the range 27 - 45% for a NLR of 0.80 ± 0.04 g N/(L·d) and a NRR between 0.22 and 0.38 g N/(L·d) (Figure 6.6.b).

However, and despite the available IC concentrations in the reactor (over 600 mg IC/L), the N/IC ratio in the feeding was still higher than 1 g/g (2.2 ± 0.9 g N/g IC). This means that the partial nitritation would be only capable of oxidizing the available ammonium below 50% values, since the necessary proportion between both substrates (N/IC ratio) was not low enough. In order to overcome this hindrance of the partial nitritation by the low high N/IC ratio in the OFMSW permeate, the feeding was supplemented with an external source of IC ($NaHCO_3$) during Stage III-B. The concentration of ammonium in the influent remained in a similar range as in Stage II-B (1275 ± 53 mg NH_4^+-N/L, Figure 6.6.a). The ammonium in the effluent stabilized on an average concentration of 561 ± 49 mg NH_4^+-N/L, while the nitrite and nitrate concentrations remained close to zero. The ratio between nitrate produced and ammonium oxidized remained below the stoichiometric value for the PN-AMX processes (0.11 g/g), similarly as in Stages I-B and II-B (Figure 6.6.c). The NLR was decreased to 0.52 ± 0.02 g N/(L·d), achieving a stable NRR of 0.30 ± 0.03 g N/(L·d), and the removal efficiency of total nitrogen was able to recover up to 60% (Figure 6.6.b).

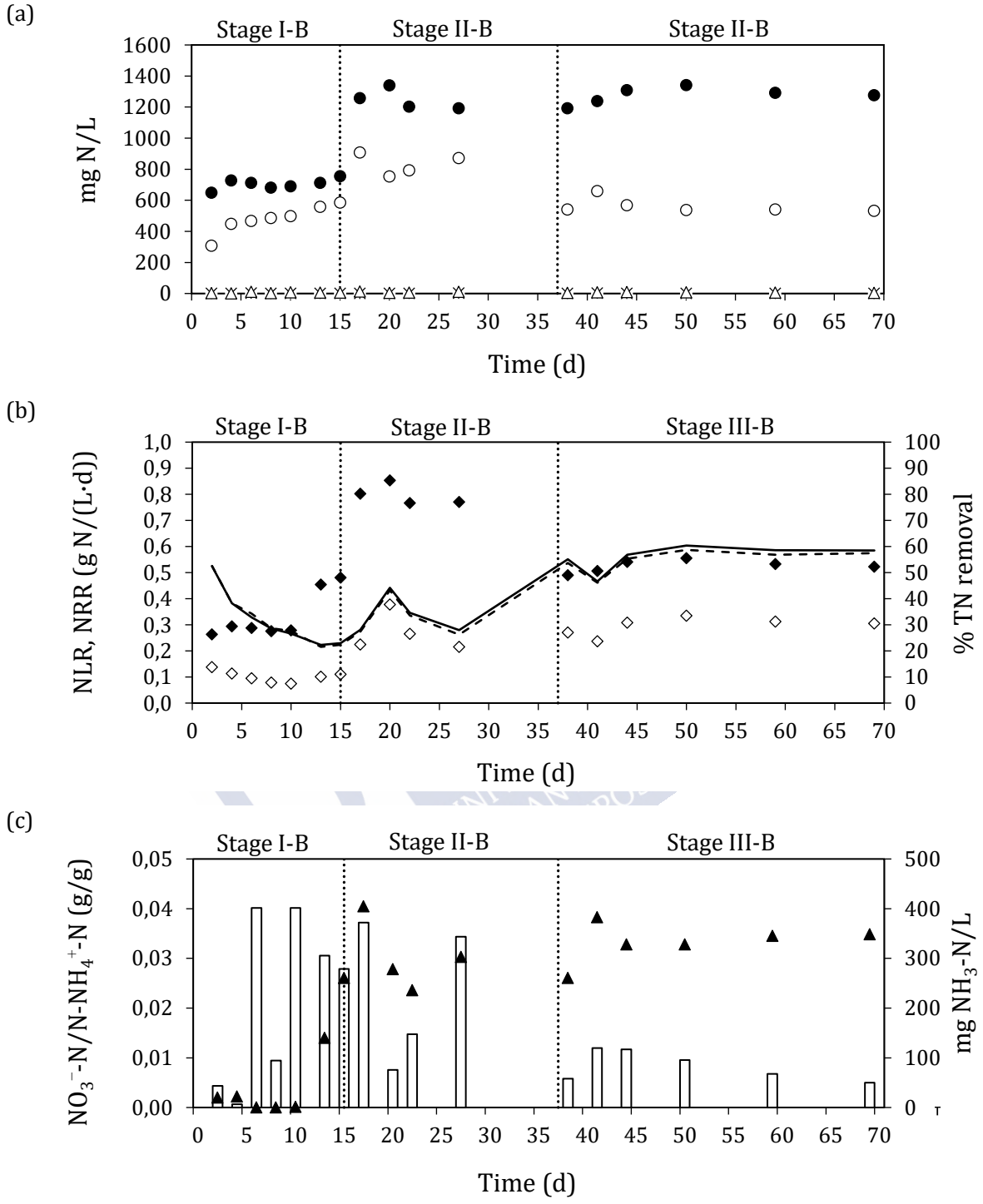

Figure 6.6. Data from operation of the PN-AMX SBR in the second laboratory experiment (Stages I-B to III-B): (a) concentration of NH_4^+-N in the influent (●), and NH_4^+-N (○), NO_2^--N (∗) and NO_3^--N (△) in the effluent (mg N/L); (b) Nitrogen Loading Rate, NLR (g N/(L·d),◆), Nitrogen Removal Rate, NRR (g N/(L·d),◇) and % of total nitrogen (TN) removal (—); (c) nitrate produced to ammonium oxidized ratio (g NO_3^--N/g NH_4^+-N, ▯), and concentration of free ammonia (mg NH_3-N/L) (▲).

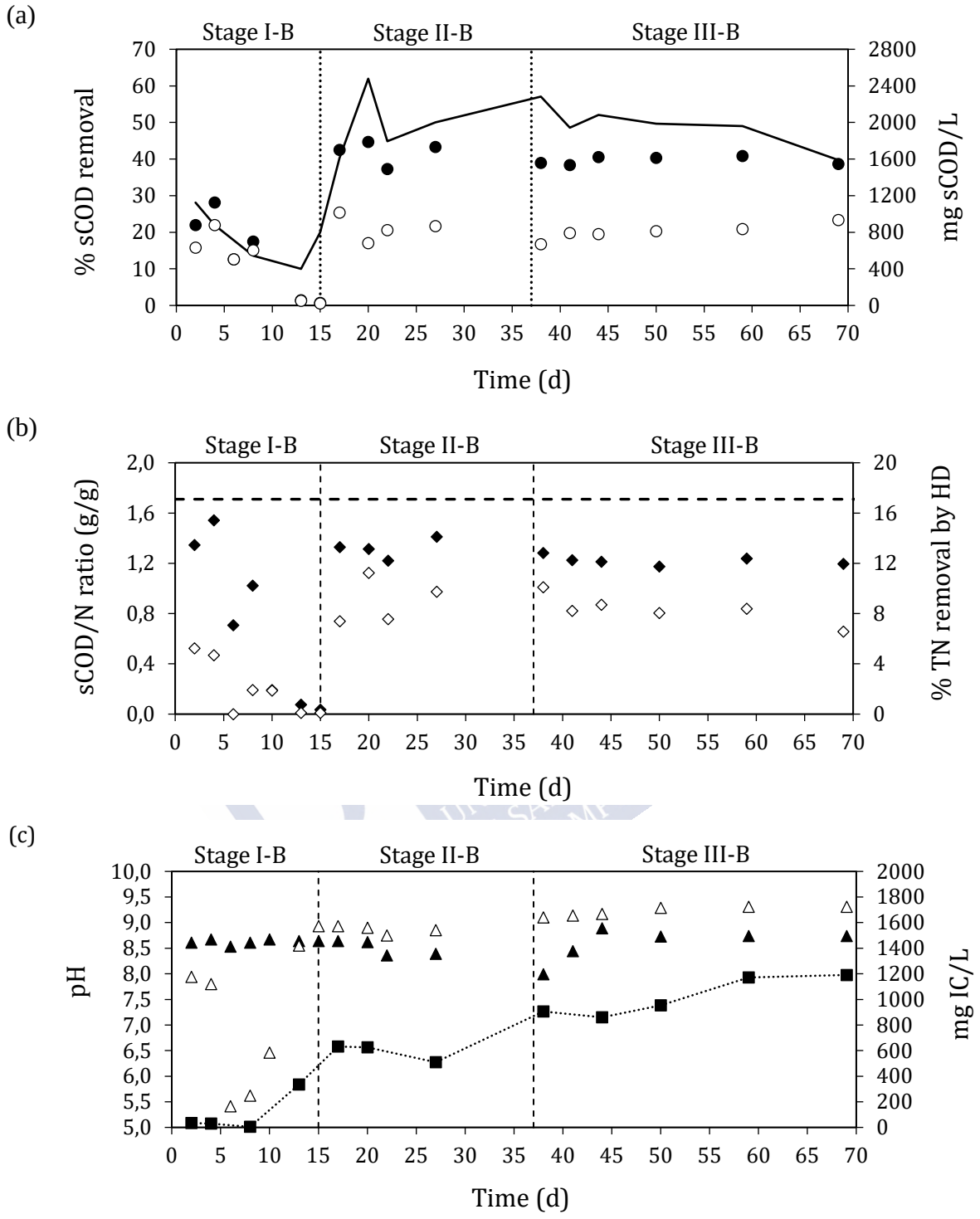

Figure 6.7. (a) Concentration of soluble COD (sCOD) in the influent (●) and effluent (○) (mg sCOD/L), and % of sCOD removal (—); (b) ratio sCOD/N in the influent (g sCOD/g N, ◆), maximum % of TN removal by possible heterotrophic denitrification (HD) (◇) and sCOD/N ratio threshold for complete denitritation (1.71 g sCOD/g N, Jenni et al. (2014)) (- - -); (c) concentration of inorganic carbon (IC) in the effluent (mg IC/L, ■) and pH of the influent (▲) and effluent (△) for the second experiment (Stages I-B to III-B).

With respect to the removal of organic matter, the decrease of the dilution between Stage I-B (1:5) and II-B (1:3) increased the sCOD concentration in the feeding to 1677 ± 129 mg sCOD/L, with an average organic matter removal efficiency of 49.3 ± 9.3% (Figure 6.7.a). The concentrations of organic matter in the influent and effluent during Stage III-B were in a similar range as in Stage II-B, achieving average removal of 49.3 ± 5.7%. On the other hand, the sCOD/N ratio remained stable and low during Stages II-B and III-B (average of 1.26 ± 0.07 g/g), with a concurrent low contribution of the HD to the removal of nitrogen (between 6.5 - 11.2%, Figure 6.7.b).

Regarding the solids, the OFMSW permeate fed to the SBR had similar solids concentrations (between 0.02 - 0.10 g VSS/L) as in the first experiment, and the solids in the effluent were below 0.15 g VSS/L during Stages I-B and III-B (Figure 6.8). As to the biomass concentration inside the reactor, this was initially high during Stage I-B due to the high quantity of inoculum available (7.9 ± 0.4 g VSS/L), but probably due to the N/IC issues it decreased, with values of 4.5 g VSS/L during Stage II-B. Later, the biomass concentration during Stage III-B was lower (2.9 ± 0.5 g VSS/L, Figure 6.8) and coincided with a slight recovery of the nitrogen removal up to 60% (Figure 6.6.b), which may suggest a better selection of the biomass inside the reactor, since the biological removal of nitrogen improved for a lower concentration of biomass.

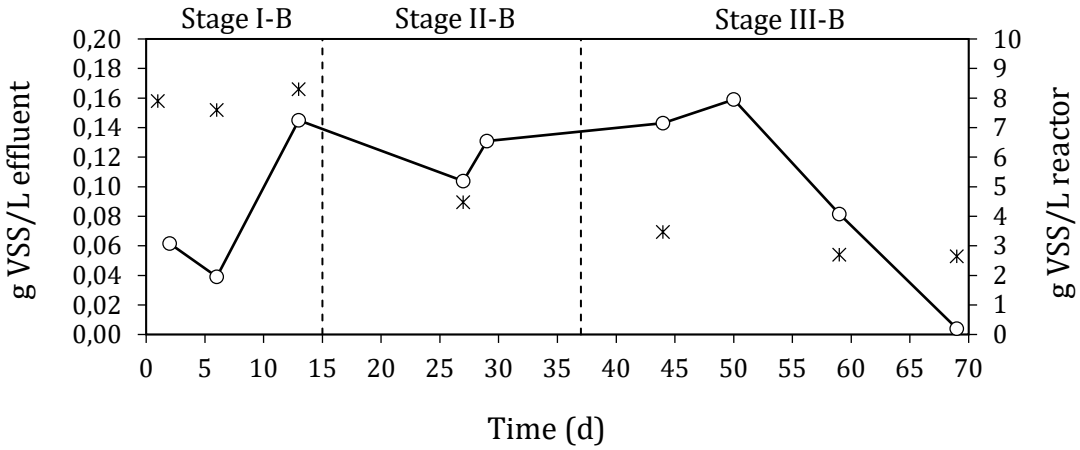

Figure 6.8. Concentration of solids (g VSS/L) in the effluent (○) and in the reactor (✳) (mg VSS/L) for the second experiment (Stages I-B to III-B).

6.5. Discussion

6.5.1. Partial nitritation and alkalinity limitations

During the first experiment, the OFMSW permeate was characterized by a low sCOD/N ratio (1.06 ± 0.13 g sCOD/g N, Figure 6.2.b) regarding the threshold for heterotrophic denitrification via nitrite

(1.71 g COD/g N, Jenni et al. (2014)). On the other hand, while the concentration of inorganic carbon (IC) as carbon source constitutes an important factor affecting the microbial interactions in the PN-AMX processes (Ma et al., 2015), the ratio between the nitrogen and the IC indicates the extent of the partial nitritation (PN) process, which might be hindered by alkalinity limitations. For a N/IC ratio of 1 g N/g IC, the alkalinity allows to oxidize only 50% of the ammonium (if enough DO is provided), while higher N/IC ratios restrict the ammonium oxidation below this 50% threshold (Pedrouso et al., 2017). Durán et al. (2014) found a 50% oxidation of ammonium for the partial nitration process treating pig slurry when adjusting the NH_4/IC ratio in the feeding to 0.82 g N/g C.

As can be seen in Figure 6.9, the N/IC ratio for the OFMSW permeate was on average 1.23 ± 0.07 g N/g IC during the first experiment, and thus the achievement of 50% of ammonium oxidation would be affected by the available alkalinity.

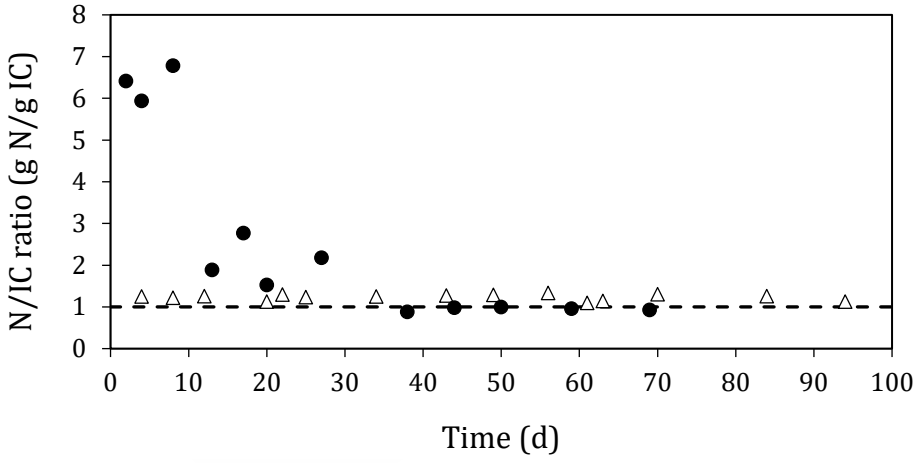

Figure 6.9. Ratio N/IC (g N/g IC) in the influent through the first (△) and second experiment (●), and N/IC ratio threshold (1 g N/g IC) below which 50% or more of the ammonium can be oxidized (- - -).

On the other hand, the application of the aerobic pre-treatment to the OFMSW permeate successfully reduced both the concentration of organic matter during Stage I-B (from 877 to 49 mg sCOD/L, Figure 6.7.a) and the sCOD/N ratio (from 1.5 to 0.1 g sCOD/g N, Figure 6.7.b), but it failed at decreasing the N/IC ratio, which was of 6.4 ± 0.4 g N/g IC on average (Figure 6.9), while values lower than 1 g N/g IC are necessary to guarantee the oxidation of 50% of the ammonium. The IC concentration in the effluent dropped down to 4 mg IC/L during Stage I-B, provoking a pH drop in the reactor down to 5.4 (Figure 6.7.c), which decreased the biological removal of nitrogen from 53 to 21% (Figure 6.6.b). During Stage II-B (days 15 - 36), the aerobic pre-treatment was not further applied to the feeding, which dilution was decreased to 1:3. However, the N/IC ratio was still high during Stage

II-B (2.2 ± 0.9 g/g, Figure 6.9), and consequently both the removal of ammonium and total nitrogen remained at 31.6 ± 8.2% on average (Figure 6.6.b) due to alkalinity limitations.

In order to overcome these limitations, the feeding was supplemented with an external source of IC ($NaHCO_3$) during Stage III-B (day 37-69), with a final dilution of the feeding of 1:3. Previously research works addressed the reversible limitation by IC upon a pulse addition of bicarbonate (Guisasola et al., 2007). The IC concentration in the feeding increased to 1374 ± 9 mg IC/L, with IC concentrations in the effluent of 1017 ± 154 mg IC/L during Stage III-B (Figure 6.7.c). The average N/IC ratio (0.96 ± 0.05 g/g, Figure 6.9) equaled the threshold for 50% limitation of the partial nitritation (1 g N/g IC), and thus the removal of total nitrogen and ammonium were able to increase up to 60% and 57%, respectively (Figure 6.6.b).

Table 6.5. Maximum removal percentages of ammonium and NRR obtained for each reactor treating OFMSW digestate (Chapter 5) and OFMSW permeate (Chapter 6).

	Chapter 5				Chapter 6	
	AS-SBR		nAS-SBR		exp. A	exp. B
Operational period	Stage II_{AS}	Stage V_{AS}	Stage III_{nAS}	Stage V_{nAS}	Stage II-A	Stage III-B
Days	34 - 96	241 - 300	60 - 76	193 - 228	75 - 94	44 - 69
sCOD/N ratio (g/g)	1.4 ± 0.4	2.9 - 7.5	1.0 ± 0.1	1.8 ± 0.1	1.1 ± 0.1	1.20 ± 0.03
N/IC ratio (g/g)	1.3 ± 0.1	0.8 ± 0.3	1.6 ± 0.1	0.8 ± 0.1	1.2 ± 0.1	0.97 ± 0.03
IC feeding (mg	666 ± 78	1942 ± 333	964 ± 19	5027 ± 351	651 ± 56	1376 ± 9
% NH_4^+-N removal	89.6 ± 9.8	88.9 ± 15.9	89.9 ± 4.2	73.1 ± 0.9	86.9 ± 5.7	57.1 ± 1.4
NRR (g N/L·d)	0.16 ± 0.03	0.032 ± 0.017	0.050 ± 0.005	0.69 ± 0.03	0.031 ± 0.005	0.29 ± 0.03

Table 6.5 summarizes the maximum ammonium removal percentages obtained when treating anaerobically digested OFMSW liquors with the PN-AMX processes in Chapter 5 and 6. Regarding the first experiment in this Chapter, the maximum ammonium removal occurred during Stage II-A (87%) with a NRR of 0.031 ± 0.005 g N/(L·d), whereas in Stage II_{AS} 89% of the ammonium was removed for a NRR of 0.16 ± 0.03 g N/(L·d), for similar N/IC ratios and IC concentrations in the feeding. Although the stripping of ammonium observed in batch tests AER1 and AER2 (due to the aeration of the OFMSW permeate) showed up to a 87% decrease in the N/IC ratio within 10 h (Figure 6.5.c), in Chapter 5 the N/IC ratios were similar regardless of the ammonia stripping pre-treatment due to the different concentrations of ammonium in each case, and the oxidation of ammonium was also similar.

On the other hand, the oxidation of ammonium reached 57% during Stage III-B (NRR of 0.29 ± 0.03 g N/(L·d)), in agreement with the N/IC ratio (0.97 ± 0.03 g/g) close to the threshold for 50%

ammonium oxidation by alkalinity limitations, whereas for Stage V_{AS} and Stage V_{nAS} (Chapter 5) higher removal efficiencies were obtained (89% and 73%, respectively) for NRR of 0.032 ± 0.017 g N/(L·d) and 0.69 ± 0.03 g N/(L·d), respectively, and a common N/IC ratio between 0.76 - 0.78 g/g.

However, it should be noticed that, although the N/IC ratio during Stage III-B (0.97 g N/g IC) was more favourable for the partial nitritation, compared to the N/IC ratio during Stage II-A (1.2 g N/g IC), the high concentration of available IC in the effluent (above 600 mg IC/L) denote that the alkalinity was not being properly consumed due to the hindering of the partial nitritation and the anammox processes, probably due to the very high N/IC ratios provided in Stage I-B. A more favourable N/IC ratio for partial nitritation implies a better performance for the first step of the PN-AMX processes, however the removal efficiency of total nitrogen (57% in Stage III-B compared to 87% in Stage II-A) may not increase if the removal of nitrogen by anammox is not properly stimulated.

In relation with the hindrance of the PN by the high N/IC ratios, the activity of the nitrite oxidizing bacteria (NOB) seemed to be negligible during both experiments. The concentrations of nitrate in the effluent were close to zero during the first (Figure 6.1.a) and second experiments (Figure 6.6.a), and the ratio between nitrate produced and ammonium oxidized was always far below the stoichiometric value for the PN-AMX processes (Figure 6.1.c and Figure 6.6.c). Although these two parameters being in a very low range may also suggest the presence of a HD processes contributing to the consumption of the produced nitrate (as well as a low activity of the nitrate-producing anammox bacteria), it also implies a low partial nitratation from the produced nitrite.

In this case, the NOB probably were hindered in the first place by substrate limitations, since the low $\%NH_4^+$-N oxidation during Stages I-A, I-B and II-B would limit the available nitrite. Besides, the related high ammonium concentrations in the effluent favoured higher concentrations of free ammonia (especially during Stages II-B and III-B, Figure 6.6.c), which is usually referred to as an inhibitory parameter to suppress the NOB activity (Li et al., 2014; Spagni et al., 2014; Wu et al., 2016).

6.5.2. Heterotrophic denitrification

The presence of moderate to high organic matter concentrations and sCOD/N ratios in the permeate from the AnMBR can promote the growth of aerobic heterotrophs (Li et al., 2016). Still, the maximum potential contribution of the HD process to the removal of total nitrogen was calculated for the first and second experiments (Figure 6.2.b and Figure 6.7.b, respectively) assuming that the consumption of organic matter was due to the HD.

Considering the sCOD/N ratios for the OFMSW permeate, the average values were 1.06 ± 0.13 g/g for Stages I-A and II-A (Figure 6.2.b), and 1.26 ± 0.07 g/g for Stages II-B and III-B (Figure 6.7.b), and

even a lower range was achieved at the end of Stage I-A (from 1.54 to 0.08 g/g) due to the aerobic pre-treatment of the permeate. The sCOD/N ratio was thus always below the threshold for the stoichiometric one for complete denitritation (1.71 g COD/g N, Jenni et al. (2014)).

In this case, the moderate sCOD/N ratios of the OFMSW permeate are in agreement with the low estimated maximum contribution of the heterotrophic denitrification to the removal of nitrogen: 7.1 ± 2.6% for Stages I-A and II-A (SDA: 0.091 g N/(g VSS·d)), and 8.6 ± 1.4% for Stages II-B and III-B (SDA: 0.201 g N/(g VSS·d)). The contribution of HD was also inferred from the low nitrate produced to ammonium oxidized ratios during the first and second experiments, which were far below the stoichiometric values for the PN-AMX processes (Figure 6.1.c and Figure 6.6.c, respectively). The presence of moderate sCOD/N ratios can favour the synergy between anammox and denitrifiers, where the latter would consume the nitrate produced by the anammox bacteria and NOB (Li et al., 2016a; Miao et al., 2016). Therefore, the HD processes may have acted here as a supplementary aid for the biological removal of nitrogen up to a certain point.

The lower sCOD/N ratios in the OFMSW permeate contrast with the characterization of the OFMSW digestate studied in Chapter 5. There, the very high sCOD/N ratios observed for the operation of the AS-SBR (in the range of 0.4 - 7.5 g sCOD/g N, Figure 5.5b) favoured the outcompetition of the anammox bacteria by the heterotrophic denitrifiers, with a potential contribution of the latter to the removal of nitrogen up to 39% (Figure 5.5.b). For milder sCOD/N ratios, as in the case of the nAS-SBR (from 0.3 to 1.9 g sCOD/g N, Figure 5.9.b), a lower influence was observed over the PN-AMX processes (potential contribution of the HD to the removal of nitrogen up to 15%, Figure 5.9.b).

With respect to the fractionation of the COD, the removal of soluble COD was 43.8 ± 7.4% for Stage I-A and 52.5 ± 6.4% for Stage II-A (Figure 6.2.a), while took values of 43.4 ± 15.4% and 49.3 ± 5.7% for Stages II-B and III-B (Figure 6.7.a), respectively. Considering the fraction of biodegradable sCOD in the OFMSW permeate (43.2%), it is clear that the removal of organic matter in the system was limited to values close to the actual sCOD available (the biodegradable fraction) in the permeate. On the other hand, the removal of organic matter was on average 18.4 ± 8.2% during Stage I-B, which means that only 57% of the available biodegradable sCOD was oxidized in the system in agreement with the high N/IC ratios (up to 6 g/g) and the low IC (< 30 mg IC/L, Figure 6.7.c).

6.5.3. Influence of salinity and conductivity

The concentration of salt in the OFMSW permeate had average values of 0.85 ± 0.23 g NaCl/L during the first experiment (Figure 6.10.a) and 1.46 ± 0.59 g NaCl/L for Stages I-B and II-B (Figure 6.10.b). Still, no detrimental influence of these salt concentrations over the removal of nitrogen by the PN-AMX processes was to be expected, since previous research studies reported IC_{50} inhibitory levels

ranging from 5.4 g NaCl/L to 13.5 g NaCl/L for anammox bacteria (Carvajal-Arroyo et al., 2013; Dapena-Mora et al., 2007).

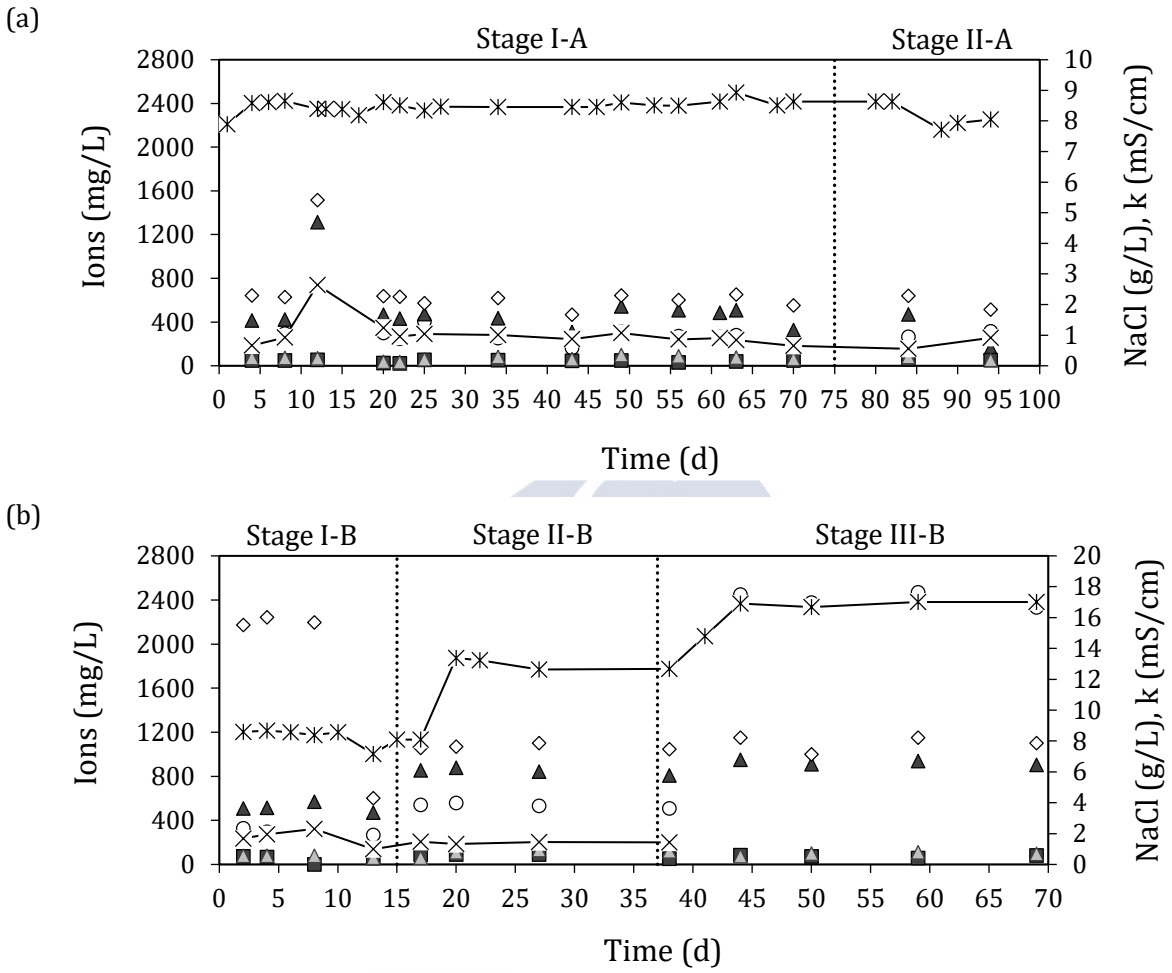

Figure 6.10. Concentration of salt (g NaCl/L, ×), conductivity (mS/cm, ✳) and the main ion concentrations (mg/L): sodium (Na^+, ○), potassium (K^+, ▲), chloride (Cl^-, ◇), phosphate (PO_4^{3-}, ■) and sulphate (SO_4^{2-}, ▲) for the feeding during the first (a) and second experiments (b).

The concentration of ions in the feeding, except for day 12 in Stage I-A, remained in the same range during both experimental periods (Figure 6.10). Besides Na^+ and Cl^-, the concentrations of phosphate were on average 44 ± 11 mg PO_4^{3-}/L for the first experiment, and 74 ± 14 mg PO_4^{3-}/L for the second experiment. Caffaz et al. (2008) observed reversible inhibition of anammox biomass (50%) when the phosphate concentration increased from 30 to 100 mg PO_4^{3-}-P/L, while Egli et al. (2001) found no inhibition for 1900 mg PO_4^{3-}/L when treating landfill leachate with a rotating biological contactor supporting the anammox process. Furthermore, IC_{50} values of 1900 mg PO_4^{3-}/L (Dapena-Mora et al.,

2007) and 2403 mg PO_4^{3-}/L (Carvajal-Arroyo et al., 2013) were reported. Therefore, the phosphate concentrations in the OFMSW permeate did not influence the PN-AMX processes. Only the potassium (484 ± 249 mg K^+/L and 762 ± 187 mg K^+/L, respectively) was in higher concentrations.

As previously addressed in Chapter 5, the conductivity rather than the single salts may be mainly responsible for the inhibition of the anammox bacteria. The conductivity of the 1:5 diluted OFMSW permeate was of 8.1 ± 1.2 mS/cm for Stages I-A, II-A and I-B. During Stages II-B and III-B, the lower dilution of the feeding (1:3) increased the conductivity to 14.9 ± 2.0 mS/cm (Figure 6.10.b). These moderate conductivity values of the OFMSW permeate surpass the reported IC_{50} values for the anammox process treating the liquid fraction of digestate from OFMSW liquors (6.1 mS/cm, Scaglione et al., 2017), and thus certain effect of the conductivity over the overall nitrogen removal process may have occurred, specially for Stages II-B and III-B. However, similarly to the conductivity found in both OFMSW digestates in Chapter 5 (Figure 5.19), no direct relation could be established between this parameter and the removal of nitrogen.

6.6. Conclusions

In the continuous operation of the PN-AMX system treating OFMSW permeate, the performance of the nitrogen removal could be correlated mainly to the nitrogen to inorganic carbon (N/IC) ratio in the OFMSW permeate. High N/IC ratios (above 4 g/g) and low IC concentrations in the permeate (108 ± 12 mg IC/L), favoured by a previous aerobic biological pre-treatment, decreased the TN removal to 21%, while TN removal efficiencies up to 70 - 80% were achieved for lower ratios of 1.2 g N/g IC (closer to the threshold for alkalinity limitation of the partial nitritation, 1 g N/g IC). The improved nitrogen removal was possible also due to a step-wise sequence distribution within the reactor cycle, which was fed only initially for each sequence to promote the oxidation of ammonium, and further provided with phases with stirring and no aeration to promote the anammox activity.

The sCOD/N, on the contrary, was lower in the OFMSW permeate (around 1 g sCOD/g N) and allowed the heterotrophic denitrification to have a possible maximum contribution to the removal of nitrogen up to 10%. The application of a biological pre-treatment with aerated activated sludge successfully decreased the concentration of organic matter and sCOD/N ratio fed to the reactor, but increased in turn the N/IC ratio above 4 g/g, limiting the alkalinity and hindering the biological processes. The supplementation of IC from an external source ($NaHCO_3$) allowed the partial nitritation to recover from 22% to 60% of ammonium removal. As a conclusion, the aerobic pre-treatment of the OFMSW permeate would not be counseled since, on one hand, improves the quality of the feeding for the PN-AMX processes, regarding the organics content, but on the other hand greatly improve the alkalinity limitations for the partial nitritation and the PN-AMX processes.

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General Conclusions

The present thesis focuses on the application of the PN-AMX processes for the treatment of industrial wastewater streams. The main conclusions of the chapters with experimental results within this thesis are detailed below:

Chapter 3. Treatment of fish canning industry wastewater with the PN-AMX processes.

In this chapter, a sequencing batch reactor (SBR) was operated at laboratory scale for the treatment of anaerobically pre-digested fish canning effluents with the PN-AMX processes and granular biomass. These effluents are characterized by a high salinity and high concentrations of organic matter, while its composition may be variable due to seasonality of the fish canning industry.

The results obtained in the SBR reactor showed that the salinity (7.3 - 8.2 g NaCl/L) and the conductivity (12 - 15 mS/cm) were the parameters responsible for the detriment in the nitrogen removal within the PN-AMX processes, from the initial values of 50% to 10%, with nitrogen removal rates between 20 and 100 mg N/(L·d). Considering the previous limitation by salinity, and the high conductivity of the fish-cannery effluent (both around the respective limiting thresholds for the PN-AMX processes), a concatenation of both factors was most probably responsible for the low performance of the process.

As a consequence of the limited activity of the PN-AMX process, and especially of the anammox bacteria, inhibitory concentrations of nitrite accumulated in the SBR. In order to stimulate the consumption of this nitrite by the heterotrophic denitrifying bacteria (HDB), which can act as a supplementary aid for the global removal of nitrogen, the feeding was supplemented with fish canning effluent before its anaerobic digestion. The concentration of organic matter reached 120 mg TOC/L at the most, with an average 20% of nitrogen removal due to the heterotrophic denitrification (HD).

In agreement with the observed results, the relative abundance of the anammox bacteria (determined by Illumina® analysis) was below 2.5% during the operation of the SBR, while the relative abundance of the main HDB reached values between 12 and 18%. This confirms, on one hand, the low presence of anammox bacteria capable of consuming the nitrite generated in the partial nitritation, and on the other hand, it shows the predominance of the HDB at the end of the operation. The feeding strategy did favour the HD process, but it was not sufficient to decrease the nitrite concentration as to stimulate the activity of the anammox bacteria. In this sense, the supplementation of organic matter is recommended only punctually, since both the higher concentrations of organic matter and the higher abundance of HDB may end up acting in the long-term as another inhibitor for the PN-AMX process.

The high concentrations of phosphate and sulphate in the fish canning effluent probably favoured the appearance of whitish precipitates on the surface of the granular biomass. The analysis of such precipitates through scanning electron microscopy (SEM) revealed that they composition was based mainly on sulfur and phosphorus. These precipitates may have contributed to hindering the performance of the PN-AMX processes by limiting the mass transfer of substrates from the liquid medium to the inner granular biomass.

Chapter 4. Treatment of pig slurry wastewater with the PN-AMX processes.

In this chapter, a sequencing batch reactor (SBR) was operated at laboratory scale for the treatment of pig slurry anaerobically pre-digested in a pilot plant located at a pig farm, with the PN-AMX processes and granular biomass. These effluents are characterized by a high conductivity and high concentrations of organic matter.

At the beginning of the operation, batch tests were performed in order to determine the effect that the complex matrix of the pig slurry effluent had over the activity of the biological processes. The batch tests revealed a decrease in the maximum specific anammox activity of 44.4%, indicating a strong deterioration of the removal of nitrogen by anammox considering its maximum potential. This information is crucial when designing the operation of the PN-AMX process for treating this type of wastewater, since only a fraction of the initial available capacity for nitrogen removal should be considered in order to take into account the effect of the wastewater over the biological processes.

As in the previous chapter, a concatenation of factors was most probably the responsible for the long-term suboptimal performance of the PN-AMX processes with respect to the removal of nitrogen. The conductivity (6 - 8 mS/cm) was slightly above the inhibition threshold for the PN-AMX processes, while the concentration of organic matter was considerable (between 180 and 230 mg sCOD/L) and favoured sCOD/N ratios up to 1.14 g/g, with estimated nitrogen removal efficiencies due to HD up to 10%.

The presence of nitrite oxidizing bacteria (NOB) was detected in the inoculum at the beginning of the operation. A strategy based on the limitation of the dissolved oxygen (DO) concentration to favour inhibiting concentrations of free ammonia for the NOB (up to 40 mg NH_3-N/L), was followed. The purpose of such strategy was to suppress the NOB activity, since their presence is one of the main inhibition sources for the anammox process in the start-up of the PN-AMX combined process. The inhibition of NOB, observed from the FISH and Illumina® analysis, was successfully achieved for an optimal DO range of 0.07 - 0.12 mg O_2/L, which shows the critical importance of maintaining a tight control of the dissolved oxygen provided to the system.

Chapter 5. Treatment of Organic Fraction of Municipal Solid Waste (OFMSW) digestate with the PN-AMX processes.

In this chapter, two sequencing batch reactors (SBRs) were operated at laboratory scale for the treatment of anaerobically pre-digested OFMSW from a full-scale treatment plant, with the PN-AMX processes and granular biomass. These effluents are characterized by very high concentrations of organic matter and nitrogen, and consequently high sCOD/N ratios, which are not favourable for the PN-AMX processes. The comparison between both SBRs was based on the presence or absence of an ammonia stripping pre-treatment prior to the anaerobic digestion step, testing the effect of such pre-treatment over the PN-AMX process as post-treatment to the produced digestate.

The results obtained for both SBRs showed that up to a 90% decrease of the maximum specific anammox activity took place during their operation, regardless of the previous pre-treatment. Therefore, the effect of the complex OFMSW digestate matrix is even stronger than the one observed for other types of wastewater streams. The performance of the PN-AMX processes was closely linked to the sCOD/N ratio of the feeding. In this sense, the feeding strategy consisted in the step-wise increase of the OFMSW proportion fed (diluted with tap water) in order to progressively adapt the biological processes to the harsh conditions of the wastewater. This strategy is crucial in order not to immediately loose the bacterial activity after the start-up. On the other hand, the ammonia stripping pre-treatment favoured lower nitrogen concentration in one of the digestates fed, and consequently higher sCOD/N ratios (from 2.8 to 8.5 g/g).

The increasing organic matter concentrations and sCOD/N ratios provoked a clear shift between bacterial populations (mainly, anammox bacteria and HDB) for sCOD/N ratios above 1.7 g/g, in agreement with the reported threshold for complete heterotrophic denitritation (1.71 g/g). In this sense, the lower sCOD/N ratios of the OFMSW digestate without ammonia stripping pre-treatment (below 2 g/g) would appeal more favourable for the PN-AMX process. Nevertheless, the implementation of previous ammonia stripping pre-treatments showed to favour higher nitrogen

removal efficiencies (up to 94%) in the related PN-AMX SBR, with a better effluent quality (below 130 mg NH_4^+-N/L). On the other hand, the higher NRR achieved without pre-treatment (687 mg N/(L·d) vs. 58 mg N/(L·d)) were only possible due to successive re-inoculations of the corresponding SBR.

Chapter 6. Treatment Organic Fraction of Municipal Solid Waste (OFMSW) permeate with the PN-AMX processes.

In this chapter, a sequencing batch reactor (SBR) was operated at laboratory scale for the treatment of OFMSW from a full-scale treatment plant pre-digested in an anaerobic membrane reactor, with the PN-AMX processes and granular biomass. Unlike the OFMSW digestates treated in Chapter 5, the OFMSW permeate treated in Chapter 6 was characterized by lower organic matter and nitrogen concentrations, with sCOD/N ratios below 1.3 g/g. On the other hand, the N/IC ratio, which limits up to 50% de oxidation of ammonium by partial nitritation above 1 g N/g IC, was higher compared to the OFMSW digestate.

The results obtained in the SBR reactor pointed out the effect of the N/IC ratio in the OFMSW permeate over the performance of the PN-AMX processes. Maximum nitrogen removal efficiencies of 87% were obtained for N/IC ratios of 1.2 g/g, close to the limiting threshold for the partial nitritation. On the other hand, and based on the strong effect of the organics content of the wastewater over the PN-AMX processes observed in Chapter 5, several pre-treatments were applied to the OFMSW permeate in order to make it more accessible to the PN-AMX processes. The best-case scenario was achieved with a biological pre-treatment consisting in the aeration of the permeate with activated sludge at pH = 6.0 during 25 - 30 h. This allowed the organic matter concentration of the undiluted permeate to decrease from 5000 to 750 mg sCOD/L.

However, this strategy should not be counseled for the long-term operation of the PN-AMX processes since it may also provoke the rise in the N/IC ratio above 4 g/g, which decreased the subsequent removal efficiency of the PN-AMX processes to 22% and required the implementation of IC from an external source to recover the nitrogen removal (up to 60% in the end).

Publications & Conferences

International Journal Publications

Pichel A., Moreno R., Figueroa M., Campos J.L., Méndez R., Mosquera-Corral A., Val del Rio A. (2019). *How to cope with NOB activity and pig manure inhibition in a partial nitritation-anammox process? Separation and Purification Technology*, 212, 396-404.

Val del Rio A., **Pichel A.**, Fernández-González N., Pedrouso A., Fra-Vázquez A., Morales N., Méndez R., Campos J.L., Mosquera-Corral A. (2018). *Performance and microbial features of the partial nitritation-anammox process treating fish canning wastewater with variable salt concentrations. Journal of Environmental Management*, 208, 112-121.

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Pichel A., Val del Rio A., Pedrouso A., Morales N., Vázquez-Padín J.R., Méndez R., Campos J.L., Mosquera-Corral A. (2015). *Evaluación microscópica de biopelículas mediante la aplicación de microsensores: Caso ELAN®*. Revista Técnica de Medioambiente, 183, 44-53.

Book Chapters

Val del Rio A., **Pichel A.**, Pedrouso A., Mesquita D.P., Campos J.L., Mosquera-Corral A. (2019). *Nitrogen removal by anammox-based processes from different anaerobically digested effluents*, Chapter 17 (Part 5) in: *Post-Treatments of Anaerobically Digested Effluents* (IWA Publishing Ed.).

Campos J.L., Mosquera-Corral A., Val del Rio A., Pedrouso A., **Pichel A.**, Belmonte M., Ruiz G., Jorquera L., Jeison D., Vergara C. (2017). *Wastewater treatment plants: from energy and resources "sinks" to "sources". A critical evaluation of new technologies implementation*. Environmental Science and Engineering, vol. 9: Environment and Energy Management: Ethics, Laws and Policies (Studium Press Ed.).

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Figueroa M., Moreno R., Ruiz C., Monsalvo V.M., **Pichel A.**, Val del Rio A., Campos J.L., Mosquera-Corral A. (June 2016). *New insights about the treatment of pig manure by anaerobic digestion and partial nitritation-anammox*. Oral presentation. 3rd IWA Specialized International Conference: Ecotechnologies for Wastewater Treatment 2016 (ecoSTP16). Cambridge, England.

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Resumen

El tratamiento de las aguas residuales es un proceso con un coste y requisitos de aporte energético relativamente bajos, mientras que la energía que contienen potencialmente las aguas residuales es 5 veces superior a la electricidad necesaria para su tratamiento. Sin embargo, los fenómenos globales como el crecimiento de la población, la escasez de agua y el cambio climático, determinan la imposición de límites de vertido cada vez más estrictos para los efluentes tratados, obligando a modernizar los esquemas actuales de tratamiento para una gestión mejorada y vanguardista de las emisiones contaminantes antes de la descarga final. Por otra parte, tanto el incremento de los precios de la energía como la sostenibilidad medioambiental a través de la recuperación de recursos de las aguas residuales han centrado la atención sobre la recuperación eficiente de su contenido energético, con el objetivo final de conseguir un tratamiento autosuficiente, e idealmente productor de energía.

Entre los efluentes líquidos más complejos en cuanto a su tratamiento se encuentran las aguas residuales industriales. En la última década, ha habido un creciente interés en la digestión anaerobia (DA) como tecnología eficiente para su tratamiento, debido a la recuperación de energía a partir del biogás generado (metano), a la baja producción de lodos y a su capacidad para desactivar microorganismos patógenos durante el proceso. El sobrenadante de la DA, o digestato, es rico en nitrógeno (principalmente, nitrógeno amoniacal), que no se oxida o recupera durante la DA. La presencia de grandes cantidades de este nitrógeno en el efluente final tratado puede provocar problemas de eutrofización y modificar el medioambiente en el cuerpo de agua final, por lo que es necesario un tratamiento adicional después de la DA y antes de la descarga final. La eliminación biológica de nitrógeno (EBN) en estos efluentes se ve favorecida por las características de los digestatos, ya que tienen una baja concentración de materia orgánica y una alta concentración de nitrógeno. La EBN se ha ido adoptando progresivamente como estrategia para la eliminación de nitrógeno en los digestatos, ya que incluye procesos más eficientes y económicamente viables en comparación con los procesos físico-químicos, y a pesar de que su relación coste-eficiencia se ha visto

incrementa en los últimos años por la mayor oferta de procesos biológicos disponibles. Los procesos biológicos convencionales para la EBN, como la tecnología de lodos activos y la combinación de los procesos de nitrificación autótrofa y desnitrificación heterótrofa, se han aplicado ampliamente en el tratamiento de las aguas residuales; sin embargo, la Directiva 91/271/EEC remarca como objetivo el desarrollo de nuevos procesos biotecnológicos para la gestión del agua.

El tratamiento convencional de las aguas residuales para la eliminación de nitrógeno, basado en la nitrificación-desnitrificación, tiene como principal desventaja la necesidad, en muchos casos, de una fuente externa de carbono para la desnitrificación, que junto con los elevados requerimientos de energía (aeración) para la nitritación lo convierten en un proceso química y energéticamente intensivo. La detección de un atajo en el ciclo del nitrógeno, representado por las bacterias anammox, permitió desde su descubrimiento hace 20 años la aparición de nuevas tecnologías basadas en la eliminación autótrofa de nitrógeno, caracterizadas por ser más eficientes a un menor coste, así como por ser más sostenibles medioambientalmente en comparación con los procesos de EBN convencionales. El proceso combinado de nitrificación parcial-anammox (NP-AMX), que añade al proceso anammox una primera etapa de nitrificación parcial para obtener el nitrito necesario para las bacterias anammox, constituye una alternativa innovadora a los procesos convencionales. Las principales ventajas que ofrece son el menor requerimiento de energía (aeración), una menor producción de lodos y la ausencia de requerimientos de materia orgánica de fuentes externas.

Teniendo en cuenta este contexto, la presente tesis doctoral se ha focalizado en estudiar la aplicación del proceso NP-AMX para la eliminación de nitrógeno en diferentes tipos de aguas residuales industriales. Estos efluentes se caracterizan por tener elevadas concentraciones de nitrógeno y materia orgánica, cuyo tratamiento requiere de una mayor investigación para asegurar el correcto funcionamiento de los procesos biológicos. Los principales objetivos a partir del desarrollo de esta tesis se relacionan con:

- Evaluar la capacidad de eliminación de nitrógeno del proceso NP-AMX al tratar aguas residuales industriales con altas concentraciones de nitrógeno, materia orgánica, sales y otros compuestos, así como alta conductividad.
- Identificación de las principales características de estos efluentes industriales que suponen un riesgo potencial para el correcto funcionamiento del proceso NP-AMX.
- Estudio de la relación existente (competición o sinergia) entre el proceso NP-AMX y otros posibles procesos biológicos para la eliminación de nitrógeno.

- Evaluar el desarrollo de las poblaciones bacterianas principales del proceso NP-AMX, consistentes en las bacterias oxidantes de amonio (AOB) y las bacterias anammox, al tratar efluentes industriales.

De este modo, en los capítulos de resultados experimentales se aborda el tratamiento de tres tipos distintos de aguas residuales industriales con el proceso NP-AMX. Los contenidos principales y objetivos específicos correspondientes a cada capítulo de la presente tesis se describen más en detalle a continuación.

En el **Capítulo 1** se presenta un resumen actualizado del contexto referente al tratamiento de las aguas residuales industriales, detallando el cambio de paradigma en los últimos años desde los tratamientos biológicos convencionales para la eliminación de nitrógeno, hasta las aplicaciones actuales de nuevos procesos como el NP-AMX. Se presta especial atención a la caracterización interna del proceso NP-AMX, así como su relación natural con otros procesos biológicos como la desnitrificación heterótrofa, sus ventajas con respecto al proceso convencional de nitrificación-desnitrificación, y las fuentes conocidas de inhibición en las aguas residuales. Por último, se resumen los últimos trabajos de investigación publicados sobre la aplicación de procesos biológicos NP-AMX o basados en anammox para el tratamiento de efluentes industriales.

En el **Capítulo 2**, se proporciona una descripción de las metodologías analíticas empleadas para determinar los parámetros convencionales usados a lo largo de la tesis para caracterizar las aguas residuales y la biomasa. Los parámetros más convencionales como el pH, la concentración de oxígeno disuelto, la demanda química de oxígeno (DQO), la concentración de especies nitrogenadas (amonio, nitrito y nitrato) o la concentración de sólidos, se midieron siguiendo las instrucciones del "Standard Methods". Además, se incluyen otros parámetros como la concentración de iones inorgánicos, la concentración de metales y la conductividad.

En cuanto a la caracterización de la biomasa granular NP-AMX, se detalla el protocolo seguido para la determinación de la actividad específica de la biomasa granular, así como su estudio morfológico mediante microscopía electrónica de barrido (o SEM) para determinar la composición elemental de la superficie granular. Se describen brevemente las dos técnicas de microbiología incluidas en los resultados: la Hibridación *in situ* con Fluorescencia (FISH) para la identificación de las distintas poblaciones microbianas (Capítulo 4), y la extracción-secuenciación de ADN y posterior análisis mediante Illumina® para determinar la diversidad bacteriana y su taxonomía (Capítulos 3 y 5). A mayores, también se presentan en este capítulo los cálculos empleados para determinar los parámetros que permiten analizar los resultados obtenidos a lo largo de la tesis.

La industria conservera representa un sector económico muy importante en Galicia, que genera grandes volúmenes de aguas residuales, caracterizados por una elevada concentración de sólidos, materia orgánica y nitrógeno. En la actualidad, la DA es la tecnología más extendida en las depuradoras asociadas a las industrias conserveras para el tratamiento de los efluentes generados, principalmente a partir de lavado de proceso y enlatado de conservas.

Por todo ello, en el **Capítulo 3** se estudió la operación de un reactor SBR a escala de laboratorio, con un volumen de operación de 1.5 L, encaminado a tratar el sobrenadante de DA del efluente en una industria conservera. Estos efluentes se caracterizan por una elevada salinidad, así como la variabilidad en su caracterización, que pueden repercutir sobre el proceso de NP-AMX. Entre los objetivos de este trabajo se encuentran la mejora en la eliminación de nitrógeno en condiciones de alta salinidad mediante el aporte puntual de materia orgánica para el proceso de desnitrificación heterótrofa, además de la limitación de la actividad de poblaciones bacterianas no deseadas, como las bacterias oxidantes de nitrito (BON) que pueden llegar a competir por el sustrato nitrito con las bacterias anammox. Se siguió además la evolución de las poblaciones bacterianas durante la operación del reactor para determinar el efecto de la salinidad o la materia orgánica.

Los resultados obtenidos durante la operación del reactor a escala de laboratorio indicaron que tanto la salinidad (7.3 - 8.2 g NaCl/L) como la conductividad (12 - 15 mS/cm) del efluente de industria conservera influyeron negativamente sobre el funcionamiento del proceso NP-AMX. La materia orgánica (en el rango de 40 - 120 mg TOC/L) no estimuló la eliminación de nitrógeno por desnitrificación heterótrofa por encima del 24%, con picos ocasionales del 40 - 60 % debido a aumentos puntuales en la concentración de materia orgánica debido a la variabilidad del agua residual. Sin embargo, la eliminación total de nitrógeno disminuyó desde el 50% inicial al 10% a largo plazo, con valores finales de eliminación y carga eliminada del 20% y 0.02 g N/(L·d), respectivamente. El análisis microbiológico reveló que la abundancia relativa de bacterias anammox fue inferior (< 2.5%) en comparación con las principales especies de bacterias desnitrificantes (12 - 18%). Por otra parte, la presencia de altas concentraciones de fosfato y sulfato en el agua residual probablemente favorecieron la aparición de precipitados blancos sobre la superficie de los gránulos NP-AMX, cuya composición elemental (medida mediante microscopía electrónica de barrido) indicó la presencia de azufre y fósforo principalmente. La formación de precipitados pudo haber contribuido a la disminución en la capacidad de eliminación de nitrógeno debido a limitaciones de transferencia de sustratos desde el medio líquido hasta el interior de la biomasa granular.

Aparte de la industria conservera, la producción y cría de animales de consumo, como cerdos y gallinas, es otra industria en alza que también genera grandes cantidades de aguas residuales. La creciente producción de cerdos, en concreto, tiende en la actualidad a su concentración en pequeñas áreas localizadas para reducir los costes de producción, lo cual incrementa la generación de residuos

asociados. Precisamente, el aumento del número de granjas de ganado porcino ha conllevado un incremento en las regulaciones medioambientales en lo referente a la aplicación de los purines generados como fertilizantes. Por lo tanto, se hacen necesarias soluciones sostenibles para el tratamiento de los purines de cerdo con respecto al nitrógeno que contienen, ya que estos efluentes líquidos se encuentran entre los más difíciles de tratar dentro de los efluentes derivados de la cría de ganado, caracterizados por altas concentraciones de materia orgánica y salinidad. Al igual que con los efluentes de la industria conservera, la DA se ha postulado como la tecnología preferente para la eliminación de materia orgánica y producción de biogás, mientras que la eliminación biológica de nitrógeno en purines, se ha centrado en los últimos años en sistemas basados en el proceso anammox.

En consecuencia, en el **Capítulo 4** se estudió la operación de un reactor SBR a escala de laboratorio, con un volumen de operación de 1.4 L, destinado a tratar el sobrenadante de un digestor anaerobio a escala piloto tratando los purines de cerdo generados en una granja de ganado porcino. Entre los objetivos de este trabajo se encuentran el estudio de la evolución en la actividad anammox en relación a la conductividad y concentración de materia orgánica del purín pre-digerido, incluyendo ensayos en discontinuo para determinar la pérdida de actividad anammox al exponerse a la matriz compleja del agua residual. Se estudió además la relación entre el proceso NP-AMX y la desnitrificación heterótrofa, así como la limitación de las BON, que constituyen uno de los mayores problemas potenciales durante el arranque de reactores con el proceso NP-AMX tratando aguas residuales industriales. La evolución de las poblaciones de bacterias oxidantes de nitrito y bacterias anammox se evaluó durante la operación del reactor mediante el análisis por FISH e Illumina®.

Los ensayos en discontinuo con purines de cerdo mostraron una disminución de la actividad específica máxima de las bacterias anammox del 44.4%. El comportamiento del proceso NP-AMX se vio influenciado por la combinación de varios factores característicos del purín de cerdo, como la conductividad (6 - 8 mS/cm) y la relación entre la DQO soluble (DQOs) y el nitrógeno (< 1.14 g/g), que indica la posible interacción o competición a largo plazo entre los procesos de NP-AMX y la desnitrificación heterótrofa. Los análisis de FISH e Illumina® mostraron la presencia de BON desde el inicio de la operación, siguiéndose una estrategia basada en la limitación de la concentración de OD y la inhibición por amonio libre (hasta 40 mg NH_3-N/L) para limitar su actividad. El rango óptimo de OD fue de 0.07 - 0.12 mg O_2/L para conseguir la inhibición de las BON, asegurando al mismo tiempo un balance satisfactorio entre la nitritación parcial y el proceso anammox, que fue capaz de eliminar el 60% del nitrógeno total con una carga eliminada de 0.1 g N/(L·d). La contribución de la desnitrificación heterótrofa a la eliminación de nitrógeno se limitó a un máximo del 10%, probablemente debido a que la relación DQOs/N estaba por debajo del ratio necesario para la desnitrificación por nitrito (1.71 g/g).

El tercer y último tipo de agua residual estudiada en la presente tesis fue la fracción orgánica de los residuos sólidos urbanos (FORSU). La eliminación de la materia orgánica de la FORSU mediante el proceso de DA, además de producir biogás durante el proceso, supone en el caso de esta agua residual una desviación importante de la cantidad de efluentes urbanos que son destinados a vertederos, cuya producción asciende a más de 2 billones de toneladas por año en la actualidad. La diferencia entre la FORSU y los lixiviados estriba, por lo tanto, en su procedencia, ya que los últimos se almacenan durante largos periodos de tiempo (del orden de meses o años) en vertedero antes de su tratamiento, mientras que la FORSU se separa y trata en instalaciones de depuración de aguas residuales tras su generación.

Sin embargo, la extendida tendencia actual a tratar la FORSU mediante la DA provoca una gran cantidad de digestatos, que normalmente se separan en fracción sólida y fracción líquida. La fracción líquida de la FORSU pre-digerida se caracteriza por concentraciones extremadamente altas de nitrógeno, materia orgánica recalcitrante e incluso metales, por lo que surge la necesidad de encontrar tratamientos más eficientes para eliminar el nitrógeno y cumplir con la legislación de límites de vertido. En este sentido, la utilización de procesos biológicos (nitrificación-desnitrificación) se ha investigado ampliamente en la última década, mientras que son muy escasos los trabajos que evalúan su tratamiento con sistemas basados en el proceso anammox.

En el **Capítulo 5** se estudió la operación de dos reactores SBR a escala de laboratorio, con un volumen de 1.5 - 1.7 L, para el tratamiento de la fracción líquida de la FORSU. El influente de ambos reactores consistió en el efluente de un digestor anaerobio (también a escala de laboratorio) tratando FORSU de una planta real; en uno de los dos digestores, la FORSU se pre-trató con stripping de amonio para reducir la concentración de nitrógeno, inhibitorio para la DA. Por lo tanto, uno de los objetivos principales en el Capítulo 5 fue el estudio del efecto de este pre-tratamiento sobre el digestato producido en la DA, y consecuentemente sobre la posterior eliminación de nitrógeno con el proceso NP-AMX. Además de monitorizar la actividad anammox durante toda la operación, se observó la evolución de las poblaciones bacterianas mediante análisis por Illumina®. Con el objetivo de facilitar la adaptación del proceso NP-AMX al efluente de FORSU, se siguió como estrategia de alimentación el aumento progresivo de la proporción de FORSU (diluida con agua) en la alimentación.

Los resultados de laboratorio mostraron una reducción a lo largo de la operación de hasta el 90% en la actividad máxima específica de las bacterias anammox, con independencia de la presencia o ausencia de un pre-tratamiento previo a la DA. El funcionamiento del proceso NP-AMX en ambos reactores estuvo relacionado principalmente con la relación DQOs/N en el agua residual, siendo notable el cambio en las poblaciones bacterianas (de bacterias anammox a bacterias desnitrificantes) por encima de un ratio de 1.7 g DQOs/g N, indicando el intercambio

de procesos dominantes para la eliminación de nitrógeno. Sin embargo, las eficiencias más altas de eliminación de nitrógeno (94%) se consiguieron para el SBR tratando FORSU con pre-tratamiento de stripping de amonio, a pesar de tratar ratios DQOs/N (2.8 - 8.5 g/g) 4 veces mayores que sin pre-tratamiento. La capacidad de eliminación de nitrógeno en el segundo caso fue mayor (0.687 g N/(L·d) vs. 0.058 mg N/(L·d)), aunque esto se debió en parte a las sucesivas re-inoculaciones que fueron necesarias en el reactor.

A diferencia de la FORSU tratada en el Capítulo 5, en el **Capítulo 6** se trató mediante el proceso NP-AMX la FORSU pre-digerida en un digestor anaerobio de membrana. La tecnología de membranas ha despertado interés en los últimos años debido a que puede reducir los largos tiempos de residencia hidráulicos (TRH) propios de la DA, disminuyendo el volumen de reacción necesario y aumentando la capacidad de eliminación con mayores cargas tratadas y producción de biogás asociada, además de menores costes de operación. En relación al post-tratamiento para la eliminación de nitrógeno, la membrana genera un efluente con menor concentración de sólidos para el proceso NP-AMX. En este caso, los objetivos de la operación de un reactor SBR de laboratorio con el proceso NP-AMX consistieron en la estrategia de operación óptima para tratar este tipo de efluentes, teniendo en cuenta los ratios DQOs/N moderados y los mayores ratios de nitrógeno frente a carbono inorgánico (N/IC), que proporciona una idea del correcto funcionamiento de la nitritación parcial, ya que por encima de 1 g N/g IC la oxidación de amonio está limitada al 50%. Además, se evaluó la viabilidad de implementar un pre-tratamiento previo al proceso NP-AMX para adecuar las características del permeado de FORSU al proceso NP-AMX, concretamente la relación DQOs/N.

Los resultados obtenidos en laboratorio indicaron el efecto del ratio N/IC en el permeado sobre la eliminación de nitrógeno, cuyo valor máximo fue del 87% para ratios N/IC (1.2 g/g) próximos al valor limitante para el 50% de la nitritación parcial (1 g/g). Por otra parte, se probaron dos tipos de pre-tratamiento antes del proceso NP-AMX: uno físico-químico, consistente en aplicar directamente aireación o agitación al permeado de FORSU, y otro biológico con lodos activos para conseguir menores concentraciones de materia orgánica, más adecuadas para el proceso NP-AMX, mediante su oxidación aerobia. La aplicación del pre-tratamiento biológico durante 30 h a pH = 6.0 permitió rebajar la concentración de materia orgánica de 5000 mg DQOs/L a 750 mg DQOs/L, con un ratio final de 1.3 - 1.6 g DQOs/g L. Sin embargo, la relación N/IC se incrementó por encima de 4 g N/g IC, provocando la pérdida inicial de actividad en el proceso NP-AMX luego de la aplicación del pre-tratamiento, con eliminaciones finales de nitrógeno de hasta el 60%.

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